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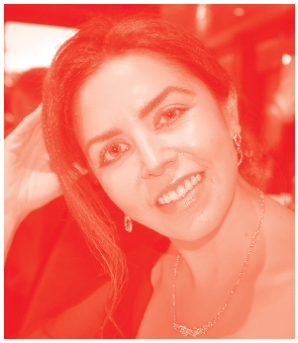

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Meet the editor

Dr. Jose C. Jimenez-Lopez obtained a BS in Biochemistry in 1998, a BS in Biological Sciences in 2001, and an MS in Agricultural Sciences in 2004, all from the University of Granada, Spain. He obtained a Ph.D. in Plant Cell Biology from the Spanish National Research Council (CSIC) in 2008. He was a postdoctoral research associate at Purdue University, USA (2008–2011), and a Marie Curie research fellow (2012–2015) at the University of Western Australia and CSIC. Currently, he is a tenured scientist at CSIC, working in the functionality, health benefits, and molecular allergy aspects of seed proteins from crop species of agro-industrial interest (mainly legumes). He is an author of more than 70 journal articles, 30 book chapters, and 2 patents. He has contributed to more than 130 international congresses. Dr. Jimenez-Lopez is an active member of different scientific societies, an editorial board member for international journals, and an editor of multiple books.

Dr. Alfonso Clemente is a senior scientist and the director of the Zaidín Experimental Station (EEZ), Spanish National Research Council (CSIC), Spain. He has carried out postdoctoral stays at the Institute of Food Research (1999–2000), John Innes Centre (2001–2002), and Sainsbury Laboratory (2003–2004) in the United Kingdom. An expert in gastrointestinal health, Dr. Clemente investigates the beneficial influence of dietary components resistant to the digestive process, using several in vitro and in vivo approaches. He has led numerous research projects with collaborations at national and international levels. During 2015–2021, Dr. Clemente was president of the Spanish Legume Association (AEL), coordinating numerous national and international activities and scientific meetings. He has been a board member of the Spanish Society of Microbiota, Probiotics, and Prebiotics (SEMiPyP) since 2016.

Contents

Preface

With more than 20,000 species, legumes (*Fabaceae*) are the third largest and most diverse plant family. They are highly desired crops worldwide; approximately 25 crop legumes are crucial for global food structures. They provide a broad variety of important and affordable sources of vegetable proteins for humans and animals while contributing to food and feed security in the perspective of an increasing global population. Legume seeds exhibit nutritional properties and health benefits and provide crucial facilities to agriculture through their capability to fix atmospheric nitrogen by microorganism symbiosis. Their other multiple beneficial roles in agroecosystems include augmenting carbon quantity in soils dedicated to agriculture, stimulating the production of rotation crops, and controlling weeds.

Despite their importance, legume production has slowed in the past 50 years, which has caused a substantial reduction in the per capita accessibility of food legumes. Thus, continuing to develop legume varieties with desirable traits is essential for coping with new challenges caused by climate change. To improve production and seed quality compounds, genetic resources from germplasms with environmentally strong genes are being used to design high-yield crops that are resistant to climate challenges.

In this framework, genomic and genetic developments are of high importance and play a crucial role in increasing pf crop production using both traditional breeding as well as cutting-edge and original biotechnological methodologies and techniques, whose uses will certainly contribute to sustainable agriculture and food security.

This book is a collection of studies on improving legume seed traits. Chapters examine genetics and genomics of legumes, seed trait research to obtain stress-resilient grains, genetic markers linked to seed quality and increased crop yield, plant-soil interactions, and more.

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Section 1

Legumes Genetics

Chapter 1

Advanced Breeding Approaches for Cold-Tolerant Chickpea and Lentil in Dryland Areas

Hamid Hassaneian Khoshro and Ramin Lotfi

Abstract

Chickpea and lentils are the two most economically important food legumes in dryland areas. They are traditionally sown in the spring of cold dryland areas of the Mediterranean regions. Therefore, the grain yield of the crop is affected by drought and high thermal stresses at the end of the season. Autumn cultivation of these crops by cold-tolerant varieties could increase grain yield up to 50%, then spring cultivation through higher availability of soil water. Breeding for cold-tolerant chickpea and lentil that is widely adaptable to autumn cultivation in cold regions and various growth conditions is the best strategic approach but requires a fine-tuned combination of advanced phenotyping and genotyping methods. However, breeding and selection of suitable cold-tolerant chickpea and lentil genotypes is complex by its narrow genetic base, which limits the sources of novel alleles. This chapter illustrates the morphological, physiological, and molecular effects of cold stress on chickpea and lentil growth and development. It will be also elaborated on conventional and advanced breeding approaches and application of advanced genotyping and phenotyping tools commonly used to develop cold-tolerant chickpea and lentil cultivars. The following, about key crop cold-tolerance traits that can be easily screened by using genotypic and phenotypic technologies are discussed.

Keywords: chickpea, cold tolerant, lentil, molecular techniques, plant breeding, physiological traits

1. Introduction

The term "stress" is defined as any disturbance that adversely influences plant growth [1–6]. Plants in nature deal with abiotic/biotic stresses. Abiotic stresses, such as low or high temperature, deficient or excessive water, high salinity, heavy metals, and ultraviolet radiation, are hostile to plant growth and development. In most crop species, suboptimal temperatures can be divided into chilling and freezing ranges. According to Graham and Patterson [7] for chickpea plant temperature below −1.5°C is the typical freezing point, and between −1.5°C and 15°C is chilling range temperatures. Temperatures up to 15°C have been demonstrated to cause flower and pod abortion in parts of the world [3, 8]. Freezing range temperatures during the seedling and early vegetative stages of crop growth are considered an important problem for winter-sown chickpea in the countries surrounding the

Mediterranean Sea, the tropical highlands, and temperate growing regions [8]. Cold-sensitive crops are damaged through temperatures below −1.5°C. Ice forming within the intercellular spaces could damage sensitive plants. The rigid ice lattice structure enlarges with reducing temperature and may creep into cellular membranes and disrupted the normal cell function [9]. The upper and lower leaves of the plant canopy, stems, meristems and roots have different responses to the freezing stress [10]. Antifreeze proteins and ice nucleators control the initial formation of ice. Tolerance to freezing is often associated with mechanisms at the cellular level, including increased membrane fluidity and osmotic adjustment [11] as well as supercooling without ice nucleation [12]. Wery et al. [11] found that selected wild *Cicer* species had more freezing tolerance than well-known cold-tolerant cultivars. The effects of cold and freezing temperatures during growth stages of legume crops need to study by observing physiological, biochemical, and molecular traits to develop cold-temperature-tolerant cultivars.

2. Cold stress effects on legume plants

2.1 Morphological aspects

Freezing range temperatures are detrimental to chickpea yield. At the vegetative stage, freezing temperatures have a severe negative effect on plant growth and development. Freezing range temperatures even during a low period can disrupt germination, decline the early growth and biological yield of the plant, and can destroy plants, especially those at the late vegetative or reproductive growth stages. During germination, chilling range temperatures result in poor crop establishment, increased susceptibility to soil-borne pathogens, and reduced seedling vigor. Walia et al. [13] demonstrated that low temperature (10°C) decreased the germination rate of chickpea seeds. The recommended threshold temperatures range for chickpea germination that varies from 5 to 35°C and the optimum germination temperature is 20°C [11]. Chickpea, along with many other chilling sensitive species, is prone to "imbibitional chilling injury" [14]. In the field, chilled seeds are often vulnerable to infestation by soil organisms, which reduces seedling survival. At the seedling stage, long periods of chilling range temperatures can retard the growth of the plant and, in severe cases, cause plant death. Isolated frost events during the reproductive stage commonly result in flower or pod abortion [3]. Less dry matter production reduces the reproductive sink that the plant can support, which, in turn, reduces potential yield. Flower, pod, or seed abortion are further symptoms of chilling range temperatures. Causal observations have indicated that freezing can reduce seed size, probably due to stress conditions affecting the mobilization of plant resources. In addition, the seed coat can be discolored [3]. Exposure at the mature pollen stage delayed anther dehiscence and induced partial pollen sterility [15]. A low period of freezing temperatures induced pollen sterility of plants. It depends on the age of the flower; older flowers are so resistant to the amount of sterile pollen than younger flowers. Pollen were completely sterilized under low temperature at young microspore stage whereas, at vacuolated microspore stage about 23.59% and at vacuolated stage 52.4% of pollen were viable and at finally mature stage 65.5% of pollen were viable [15]. Chilling stress at reproductive stage could negatively affect flower number, pod set, seed growth and development in chickpea [3, 16]. In comparison to that, low temperature impairs seed filling processes, which influence seed size of chickpea [16].

2.2 Physiological aspects

Low-temperature stress (5°C for 3 days) inhibited root growth and the capacity for water and mineral uptake to subsequently impact the nutritional influences on plant growth [17, 18]. Photosynthesis is the principal process of capturing light energy to form carbohydrates and is sensitive to low temperatures [19, 20]. Chlorophyll (Chl) fluorescence is a direct tool for detecting photosystem II (PSII) efficiency, as the ratio of Fv to maximal fluorescence emission (Fv/Fm) [21, 22]. Photo-inhibition could decline the efficiency of the electron transport chain during the light phase of photosynthesis, and this event disrupts photosynthetic apparatus in response to stress; its key characteristics are a reduction in maximum potential quantum efficiency of PSII and dissipation of light energy as heat. Despite the reduction in photosynthetic capacity, it is often accompanied by enhancement of sugar accumulation, which is a typical stress response in all plants [21–24]. In the northern hemisphere, low temperatures during the winter and early spring are usually followed by intense PAR. These conditions can cause degradation of the thylakoid structure and distortion in light-dependent photosynthetic reactions [25]. Cold stress also affects ChlF parameters. For example, a decrease was observed in chlorophyll content, OEC efficiency on the donor side of PSII, photochemical quenching, and efficiency of open PSII reaction centers exposed to cold stress [26]. Some plant species are known for their tolerance to low temperatures, showing less photoinhibition of PSII. For example, under cold stress plants show only small modifications in ChlF parameters [27]. Low temperatures (17.6/4.9°C; day/night for 26 days during reproductive phase) resulted in a reduction in relative leaf water content, possibly due to a decline in root hydraulic conductivity, oxidative and membrane damage, and chlorophyll loss [28]. Low temperatures (5/5°C for 4 days) also reduced the leaf water content because the stomata are unable to close [29]. Generally, cold stress causes damage to PSII and reduces the stability of chloroplast membranes and photosynthesis. We conducted a study on cold-tolerant of 24 wild chickpea genotypes in DARI, Iran. According to the field result, those genotypes were divided into three groups as a response to cold stress (3 sensitive genotypes, 11 tolerant genotypes, and 10 resistant genotypes). Four selected genotypes were evaluated under 22°C, 4°C, and −4°C temperatures in a controlled cold room by chlorophyll a fluorescence (ChF) parameter. As a general phenomenon, at −4°C Fm, Fv/Fm, Fv/Fo, and PIabs significantly reduced. However, ABS/RC and Fo/Fm were increased. Maximum Fm and Fv/Fm and minimum ABS/RC were recorded in the ILWC109 genotype, similar to Aana as a newly released cold-tolerant chickpea variety (**Table 1**). It seems, ILWC109 genotype under −4°C has been could increase the number of active RC of PSII and by absorbing photons, the electron transfer chain is done more efficiently (under press by the authors). This claim is confirmed by the improvement of Fv/Fm and PIabs under −4°C.

Chlorophyll a fluorescence (ChF) allows us to evaluate the photosynthesis efficiency of plants. It is useful to study the effects of environmental stresses on plants' photosynthetic function of plants. Therefore, chlorophyll a fluorescence could help us to identify different stresses effects on plant growth, health, or integrity of the internal apparatus during photosynthesis [30, 31]. The fast ChlF technique also represents a useful tool to monitor PSII thermostability. The most efficient approach is to estimate the critical temperature, i.e., the threshold level above which there is a sharp increase/decrease of the observed parameter [32]. Low temperature affects the activity of enzyme ribulose activate (RCA), changes the availability of large and small subunits of rubisco, disrupts PSII oxygen-evolving complex (OEC), and damages the structure and functioning of D1 and D2 polypeptides of PSII [33]. Georgieva and Lichtenthaler [34] found on two pea cultivars that ChF and the

Treatments	PIabs	ABS/RC	Fv/Fo	Fv/Fm	Fo/Fm	Fm	Fo
4°C	2.75b	1.07b	1.86b	0.64b	0.36b	694.25b	244.25a
−4°C	0.94b	1.42a	1.06c	0.51c	0.49a	481.25c	234.25a
22°C	9.75a	0.91b	3.71a	0.79a	0.21c	1134.13a	242.50a
ILWC109	3.14a	1.16a	2.27a	0.672a	0.329b	830.67a	251.16a
ANA	5.16a	1.10a	2.28a	0.65ab	0.35ab	770ab	233.33a
ILWC119	5.89a	1.01a	2.31a	0.653ab	0.348ab	762.50b	231.66a
ILC533	3.72a	1.25a	1.95a	0.597b	0.403a	716.33b	245.16a

Different letter in each column indicates significant difference at $p \leq 0.05$.

Table 1.
Chlorophyll a fluorescence parameter changes of chickpea genotypes under different temperatures.

Chl/Car ratio reduced, while the Chl a/b ratio increased under cold stress. In soybean plants, photosynthetic efficiency declined by more than 50% when subjected to only one night of chilling treatment [35, 36]. Respiration in plants is a temperature-sensitive process and an initial increase in response to chilling has been reported [37]. A 68% decrease in cellular respiration was reported in chickpea [38] at freezing range temperature (5°C/13°C), possibly due to altering in mitochondrial structure, less kinetic energy, and damage structure of housekeeping proteins and enzymes related to cytochrome activity, ubiquinone synthesis, and phosphorylation reactions related to ATP-dependent metabolism [39]. Freezing tolerance is related to the process of cold acclimation in plants. Acclimation is a process resulting from both metabolic and physiological alterations in plants during low temperatures [40]. Cellular and metabolic changes occur during cold acclimation include increasing of sugars, soluble proteins, prolines, and organic acids as well as the appearance of new isoforms of proteins and altered lipid membrane composition [41, 42]. Autumn planting chickpea is exposed to decreasing photoperiods and temperatures during the fall session to early winter. Therefore, seedlings of fall-planted chickpea have a possibility of acquiring some degree of tolerance to moderate subzero temperatures.

2.3 Biochemical aspects

Each plant has different enrichment pathways in different periods of cold stress. In cold-tolerant chickpea genotypes, the content of unsaturated fatty acids increased during low-temperature exposure (10°C for 5 days followed by 4°C for 2 days), which possibly contributed toward the maintenance of membrane integrity during cold stress. Reactive oxygen species (ROS) are produced in response to cold stress in chickpea [43] and damage vital molecules in cells, including membranes. Generally, lipid peroxidation and hydrogen peroxide concentrations are measured as markers of temperature-induced oxidative stress [44]. A positive correlation was observed between lipid peroxidation and malondialdehyde (MDA) concentration in *Cicer* occidentalis [45]. Plant cells have different mechanisms (anti-oxidative) to combat oxidative damage by activating ant oxidative systems that include both non-enzymatic (e.g., tocopherols, ascorbate, proline) and enzymatic (e.g., superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)) [46]. A few studies in chickpea have identified an increase in the double bond index due to enhanced lipoxygenase (LOX) activity, suggesting that increased LOX activity plays an important role in providing cold tolerance in chickpea [47]. The upregulation of various types of antioxidants has been correlated with cold tolerance in chickpea [48].

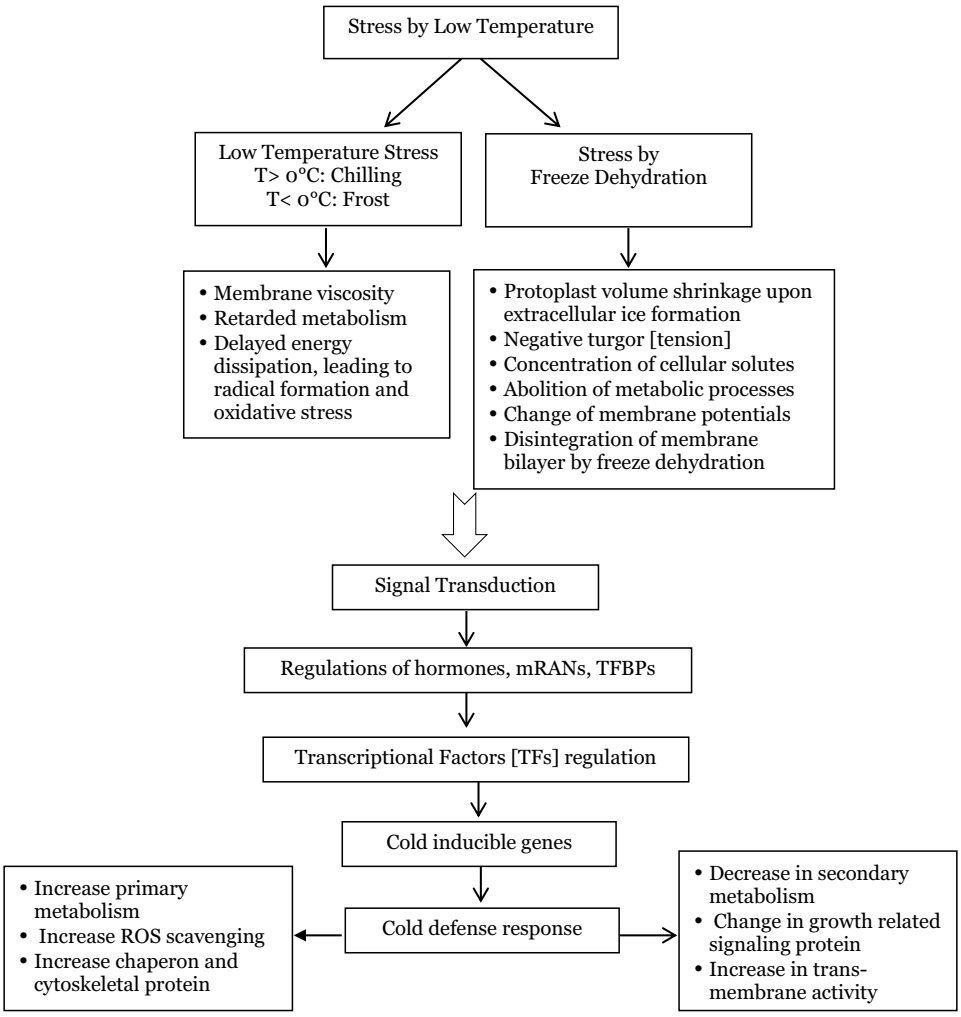

Figure 1.
A schematic diagram of cold stress response in chickpea and lentil.

Glycine betaine (GB) protects the activities of enzymes and proteins and stabilizes membranes and photosynthetic apparatus under chilling (12–14/3–4°C day/night) and freezing stress at reproductive stages [38]. Cold stress (12–14/3–4°C day/night at bud stage) decreased the endogenous GB concentration in chickpea leaves and flowers, resulting in the loss of pods [48]. Exogenously applied GB to chickpea plants at bud and pod filling stages during cold stress improved flower function, pollen germination, pollen tube growth, stigma receptivity, and ovule viability, leading to floral retention, pod set, and pod retention [38]. Also, the application of GB at reproductive stages improved grain yield/plant, the number of grain/100 pods. Low-temperature tolerance induced by GB may be related to an enhancement in relative water content (RWC), chlorophyll and sucrose, and a decrease in ABA and active oxygen species (MDA and hydrogen peroxide) [18, 45].

2.4 Molecular aspects

Several studies display those genotypes of chickpea and lentil has different molecular responses under low-temperature conditions [49–52]. This event needs an enormous gene expression reprogramming, which results in the adjusted

metabolic-structural alterations. However, the efficient adjustments are dependent on suitable cold signal transduction. Cold stress signal perception that is carried out by different pathways is the first stage. The cascades of transcriptional are the next players, which act through ABA-independent and ABA-dependent pathways to persuade cold-regulated (COR) gene expression, and the result is increasing in the levels of hundreds of metabolites, in which some of them are recognized to have defensive effects against the damaging effects of cold stress and some like reactive oxygen species (ROS), photosynthetic metabolites, and soluble sugars are thought to operate as signaling molecules and regulate specific COR genes [53, 54]. The different aspects of these phenomena are displayed in **Figure 1**. Different receptors at the cellular level are involved in receiving the external signals and, in turn, transfer them intracellularly. Thermal reactions in plants in the face of cold stress include molecular regulation and complex intracellular machinery. Two key transcriptional pathways are activated in reaction to cold stress, CBF/DREB-independent and C-repeat (CRT)/dehydration responsive element (DRE)-binding factor (CBF/DREB)-dependent [55]. The transcription factor, CBF, operates as a master regulatory player and is induced by the binding of trans-acting factors to the promoter regions of the CBF gene [53]. The constitutive expressed ICE1 (Inducer of CBF Expression 1) binds to the corresponding cis-elements on the CBF promoter and elicits the ICE1-CBF cold-responsive pathway, which is conserved in diverse plant species [53, 55].

3. Breeding strategies for improvement of cold tolerance

3.1 Conventional breeding

Conventional breeding involves crossing, the selection from landrace genotypes, simple backcrosses to a recurrent parent forms the backbone of breeding and has been widely used to introduce novel traits within breeding programs and produce chickpea and lentil cultivars suitable for targeted environments and cropping systems. Through conventional breeding, lines of varying maturity can be selected that are suitable for production in different agroecological regions. In the last 10 years at DARI, significant improvement has been achieved in crop yield and productivity through conventional breeding, which has donated to the development of high-yielding chickpea cultivars tolerant to cold stress and suitable to autumn sowing in cold regions of Iran such as FLIP 00-86C (Saral), Flip05-42C (Soufi), FLIP 02-51C (Nosrat), x03TH148 (ATA), and x03TH130 (ANA). These cultivars have been selected from the ICARDA breeding materials and registered as new cultivars [51, 52, 56].

3.1.1 Screening for freezing tolerance in the field

Based on survival and killing percent, various scales including 1–3, 1–5, or 1–9 have been developed and used by numerous workers. Attempts were made to develop a more reliable field screening technique for evaluation of cold tolerance in chickpea and lentil at ICARDA, Tel Heldya, Syria, and the main research site of ICARDA at Aleppo, Syria [57], and a screening procedure was developed. They also developed a more precise 1–9 scale (**Table 2**), using a combination of percent plants killed and visual damage on leaflets and branches on individual plants, which can be used to evaluate even individual plants.

Later, Saccardo and Calcagno [58] used a 0–5 scale (0 = all plants killed; 5 = all plants survived) to screen chickpea material for cold tolerance and to develop lines for winter sowing in Italy. They identified 27 lines as cold-tolerant, ones at the site where the minimum temperature was −12°C and the plant survival rate

Scale	Category	Reaction
1	—	No visible symptoms of damage
2	Highly tolerant	Up to 100% of leaflets show withering and drying, no killing
3	Tolerant	11–20% leaflets show withering and upto 20% of branches show withering and drying, no killing
4	Moderately tolerant	21–40% leaflets and up to 20% of branches show withering and dryings, no killing
5	Intermediate	41–60% leaflets and 21–40% branches show withering and drying, up to 5% plant-killing
6	Moderately susceptible	61–80% leaflets and from 41 to 0% branches show withering and drying, to 25% plant-killing
7	Susceptible	81–99% leaflets and 61–80% branches show withering and drying, 26–50% plant-killing
8	Highly susceptible	100% leaflets and 81–99% branches show withering and drying, 51–99% plant-killing
9	—	100% plant-killing

Table 2.
Scoring of cold tolerance in field conditions in chickpea and lentil [57].

was 50–70%. Wery [59] and Kanouni and Khalily [52] reported variation among the chickpea cultivars, which were evaluated for frost resistance (minimum temperature −10°C to −18.5°C) and suggested that the phenological stage as most important in determining the response of the crop to cold (**Figure 2**); cold resistance decreased with progress in growth from germination to the flowering stage. They used a "frost resistance ratio" (the number of plants at harvest/the number of plants that emerged) as a parameter for cold tolerance and grouped the genotypes in following categories: "fall type" (frost resistance); "winter type" (frost-tolerant); and "spring type" (susceptible to frost) and also confirmed that early sowing dates are more suitable for screening for cold tolerance under Mediterranean areas.

3.1.2 Screening under controlled conditions

In addition to field screening, there are several controlled conditions and laboratory-based tests available for the identification of genotypes with tolerance to cold stress. Some of the more common techniques applied in legumes and other plants are summarized (**Table 3**). Whereas these techniques enable segregation of germplasm with high tolerance to special temperature regimes, they do not take into account the other stresses caused by overwintering, for instance, ice heaving or snow cover, and results accordingly will necessary to be acknowledged by screening in the field. Laboratory-based methods may find a wide-ranging application in distinguishing genotypes that have the tolerance to chilling at the stages of reproduction, since conditions of the field for this stress are very replicable. These can also be suitable in screening a restricted number of parental genotypes for a given trait, such as pollen vigor at chilling range temperatures. Appropriate genotypes identified from this screening can then be used in a hybridization program to generate progenies with variable tolerance to either freezing or chilling stress. Recently at DARI, Heidarvand and Maali-Amiri [18] identified two chickpea Sel95Th1716 and Sel96Th11439 as chilling tolerant based on controlled environment and laboratory-based screening techniques. Clarke et al. [69] has developed a method for screening

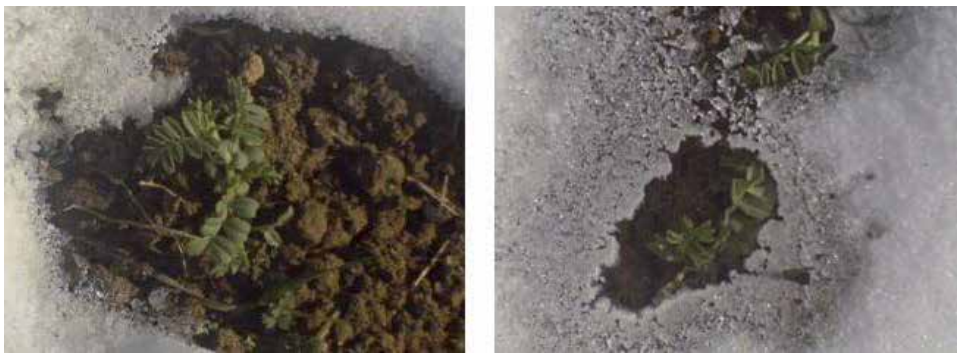

Figure 2.
Saral (FLIP 00-86C) is an Iranian new chickpea cultivar of tolerance to freezing at the seedling stage that can withstand at temperature of −22°C with snow cover in field condition [52].

Technique	Methodology	Example reference/s
Controlled environment frost screening	Plants are subjected to gradually decreasing temperature for 3 weeks, which is increased when 50% of plants show frost damage	[51, 60]
Controlled environment chilling screening	Plants are subjected to chilling temperatures during the flowering period and assessment is based on pod and seed set	[61]
Chlorophyll fluorescence and photosynthesis	Based on the fact that fluorescence emission is storing when leaves are irradiated after a dark period but is reduced if stress has damaged the cells	[28, 33, 39, 62]
Ion efflux	Measurement of ion efflux in leaves	[63]
Controlled freezing tests	Freezing whole plant/parts under a specific regime and then assessing for visible injury	[64]
Triphenyl tetrazolium chloride test (TTC)	A cell viability test based on the reducing capacity of living cells. Healthy, non-injured cells can reduce TTC better than injured cells	[65]
Leachate test	Based on the amount of naturally occurring compounds that diffuse from cells following cold exposure. Larger amounts of leachate are indicated by greater electrical conductivity	[66]
Plasmolysis test	Based on the fact healthy cells plasmolyse in a hypertonic solution such as calcium chloride, whereas injured cells do not	[67]
Pollen tube growth	Cold-sensitive genotypes yield pollen with reduced tube growth and fewer pollen tubes reaching the ovule	[68]
Osmoprotectant, membrane integrity, and enzymatic activity	There is a close relationship between cold tolerance and osmoprotectant (such as sugar, prolin, proteins, fats) metabolism	[18, 43, 45, 69]

Table 3.
A summary of controlled environment and laboratory-based screening techniques for the identification of chickpea and lentil tolerance genotypes to cold stress.

of pollen tube growth to recognize germplasm with chilling tolerance at the stages of reproduction. This method compares pollen tube growth of diverse genotypes at changing temperatures and has been applied to select reputed chilling tolerant lines as parents in the legumes breeding program. Other laboratory-based methods for

identifying tolerant genotypes can be to measure ROS-scavenging systems, including both enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), and non-enzymatic antioxidants such as ascorbate, proteins, fats, and proline [18, 45].

3.2 Molecular breeding

3.2.1 Marker-assisted breeding

Molecular markers are now considered better than physiological and morphological characters because of unaffected by environmental factors, theoretically unlimited, being stable, and simply detectable without distinction of growth and stages of development. They are also ideal for the identification of QTLs, genetic diversity analysis, tagging of useful genes, fingerprinting, construction of genetic and physical maps, evolutionary studies, positional cloning of useful genes, and marker-assisted selection [70–72]. Molecular markers are reaching a stage where they can be applied cost-effectively in breeding programs. QTL analysis, genomics research, and genotyping platforms are used to speed up the breeding process through exploiting variation at the genome level [73]. Several studies reveal the successful application of molecular markers in the improvement of chickpea and lentil cold tolerance cultivars [74–76]. Clarke and Siddique [77] found that molecular markers based on amplified fragment length polymorphisms (AFLPs) have been linked to the trait using bulked segregant analysis for F2 progeny of a cross between the chilling-sensitive cultivar amethyst and the chilling-tolerant ICCV 88516 [77]. Putative markers linked to traits for both chilling sensitivity and chilling tolerance prevail the limitations of the dominant AFLP marker system. Six pairs of specific 18–24-mer primers (AFLP-based markers) were applied to amplify the defined DNA fragment from genomic DNA of individual F4 progeny with known phenotypes in an effort to develop Sequence Characterized Amplified Regions (SCAR) markers [78]. The foremost promising primers were based on a 560-bp fragment containing a simple sequence repeat (SSR), with 10 repeats within the tolerant parent and 9 within the susceptible parent [77]. Their results also showed three-base differences on a vertical acrylamide gel, which was very suitable within the selection of chilling-tolerant progeny resulting from crosses between ICCV 88516 and amethyst [77]. Results of Amini et al. [79], based on cDNA AFLP analysis of transcripts, represented different groups of genes involved in metabolism pathways, cellular defense, cell connections and signaling, transcriptional regulation, and chromatin architecture in chickpea during cold stress.

A new method developed for marker-assisted breeding in *lupins* [80] could also be considered for chickpea and lentil in the future. Microsatellite-anchored fragment length polymorphism (MFLP) is highly efficient in producing DNA polymorphisms, and many MFLP markers can easily be converted into sequence-specific, simple PCR-based codominant markers. Difficulties in screening and breeding for tolerance to low temperatures are further confounded by low genetic variability within cultivated chickpea [81, 82]. Relatives of chickpea among the wild *Cicer* species offer a valuable genetic resource to overcome these limitations [8, 83, 84]. Tolerance to cold has been reported in five annual and one perennial species [3, 83, 85]. The original collection and many selections of annual *Cicer* species held in world gene banks were analyzed using DNA molecular markers, which are not affected by environmental influences, providing useful data for the selection of suitable parents for crosses [84, 86]. To a certain extent, it will also be possible to use chickpea-derived Sequence Tagged Microsatellite Site (STMS) markers for the marker-based analysis of wide crosses because many STMS can

be transferred between *Cicer* species [87]. Barriers in wide crosses are also being addressed through international collaboration with the aim to use embryo rescue to overcome incompatibility [77]. In lentil plant, Murray et al. [64] reported 12 QTL for winter hardiness and also, their results indicated that winter hardiness is influenced by several genes and the cumulative effects of cold stress. Target-induced local lesions in the genome (TILLING) of chickpea were used for functional validation of abiotic stress-responsive genes. A TILLING approach based on next-generation sequencing has been used in the mining genes associated with cold tolerance [88, 89]. Glaszmann et al. [90] used eight chickpea genotypes from different origins as parents for the development of a Multi parent advanced generation intercross (MAGIC) population. MAGIC population is one among a next-generation multiple mapping population, which comprised 4–20 parents in cross-combination and source of increasing genetic variability. The use of a MAGIC population is helpful because the inclusion of several parents confirmed the segregation of deployment for understanding complex traits, QTLs for multiple traits, and therefore the detection and description of unique genes [90].

3.2.2 Transcriptomics

Transcriptomics deals with the analytical study of the transcriptome that is the transcribed component of the genetic material. Sequence information and identification of novel genes for agronomically important traits can be done using a number of methods, including EST databases [91]. Next-generation sequencing and Sanger sequencing methods have been used for transcriptomic studies of chickpea. Initially, EST abundance was assessed for development-related expression, tissue-specific expression, and stress-responsive expression. Chickpea genotypes were grown under cold; salt and drought stresses and complementary DNA libraries were generated, which comprised 20,162 ESTs [92]. Gene discovery is very limited in chickpea, and few efforts have been made to identify the ESTs associated with stress responses through transcriptomic studies [92]. Mantri et al. [93] studied the transcript profiling in chickpea genotype under drought cold and salinity stress and concluded that transcriptional change of more than twofold was observed for 109, 210, and 386 genes after drought, cold, and high-salinity treatments, respectively. Deokar et al. [94] studied the differential downregulation and upregulation of the transcriptome in tolerant and susceptible chickpea genotypes subjected to abiotic stress.

In silico expression, studies were carried out to know the differential expression of tolerant and susceptible chickpea genotypes under abiotic stress [92]. Microarray, suppression subtractive hybridization, EST sequencing, and super serial analysis of gene expression (SAGE) have been used for functional genomics analysis of chickpea genotypes in stress responsive conditions [95, 96]. Sharma and Nayyar [96] used DDRT-PCR analysis to identify anther genes involved in cold tolerance in chickpea genotype ICC16349 (cold-tolerant). Their results showed cold stress altered expression of 127 ESTs in anthers, about one-third (35) belonged to several functional categories such as transcription, pollen development, ion transport, translation, signal transduction, carbohydrate metabolism, energy, and cell division. More than two-third (92) of them were novel with unknown protein identity and function. The combination of next-generation sequencing techniques with SAGE is cumulatively known as deep SuperSAGE, which makes the tool even more precise. Transcriptome analysis of chickpea roots was carried out using deep SuperSAGE under normal and abiotic stress conditions and 17,493 unique transcripts were identified which were stress responsive [97].

4. Conclusion

Chickpea and lentil improvement programs targeting the insulation of varieties against low temperature/cold stress have been initiated by many centers globally. In Iran at the Dryland Agriculture Research Institute (DARI), Saeed et al. [54], Kanouni and Khalily [52], and n and Maali-Amiri [18] identified and introduced chickpea genotypes namely FLIP 00-86C (Saral), FLIP 02-51C (Nosrat), x03TH148 (ATA), x03TH130 (ANA), Sel95Th1716, and Sel96Th11439 as chilling tolerant based on field screening and controlled environment and laboratory-based screening techniques at the vegetative stage where plants were exposed to −14°C to −25°C (**Figure 3**). Screening against low temperature has been taken up vigorously in recent years. At the Center for Legumes in Mediterranean Agriculture (CLIMA), in Australia, chilling tolerance has transferred from ICCV 88516 and two desi chickpea varieties WACPE2075 (Sonali) and WACPE2095 (Rupali) have been developed [77]. Breeding efforts made at ICARDA, Syria, have demonstrated the release of more genetic variability for flowering at low temperatures using cultivated x wild *Cicer* crosses. This shows that genes responsible for flowering at low temperatures should be transferred from wild to cultivated species, *Cicer arietinum*. Cold tolerance at flowering can also be achieved through accelerated breeding programmed based on haploid selection. Development and identification of molecular markers and QTLs offer promise for mitigating low-temperature stress at the genetic level. Molecular markers-assisted breeding can be a viable option in targeting the desired gene(s) or QTLs. Good scope exists for the exploitation of transgenic technology in the development of low-temperature/cold-tolerant genotypes. Per se, tolerance to abiotic stresses appears to be a difficult research aim to be tackled by conventional breeding due to several technical limitations. In changing climatic conditions where the crop has to face abrupt low temperature during the reproductive phase, concerted efforts for the development of low-temperature/cold-tolerant chickpea varieties are needed. An integrated approach involving molecular biologists, conventional breeders, physiologists, and agronomists should be adopted to mitigate the low temperature/cold stress for better crop productivity. This may include defining the target environment, development of reliable screening techniques, identification of desirable traits and donors, transferring the targeted gene[s] in desirable agronomic

Figure 3.
ANA (x03TH130) an Iranian new chickpea cultivar that can withstand at temperatures of −24°C with snow cover in field condition.

backgrounds. Critical assessment of cold-temperature genotypes under target areas (proper phenotyping) will certainly help in the identification of high-yielding chickpea varieties for cultivation in low-temperature/cold-prone areas.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 2

Advanced Breeding Approaches for Developing Cowpea Varieties in Dryland Areas of Limpopo Province, South Africa

Joseph Nwafor Akanwe Asiwe

Abstract

Cowpea is an important grain legume because it is a major source of cheap dietary protein. In the last four decades, the production of cowpea in South Africa is limited by lack of improved varieties that exhibit good agronomic traits and pest resistance. The purpose of the study was to develop cowpea varieties with important economic traits to meet the needs of the farmers. Germplasm lines were evaluated in field trials to select the promising lines that were used in hybridization to develop well-adapted elite genotypes. Pair-wise crosses were made to develop broad-based segregating populations. Pedigree, recurrent selection methods were used to advance the promising genotypes. Ten genotypes expressed high grain yield with combined pest resistance (aphids, bruchid, virus, leafhopper and bacterial blight). The implication of this achievement is that farmers can attain more grain yield per land area with reduced cost of pest control and increase the profit margin of the farmers. Furthermore, four elite varieties (JanaFod, ARC-GCI-CP76, UL-58 and UL-1010) have been registered and awaiting commercialization. The future activity is to commercialize the varieties to enhance uptake and availability of seeds to seed growers, farmers and consumers.

Keywords: breeding lines, fodder, grain yield, pest resistance, quality traits, *Vigna unguiculata*

1. Introduction

Cowpea is an important grain legume because it is a major source of cheap dietary protein which ranges from 23 to 32% [1–3] and 64% carbohydrate [4] that complements the over dependence on low-protein staple cereals and potatos in South Africa. The largest production of this crop is in sub-Saharan Africa, where it is a staple food crop and feed for animals [5]. Cowpea can be prepared in different forms (boiled as pudding and soup, steamed as *moin moin*, fried as *akara* to meet the dietary needs of the consumers. It is an important income earner to all the stakeholders in the value chain [6–8]. Cowpea is tolerant to drought, and this makes it a very versatile crop in drought-prone regions of the tropics. Cowpea has the ability to fix atmospheric nitrogen [9, 10] for its growth thereby reducing its nitrogen demand. This makes cowpea a low input crop and a good companion crop in most intercropping systems [6, 8]. However, the production of this crop

is limited by lack of improved varieties which are resistant insect pests, diseases, parasitic weeds and other abiotic stresses. The purpose of the research was to develop cowpea varieties that would overcome these constraints through a well-designed breeding programmes and activities. Cowpea is one of the neglected orphan or underutilized grain legumes in South Africa that has great potentials for enhancing food security and nutrition for the rural masses where poverty and starvation are a reality.

1.1 Word cowpea production

According to DAFF [11] the world annual cowpea grain production is about 3 million tons that is produced on 12.5 million hectares but only a small proportion enters the international trade. West and Central Africa is the leading cowpea producing regions in the world, producing 64% of the estimated 3 million tons of cowpea seed that is produced annually. Nigeria is the world's leading cowpea-producing country followed by Brazil. Other countries in Africa include, Senegal, Ghana, Mali and Burkina Faso. Ghana, Niger and Cameroon are significant producers. The major production areas elsewhere in the world are Asia (India, Myanmar) and America (USA, Brazil, West Indies). Conservative estimates suggest that greater than 12.5 million ha are planted annually to cowpea around the world. Of this area, about 9.8 million ha are contributed from West Africa, making it the region with the largest production of cowpea in the world [2, 11].

1.2 Cowpea production in South Africa

DAFF [11] reported that small-scale farmers are the major cowpea producers in South Africa under rain-fed farming conditions but there are no records regarding the size of area under production and yields produced. However, [12] reported that the land area used by farmers to produce cowpea ranges between 0.5 and 2.0 hectares per farmer. Farmers that cultivate land area up to two hectares make use of tractor or motorized implements [7] to save cost and to produce more above their family consumption and the excess is preserved for sale. The major cowpea producing areas in South Africa are Limpopo, Mpumalanga, North-West and KwaZulu-Natal [11]. A study conducted by [7] showed that farmers grow cowpea for consumption and as source of income.

Farmers prefer important traits such as seed color, seed size, growth habit and early maturity varieties. This suggests the great opportunity that exists for the development of cowpea varieties with divers coat colors and high potentials for their demand and marketability. Based on the duration of rainfall, some farmers choose early maturing varieties, as this will assist the crop to escape moisture deficits and frost damage while others choose late maturing types because they are more interested on the fodder for livestock feeding. On the other hand, KwaZulu-Natal farmers preferred cowpea varieties based on growth habit [12]. The diverse preferences by farmers call for the need to develop varieties with different agronomic and quality traits. The purpose of the research was to develop cowpea varieties that would overcome the various limitations encountered by farmers and to meet their needs. To develop varieties that will meet the needs of farmers and consumers, a well-tailored need assessment survey was conducted in some of the cowpea production areas between 2006 and 2007 [12]. Through this survey, the dire needs of farmers and quality trait preferred by consumers were documented and used as the breeding objectives [13, 14]. Therefore aim of the research was to develop cowpea varieties that would overcome these constraints through a well-designed breeding programmes and activities while the specific objectives include:

1. Development of early maturing varieties (70–90 days). These are grain type of cowpea which are commonly cultivated in regions with short rainfall duration (**Figure 1**). In higher rain region, they can be used for double cropping (first cropping from October–December and second cropping from January to March). They are best varieties to use to evade terminal drought.

2. Development of Medium maturing variety (91–100 days). These are dual-purpose cowpea types. They are good for grain and fodder for animal feed (**Figure 2**).

3. Development of late maturing varieties (101–120 days). They are mainly for fodder and leafy vegetable production with limited seed production. The medium and the late cowpea types are of high value for integrated livestock production and rural livelihood in rural communities (**Figure 3**).

4. Development of high yielding varieties with multiple trait characteristics (adaptation and pest (insect and disease resistance). The prevalent insect pests include aphid, leafhopper, cowpea bruchid and blister beetles [13] and the diseases are bacterial blight, anthracnose as well as nematodes. Multiple pest resistance genes have been incorporated in most of the elite cowpea lines.

5. To develop high consumer quality trait varieties such as seed size, color, low cooking time and high protein content. To incorporate these quality traits in one commercial variety is practically impossible. Therefore, development of high yielding and pest resistance cultivars with different quality traits is the practice. Seed coat color and texture is an important consumer preferred quality traits. Large seed size with bright coat color command a high premium price in South Africa.

6. Common insect pests of cowpea

The common insect pests of cowpea include, cowpea aphids, leaf hopper (**Figure 4**), bruchid, blister beetles and pod-sucking bugs (**Figure 5**) [13]. The economic importance of these insects vary from one location to another depending on the climatic variables that promote their abundance and the presence of their alternate hosts. It is important to note that the presence of winter for at least 4–5 months in some provinces has reduced drastically the carryover effects of some of these pests from one cropping season to another thereby reducing insect spectra and early incidence in South Africa unlike West Africa where there is no winter. Most of the breeding parents used for the development of the elite genotypes in South Africa were introduced from IITA-Nigeria and they possess multiple resistance to both aphid and bruchid, and were incorporated into the elite lines.

Figure 1.
Two different grain cowpea varieties.

Figure 2.
Two dual-purpose cowpea varieties.

Figure 3.
Two fodder (late) cowpea types (top and bottom-Janafod).

2. Materials and methods

One the major constraints to cowpea production in South Africa is lack of improved varieties. This was identified in the need assessment survey [7, 12]. To solve this constraint, international improved varieties were introduced in 2005/6 from International Institute of Tropical Agriculture (IITA), Nigeria for adaptive breeding purposes. Some of the varieties possess economic traits (high yielding, pest resistance and quality). The introduced varieties formed the breeding stock

Figure 4.
Susceptible cowpea-IT00K-1263 to leafhoper infestation (left) resistant cowpea-UL-1010 (right).

Figure 5.
Insect pest of cowpea: Anaplocnemis curvipis (upper left), Mylabris spp (upper right), seed damaged by bruchid (lower left) and cowpea aphid (lower right).

for the development of new adapted germplasm in South Africa. Based on the screening outcomes, promising varieties were selected for pair-wise crosses with promising local South African germplasm lines such as Glenda and Betchuana white to develop broad-based F_2 population which was used to form the various segregation populations for the specific objectives. Some of the varieties possess economic traits (high yielding, pest resistance and quality). The introduced varieties formed the breeding stock for the development of new adapted germplasm in South Africa. Based on the screening outcomes, promising varieties were selected for pair-wise crosses with promising local South African germplasm lines such

as Glenda and Betchuana white to develop broad-based F_2 population which was used to form the various segregating populations for the specific objectives. Some of these genotypes were screened in hot spots (Bela-Bela and Taung) for adaptation and important traits (yield, aphid resistance, and diseases (bacterial blight and anthracnose) and advanced to subsequent generations. Between 2005 and 2007, intensive screenings were conducted on the segregation populations using Pedigree method. From F_4-F_8, segregating populations were subjected to selection and advancement (using pedigree selection method) in a replicated field trials for adaptation and validation of important economic traits [7, 14, 15]. From ($F_{9–10}$)- an advanced fixed generation with promising genotypes from various traits for various specific objectives were tested in multiple locations for G X E [16–18] for adaptation. During the field evaluation processes, the populations were also subjected to aphids, bacterial blight, anthracnose screenings under natural infestation, and bruchid screening in the laboratory. Promising varieties selected from the evaluations were tested over seasons.

2.1 Data analysis

All the data collected were subjected to analysis of variance (ANOVA) procedure using Genstat Version 20 software. Means were separated using Duncan Multiple range Test (DMRT) at $P < 0.05$.

3. Results and discussion

3.1 Common insect pests of cowpea

The common insect pests of cowpea include, cowpea aphids, leaf hopper (**Figure 4**), bruchid, blister beetles and pod-sucking bugs (**Figure 5**) [13]. Most of the breeding parents used for the development of the elite genotypes in South Africa were introduced from IITA-Nigeria and have multiple resistance to both aphid and bruchid, and were incorporated into the elite lines (**Table 1**). This suggests that the parental lines used to develop the elite breeding lines have high heritability and were able to transmit the genes to their offspring [18]. The implication of the multipest resistance is that farmers can grow the varieties with reduced cost of pest control thereby enabling the farmers to maximize profit.

3.2 Development of early maturing varieties (70: 90 days)

The performance of the extra-early and early maturing varieties developed are shown in **Table 2**. Early maturity was also bred with good quality traits (seed size and color), plant type (erect or semi-erect), high yield, as well as pest resistance [10, 19]. This is to increase the acceptability and adoption of the varieties. In addition, early maturing varieties are regarded as "climate smart" and water use efficient varieties [1, 15]. Farmers in drought-prone regions of Limpopo Province can successfully grow such varieties within the short rainfall duration in their environment. The varieties are also regarded as the grain type cowpea (**Figure 1**).

3.3 Development of medium maturing varieties (91: 100 days)

Medium maturity cowpea varieties were developed for regions with higher rainfall 600–750 mm per annum with summer rainfall duration of 3–4 months. Medium cowpea types are characterized with good grain and fodder yield and are

			Resistance to				
Pedigree	**Grain yield (kg/ha)**	**Fodder yield (kg/ha)**	**Aphid**	**Viruses**	**bruchid**	**Leafhopper**	**Bacterial blight**
IT98K-962 X IT97K-499-35	1740.50^{bc}	1987.70^{cd}	R	R	R	R	R
IT98K-962 X IT98K-205-8	1928.50^{b}	2679.70^{b-d}	R	R	MR	R	R
IT98K-962 X TVX 3236	1557.90^{cd}	3611.00^{a}	R	R	MR	R	R
IT97K-497-2 X IT98K-962	1670.60^{b-d}	2796.50^{a-c}	S	R	R	R	R
IT97K-497-2 X Oloyin	1561.70^{cd}	2658.10^{b-d}	S	R	MR	S	MR
IT97K-497-2 X IT82D-889	1675.40^{b-d}	2659.50^{b-d}	S	R	MR	R	R
IT00K-1217 X IT98K-962	2595.20^{a}	2633.10^{b-d}	R	R	R	R	R
IT98K- 205-8 X Oloyin	1441.20^{d}	2283.10^{b-d}	R	R	MS	R	MR
IT98K-205-8 X IT98K-406-2	1807.50^{bc}	2488.60^{b-d}	R	R	R	R	R
IT90K-76 X Oloyin	1891.70^{b}	3022.00^{ab}	R	R	R	R	R
BW (Local check)	1858.70^{b}	1934.20^{d}	S	S	S	R	R
Grand mean	1793.5	2614					
P-level (P < 0.05)	0.001	0.001					

**R = resistant, MR = medium resistance, MS = Medium susceptible, S = susceptible (Singh et al., 1997).*

Table 1.
Yield and pest resistance of elite cowpea breeding lines.

often regarded as the dual-purpose cowpea (**Table 3** and **Figure 2**) [20, 21]. They are also suitable for livestock integration. This type of cowpea also combine good quality traits (seed size and color) with, high yield, plant type (semi-erect) and pest resistance. The list of medium maturity cowpea are shown in **Tables 2** and **4**. Dual-purpose cowpea varieties under good rainfall distribution produce grain yield far above the grain type because it takes extra time to develop more photosynthetic apparatus such as leaves, canopy, branches and height which enables the varieties to produce flowers and more pods which are translated into high grain yield [19–21]. The high fodder yield is generated from the branches and leaves [2, 5].

3.4 Development of vegetable cowpea varieties

One of the ways that cowpea contribute to food security and nutrition is through the pods and leaves (**Figure 3**) which are eaten as vegetable to relish meals. This is an important cowpea menu in South Africa. It is locally called "*Morogo*". Crosses made between IITA varieties such as IT82D-889, IT81D-1228-10 that exhibit long pods (30 cm) with TVu 13,464 (short pods with high pod load) produced genotypes with longer pods 50–75 cm (**Figure 6**). The varieties can be harvested 3–4 times

Variety	Potchefstroom			Taung		
	Grain yield kg/ha	Maturity	100seed weight (g)	Grain yield kg/ha	Maturity	100seed weight (g)
99 K-494-6	3064.6	99.22	16.49	3206.2	92.84	17.24
Pan-311	2913.3	80.22	14.58	1873.1	92.59	13.56
IT00K-1217	2894.6	92.55	15.05	1719.7	92.59	15.5
TVu 13,464	2722.9	85.88	12.96	1968.9	91.84	12.96
97 K-1069-8	2377.9	97.55	15.83	2296.2	99.59	15.43
97 K-1069-1	2321.1	94.22	16.99	2708	99.09	17.89
95 K-1491	2287.2	91.55	18.55	1972.7	94.59	18.68
83D-442	2266.7	91.55	13.18	2369	95.84	12.29
97 K-568-18	2227.2	97.22	16.91	1784.7	98.09	18.12
98 K-530-1	2180.8	95.88	18.88	1642.6	97.59	17.75
93 K-452-1	1823.7	91.55	15.05	1908	93.59	16.85
S.E.M	*311.3*	1.44	*0.71*	*291.2*	1.25	0.61

Table 2.
Performance of early and medium maturing cowpea varieties evaluated in two locations.

depending on duration of rainfall or irrigation. Under irrigation production, the crops needs propping of the vines to raise the pods off from the ground to reduce damage during harvesting or damage by soil borne diseases. Fresh pod yield can vary from 5 to 8 tons ha^{-1}.

Variety	Grain yield kg ha^{-1}	Fodder Yield kg ha^{-1}	Maturity (days)	100 seed weight (g)	Harvest index
TVU 5138	2799	5529	99.25	20.96	0.506
Bechuana white	2384	5520	103.50	14.74	0.441
TVU 8464	2010	3669	97.50	14.09	0.541
TVU 13004	1993	5101	103.75	15.26	0.410
TVU 14190	1969	4912	99.50	18.71	0.429
TVU 8016	1960	5466	95.00	18.43	0.363
TVU 2095	1639	4202	104.25	18.16	0.402
TVU 5146	1610	4311	101.75	20.42	0.387
TVU 3416	1541	6286	110.25	14.27	0.241
GLENDA	1496	4293	103.50	12.50	0.362
TVU 3391	1419	5862	112.25	12.09	0.248
TVU 13932	979	6444	144.25	16.52	0.164
TVU 1836	694	2350	103.00	14.52	0.179
TVU 9671	512	2532	109.25	19.50	0.206
P Level ($P < 0.05$)	0.001	0.002	0.001	0.001	0.001

Table 3.
Yield of dual-purpose cowpea varieties.

Variety	Grain yield (Kg ha –1)	Fodder yield	100 seed weight (g)	maturity (days)
6--1--1	2610.1b	8296.4a	22.67a	95.0b
6--4--1	3039.9a	5036.8c	19.29ab	95.0b
6--4--4	3059.0a	6222.2ab	19.25ab	96.0b
6—2—1	1895.1d	2601.3e	19.08c	93.0b
6—2—2	2142.6c	2800.1d	19.89ab	95.0b
6—3—1	2080.0c	3021.98d	19.20c	91.0b
JanaFod	1650.34e	6124.67ab	11.02 d	118a
P Level (P < 0.05)	0.001	0.0001	0.002	0.04

Early maturity = 80–90 days, Medium maturity 91–100 days, Late maturity = >101 days.

Table 4.
Yield of some advanced medium maturity elite cowpea breeding lines.

Vegetable varieties offer a regular source of income to farmers as they have easy buyers of their produce in the rural markets who sell the green pods with other leafy vegetables, spinach and lettuce. The adoption of these varieties will increase family intake and improve their diet and nutrition reduce malnutrition in rural communities [6, 7].

3.5 Development of cowpea varieties for intercropping

Intercropping is an integral part of cropping system in many provinces of South Africa particularly by smallholder farmers where communal land is limiting and drought-prone. In South Africa, many small scale farmers practice intensive crop production to reduce the risk of crop failure and maximize profit per unit area [6]. Additional reason for intercropping is to reduce pest

Figure 6.
Four advanced vegetable cowpea types with long pods ranging from 30 - 75 cm.

incidence. Specific varieties were developed with particular plant architecture and canopy structure designed for alternate row and double row cereal-cowpea intercropping system for maximization of land equivalent ratio (LER). Cowpea with narrow leaf blade and canopy width has been developed for alternate row intercropping system with cereals (**Figures 7** and **8**). They require reduced inter- and intra-row spacing for maximization of plant density per hectare. The varieties developed for intercropping particularly the early maturing varieties can always fit in any intercropping system. The implication of this is that the varieties increase the productivity and profitability of the farmers using poor marginal soils (**Figure 8**) [8, 14].

3.6 Development of cowpea varieties with combined pest resistance

One of the ways to reduce over dependence on chemical spray for the control of prevailing pest spectrum of cowpea is to develop varieties with increased host plant resistance. This has been achieved by deploying varieties with combined insect pest resistance as breeding parents to develop new germplasm with medium to high resistance to different insect pests (**Table 1**). The promising genotypes were screened for resistance for each insect pest for confirmation of resistance using appropriate screening technique [22, 23]. The elite lines with combined pest resistance have the advantage of requiring minimum insect spray. This will ultimately reduce production cost and increase the profit margin of the farmers.

3.7 Development of late maturing varieties (101: 120 days)

Few varieties were developed for late maturity. They are photosensitive and suitable for fodder and leafy vegetables (**Table 5** and **Figure 3**). The fodder yield is very high with low grain yield. Some varieties if planted in October/November may not produce seed until the month of March when day length is shorter. To produce seed of such varieties, planting should be done during the first week of January while for fodder production planting can be done in the months of October or November. JanaFod is one of the late maturing varieties (**Table 6** and **Figure 3**) developed for fodder and could produce 6000 kg ha^{-1} of haulm [2]. The advantage of producing late maturing varieties is that it will enhance hay/fodder production particularly by commercial farmers who can use irrigation in their production system. The fodder

Figure 7.
Narrow leaf cowpea types developed for high density monocropping and intercropping.

Figure 8.
Cowpea shows its ability to meet its nitrogen requirement as compared to maize.

produced can be bailed and sold to other farmers during offseason or farmers who cannot produce fodder for their animals. The fodder production from the developed varieties will enhance feed security for livestock industry in South Africa.

3.8 Development of cowpea varieties for quality traits

Important quality traits apart from the nutrient elements addressed through breeding programme include, seed coat color, texture and size. These traits influence consumer preferences and demand pull [24]. Fortification of the varieties with nutrients such as protein, zinc and iron is an integral part of our breeding activities. Elite varieties are subjected to nutrient analyses in search of varieties with higher nutrient contents to be used as breeding parents (**Tables 7** and **8**). To meet the needs of consumers, different seed coat, and eye colors (**Figure 9**) with different

Variety	Grain yield kg/ha	Fodder yield kg/ha	Maturity	100-Seed weight (g)	Harvest index
TVu 3310	3947	21,293	140.937	10.30	0.1539
TVu 13,437	525	13,524	123.604	11.93	0.0560
TVu 1878	2371	10,584	134.270	17.41	0.2235
TVu 7530	2076	6255	89.134	14.33	0.4315
TVu 11,955	2220	6065	92.937	14.30	0.4213
TVu 1645	3170	6026	94.604	9.49	0.6635
TVu 1979	1940	5966	92.937	11.85	0.3352
Bechuana white	2223	5005	103.270	14.88	0.4424
TVu 13,953	127	4669	136.937	*	0.0215
TVu 14,719	2424	4323	98.604	12.71	0.5576
IT00K-1060	1110	4280	103.937	18.42	0.2942
TVu 7757	1886	3053	88.604	11.16	0.6177
Glenda	1179	3019	106.937	13.72	0.4033
P-level ($P < 0.05$)	0.010	0.01	0.001	0.001	0.002

Table 5.
Performance of fodder cowpea varieties evaluated at Taung.

Variety	Fresh pod weight	Maturity	100-seed
	Kg ha^{-1}	(days)	weight (g)
TVu 1916	5143a	99b	13
TVu 1727	2743b	106a	18.4a
TVu 15654	2183c	89c	18a
TVu 6439	1888d	96b	15.3d
TVu 14868	1303e	92c	16.1c
Tvu 14,868	1232f	90c	16c
TVu 2852	825 g	98b	15d
TVu 6477	721 g	107a	14.4e
TVu 14861	684 h	92c	15.8d
P Level (P < 0.05)	0.001	0.001	0.001

Table 6.
Performance of vegetable cowpea varieties.

coat textures (smooth, rough and wrinkled) were developed. Consumers' feedback suggests that rough and wrinkled seeds cook faster because they imbibe water faster during cooking as compared to smooth–coated varieties. These quality traits were achieved by crossing parents with different coat colors, eye colors and seed coat textures as well as seed size. The variation in nutrient content indicates variation in the genetic makeup of the varieties. The fortification of the varieties with nutrients especially zinc and iron will enhance the nutrition of the consumers and in addition, it offers opportunity for the varieties to be used for further crop improvement to generate new genotypes with higher nutrient contents. The different quality traits exhibited by the varieties give the farmers the opportunity to make choice and select their preferred varieties. This will improve their intake and nutrition and reduce malnutrition [7]. The availability of the varieties will enhance food security and nutrition in South Africa.

3.9 Cowpea varieties registered and released

As many genotypes are in the pipeline of development and selection, some of the advanced breeding lines that have been test in multiple locations and seasons were submitted for registration with the intension to release them for commercialization. In the light of this, four cowpea varieties have been registered for a release at the National Department of Agriculture (DAFF), Genetic Resources, Pretoria. The varieties are:

1. JanaFod (ARC-09-001, ZA 20125043) cream cowpea

2. ARC-GCI-CP76 (VL 2009/7536) brown cowpea

3. UL-589 (VL 2017/10266) white cowpea

4. UL-1010 (VL 2017/10267) white cowpea

In addition, six early/medium maturity cowpea varieties have been submitted for registration at the National Department of Agriculture (DAFF), Genetic Resources, Pretoria and they include

Genotypes	Maturity days	Maturity periods	Seed weight	Seed size	Seed color	Eye color	Coat Texture
IT98K-962 X IT97K-499-35	94	Early	20.46	Large	White	Black	Wrinkled
IT98K-962 X IT98K-205-8	91	Early	18.30	Large	White	Black	Wrinkled
IT98K-962 X TVX 3236	96	Early	18.61	Large	White	Brown	Wrinkled
IT97K-497-2 X IT98K-962	93	Early	22.70	Large	White	Black	Rough
IT97K-497-2 X Oloyin	95	Early	18.60	Large	Cream	Brown	Smooth
IT97K-497-2 X IT82D-889	95	Early	20.52	Large	Brown	Brown	Smooth
IT00K-1217 X IT98K-962	96	Early	22.08	Large	White	Black	Smooth
IT98K- 205-8 X Oloyin	95	Early	19.28	Large	Brown	Black	Rough
IT98K-205-8 X IT98K-406-2	89	Early	19.39	Large	White	Black	Rough
IT90K-76 X Oloyin	94	Early	21.86	Large	White	Brown	Wrinkled
BW (Local check)	95	Early	15.67	Medium	White	Gray	Smooth

Early maturity = 80–90 days, Medium maturity 91–100 days, Late maturity = >101 days. Large seed = above 18 g, Medium size = 12–18 g.

Table 7.
Some quality traits (seed size, seed color, eye color and coat texture) of elite cowpea breeding lines.

Variety	CP (%)	Zn (ppm)	Fe (ppm)
Bechuana W.	20.30a	16.55abc	49.95abc
Glenda	24.70a	36.73abc	79.43abc
IT00K-1060	25.72a	36.50abc	107.08abc
IT00K-1263	25.26a	26.88abc	94.65abc
IT84S-2246-4	24.35a	18.05abc	123.30abc
IT86D-1010	19.03ab	59.57ab	145.77ab
IT86D-719	24.05a	59.75ab	150.55a
IT95K-1156-3	25.00a	38.80abc	113.70abc
IT95 K-1491	27.05a	42.35abc	108.60abc
IT97K 390–2	25.45a	60.45a	121.55abc
IT98K-1105	25.30a	15.75abc	47.35abc
IT98K-463-6	29.85a	17.13abc	55.67abc
IT98 K-530-1	23.23a	34.00abc	133.80abc
IT98K-690	26.63a	30.97abc	130.03abc
IT99K-316-2	22.25a	44.20abc	110.65abc
IT99 K-494-6	26.60a	46.3abc	94.33abc

Variety	CP (%)	Zn (ppm)	Fe (ppm)
IT99K-529-1	27.90a	18.60abc	47.20abc
JanaFod	26.40a	39.40abc	20.00c
TVu 13464	21.25a	38.55abc	108.45abc
P-Level	0,04	0,04	0,03

Table 8.
Nutrient contents of improved cowpea varieties.

Figure 9.
Different seed coat colors bred for south African consumers.

1. UL-11
2. UL-12
3. UL-13
4. UL-14
5. UL-15
6. UL-16

These varieties upon registration and release will enhance the food and nutrition security of people in South Africa. Farmers will have seed of improved and pure varieties available to plant, and as they cultivate these varieties their profit margin will increase with better nutrition. This will also create jobs for all the value chain in cowpea production [7].

4. Conclusions

Cowpea production in South Africa is limited by lack of improved varieties that exhibit good agronomic traits and pest resistance. In the last decade and a half, significant breeding efforts as shown in the results of this study have attained great achievements in cowpea improvement to address the limitations in cowpea production. Several elite cowpea genotypes in the pipeline of development have been achieved, varieties that exhibit good agronomic and quality traits to enhance intake and nutrition in the rural communities have been developed 10 genotypes expressed high grain yield with combined pest resistance (aphids, bruchid, virus, leafhopper and bacterial blight). The implication of this achievement is that farmers can attain more grain yield per unit land area. In addition, the cultivation of these genotypes will reduce the cost of pest control and increase the profit margin of the farmers. Another important achievement of the study is that four elite varieties (JanaFod, ARC-GCI-CP76, UL-58 and UL-1010) have been registered while six varieties (UL-11, UL-12, UL-13, UL-14, UL-15 and UL-16) have been submitted for registration. The future activity is to commercialize the varieties to enhance uptake and availability of seeds to seed growers, farmers and consumers. The availability of seeds of these varieties will increase cultivation by farmers, enhance food security and nutrition and reduce malnutrition in South Africa. Since breeding is a continuous process, some of the varieties and other promising genotypes will be used through recurrent selection to develop new germplasm that are more adapted to the region as well as being climate smart.

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Chapter 3

Genetic Transformation in Agro-Economically Important Legumes

Esmerald Khomotso Michel Sehaole

Abstract

Over the past few years, many cultivated plants have been under scrutiny for their potential role in economic, agroecological, nutritional, and scientific innovation sectors, especially in various developing countries. This was aimed to identify plants that have the potential to alleviate food insecurity, improve agroecosystems while benefiting the producers financially as well. Such important crops have been studied and are continuously undergoing improvements to produce cultivars that confer biotic and abiotic stress tolerance, enhanced shelf-life, nutritional quality, and environmental benefits. This chapter reviews the benefits provided by globally cultivated legumes, the challenges faced during their propagation, the methods used to enhance these crops, and the constraints they undergo during genetic improvement. It further analyses the strategies that have been employed thus far to optimise genetic transformation.

Keywords: leguminous crops, transgenes, optimisation, gene transfer, transformants

1. Introduction

For over 2 decades now, genetic transformation has been an ongoing method explored to improve various kinds of plants for nutritional quality, enhanced field performance, and yield. Amongst plant groups that have been extensively employed for this purpose is the family *Leguminosae* which includes grain, forage, and miscellaneous legumes [1]. The legume family, Fabaceae, houses within it 20,000 species, which makes it the third-largest family of Angiosperms and the second-largest family of domesticated plants [1–3]. The species of plants found in this family range from herbs, climbers, tree species as well as shrubs of which only 11 species are globally cultivated for various uses [3, 4].

Amongst the vast array of legume species identified thus far, there are several which are classified as important crops because of the role they play in subsistence farming and agroeconomic commercialisation. They include chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.), cowpea [*Vigna anguiculata* (L.) Walp.], faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.), pea (*Pisum sativum* L.), peanut (*Arachis hypogaea*), pigeon pea [*Cajanus cajan* (L.) Millsp.], and soybean [*Glycine max* (L.) Merril] [3, 5–9]. These grain legumes are said to play an imperative role in nutritional and food security as a result of their inexpensive cultivation and amenable cropping systems for household farming [4].

Amongst them, there are legume species that have been employed as model systems, i.e., barrel medic (*Medicago truncatula* Gaertn.), *Lotus japonicus,* and in some instances soybean, whose role in legume research has proven beyond valuable [3, 5]. Their economic importance and intrinsic characteristics have been the main drivers behind their use in studying leguminous plants, through the use of genomic technologies and comparative gene mapping studies [10].

The continued studies on globally cultivated legumes are mainly driven by their imperative benefits to the environment, human and animal health as well as in the economic growth of the countries that produce them commercially [3]. This is largely attributed to the myriad nutritional components which make up the different legume species. They are rich in proteins, dietary fibre, carbohydrates, essential mineral nutrients, phytochemicals, and vegetable oil (in oilseed legumes) and consist of a relatively low lipid content [11–13]. Furthermore, legumes consist of high concentrations of antioxidants, isoflavones and are widely renowned for their low glycaemic index (GI). As a result, they provide various health benefits to both humans and animals through the prevention, reduction, or alleviation of various diseases [3].

1.1 Domestic benefits of important agroeconomic legumes

Amongst other legume crops, cowpea, soybean, and faba bean have been used domestically over a number of years as staple foods, vegetables, and major constituents of plant-based diets, thus providing an affordable protein source [5, 7, 14, 15]. They have also been utilised indigenously to make legume flour, which is used to make many traditional dishes in various rural communities. These nutritious pulses and oilseeds form part of myriad healthy eating plans including '...the Mediterranean style of eating, the DASH eating plan, vegetarian and vegan diets and lower-glycaemic-index (GI) diets...', as mentioned in Polak et al. [16]. The flexibility of these crops to blend in a range of eating plans is a result of the essential minerals found in them, necessary for the metabolic pathways taking place within the human body.

Other legumes, such as alfalfa (*Medicago sativa* L.) and trefoil (*Trifolium* spp.), serve as major sources of feed, especially in temperate regions along with *Vachellia* spp. and *Leucaena* spp., which have also been used as feed for livestock in various sub-Saharan countries [3, 5]. As mentioned above that legumes range between various plant types, legume trees are also explored as sources of timber, expensive woods, and lumber in tropical areas and as additional feed in arid environments [3].

1.2 Nutritional benefits

As a result of the high protein content of legumes, they are potentially able to eradicate malnutrition and decrease the rising rate of poverty in developing countries [1, 4, 5]. They offer an affordable yet nutritional source of protein to rural communities, which are said to be the hardest hit by protein-energy malnutrition (PEM) [13]. Legumes also consist of biologically active molecules that scavenge unstable oxygen radicals (ROS), antioxidants, which are suggested to greatly contribute to the prevention of various types of cancers, heart-related and other neurodegenerative diseases [11].

Additionally, legumes have a hypoglycaemic effect which reduces blood glucose levels. Consequently, this decreases the levels of insulin in the blood, making legumes suitable for daily dietary intake in diabetics [3, 16]. Foyer et al. [11] further mention that the inclusion of legumes in daily diet has been proven to significantly reduce mortality, therefore emphasising the benefits provided by these crops to

the human body. The anticarcinogenic properties of legumes are attributed to isoflavones, which are phytonutrients that mimic oestrogen properties and are said to hold great potential for the production of plant antibodies (plantibodies) and vaccines, that protect against microbial infection [17, 18].

Lastly, legumes are rich in micronutrients, such as calcium, chromium, copper, iron, selenium, and zinc. These mineral nutrients are important components of enzymes and antioxidants, macro- and micro-nutrient metabolism, synthesis processes as well as plasma membrane stabilisation [3, 4]. These nutrients therefore make legumes unique in the important role they play, not only in human and animal nutrition, but in the environment as well.

1.3 Agricultural and environmental benefits

One of the major benefits of leguminous plants is their ability to fix atmospheric nitrogen into bioavailable forms through their symbiosis with nitrogen-fixing microorganisms called diazotrophs [5, 7, 12]. This occurs in nodules formed on legume roots. The unique legume-diazotroph relationship enables the conversion of free nitrogen gas (N_2) from the air into ammonia (NH_3), which can either be incorporated into the plant's protein synthesis pathway or be used by nitrogen-deficient plants as an alternative source. Because this process avails biologically active nitrogen (N) to the ecosystem, it acts as an alternative source of nitrogen to plants grown in areas of limited soil nitrogen [3].

Tran and Nguyen [3] highlighted that this symbiosis has a dual effect, where it reduces the cost of nitrogen fertiliser and confers an effective, biological mechanism of environmental nitrogen control, thus reducing air pollution. For this reason, legume crops are considered to offer both sustainability in farming systems and efficient scavenging of atmospheric nitrogen. In this way, it benefits both the economy, through reduction of fertiliser costs and the environment, by recycling N, which would otherwise contribute to climate change if not effectively managed [19].

Pulse legumes are suggested to be important components in cropping systems, such as intercropping, crop rotation, and agroforestry systems, because of their ability to increase biological diversity [5, 12, 20]. Such multiple cropping methods are said to enable minimal resource utilisation, multiply yield and reduce the possibility of crop failure. Furthermore, deep-rooted grain legumes such as pigeon pea and Bambara bean tend to provide more benefits to their companion crops, which directly impacts crop success in the field and ultimately contributes to food security [5].

1.4 Commercial and industrial benefits

Legumes are not only used for pharmaceutical and domestic purposes but they, along with their derivatives, have tremendous importance in the production of commercial and industrial products. MaClean et al. [12] mention that cowpea has potential uses in the textile and cosmetic industry because of its richness in B-vitamins, various mineral elements, and lysine. Furthermore, legumes, such as lentils, soybean, and peas (*P. sativum*) along with lucerne, have been extensively employed in industries for the production of ethanol-biofuel and oil derivatives, such as biolubricants [2, 21]. They have been extensively explored in the industrial production of biodegradable products, such as dyes, inks, and plastic [3].

1.5 Challenges associated with conventional legume cultivation

As mentioned above, legumes constitute some of the highly domesticated species, produced for various purposes. With the continuously increasing human

population, there is an associated increased demand in the production of food crops to counteract food insecurity [22]. Unfortunately, the problems facing legume agriculture are becoming exacerbated, not only by the consequences of climate change but also through various anthropological activities that continue to rise as a result of population expansion and industrial revolution [23].

Rainfall has become unpredictable in terms of both intensity and seasonality, temperatures have drastically increased, and pest outbreaks are becoming more and more severe [14]. On the other hand, land degradation, industrialisation, deforestation, and the use of agrochemicals become perpetuated to accommodate human populations that have settled into the natural environment [23]. Consequently, there is a decline in soil fertility, water, and nutrient availability, which ends up severely affecting legume production and yield [24]. The resultant reduction in biomass and crop losses tend to result in the production of low-quality plants which are either diseased or are unable to survive long periods of storage [24–26].

On its own, climate change continues to threaten the metabolic productivity of legumes and other equally important crops. Problems, such as biological invasion at planting fields, have become exacerbated, leading to the infection of legume plants by bacterial, viral, fungal, and insect pathogens [27–29]. These pathogens cause diseases, such as wilt and blight, which have a negative impact on the production of quality crops. Mangena [14] mentions that because of the sessile nature of plants, they are unable to evade the environmental fluctuations in their ecosystems, such as temperature extremes, harmful ultraviolet radiation, soil salinity, prolonged drought periods, and pest outbreaks. As a result, they have evolved innate survival mechanisms, such as physical (e.g., spines and thorns on branches) and chemical defences (e.g., production of protease inhibitors and lectins), which protect the plants' biosynthetic machinery from damage [27, 29]. Although these defence mechanisms protect the crops throughout their life cycles, the severity of environmental conditions renders them ineffective to a certain extent.

2. Conventional breeding of important leguminous crops

A vast array of traditional methods has been explored to optimise the performance of legumes under environmental fluctuations in their planting fields. Inoculation of the soil with arbuscular mycorrhizal (AM) fungi, growth-promoting microbes as well as rhizobial communities have been utilised to improve micronutrient availability, growth, and development of the crops, to enhance nodulation and subsequently, nitrogen fixation [30, 31]. Other traditional methods, some of which are still being applied to date, including the optimisation of cropping systems, have also been proven to play an imperative role in the propagation of stress-tolerant crops [18].

The complexity of some legume genomes has led to the development of many high-throughput conventional systems of propagation, which have also shown great importance. Amongst others, the methods employed include traditional backcrossing, mutation breeding, pedigree breeding, single pod and single seed descent (SPD and SSD), bulk-population method, hybridisation, and polyploidisation breeding [32–35]. One of the widely explored conventional improvement techniques is biofortification. As described by World Health Organisation [36], biofortification is a method of crop improvement that focuses on enhancing the nutritional content of crops using either traditional breeding, agronomic or classical breeding approaches. It differs from conventional fortification in that the methods are used to target the gene level for enhancement so the plant may express desired genes during growth and development [36].

However, due to the limiting properties of the crops, such as self-pollination, recalcitrance, and narrow gene pool, the success of conventional improvement programmes has been limited [1]. This results in sexual incompatibility between most potential hybridisations, which ends up restricting traditional breeding methods from expanding the gene pool of wild relatives, from which new cultivars can be developed [37]. Another limiting factor of traditional approaches pointed out by Jha and Warkentin [38] and Hefferon [39] is environmental harm as a result of regular applications of fertilisers. This can have a direct negative effect on the availability of other nutrients in the soil, ultimately leading to deficiencies. Other problems include the sensitivity exhibited by some crops to certain minerals, difficulty in targeting and mobilising some minerals to certain edible plant organs as well as the inability to cater for *de novo* synthesised bioactive molecules [39].

3. Recombinant DNA technology employed in legume transformation

To overcome the constraints faced by conventional methods of legume improvement, biotechnologists have over the years devised ways to improve the qualities of these crops using molecular breeding approaches [8, 25, 40, 41]. The various methods employed in recombinant DNA technology for the enhancement of legume qualities are summarised in **Table 1**. These methods have enabled biotechnologists to overexpress, downregulate, or suppress the expression of target genes in the genomes of various legume species. *M. truncatula* and *Lotus japonicus* have played an imperative role in this regard, by providing model systems through which complex plant biochemical pathways can be extensively studied and manipulated using genetic transformation [57]. These model systems exhibit unparalleled amenability to genetic transformation as a result of their relatively small genome sizes (approximately 550 Mbp), short life cycles, and their ability to grow easily under variable environmental conditions [10].

3.1 RNA interference

RNA interference (RNAi) is described as a mechanism of gene silencing that employs the incorporation of sense or antisense RNA into a host plant's genome to silence the expression of a gene or a family of genes and down-regulate antinutrients, allergens, and toxins [3, 39]. This method employs a mechanism of RNA degradation by the host plants' biosynthetic machinery, i.e., micro-RNA (miRNA), small interfering RNA (siRNA), and endoribonucleases called Dicer [58]. Cleavage of double-stranded RNA and subsequent degradation occurs through a multiprotein complex called the RNA-induced silencing complex (RISC). This complex is formed by a ribonucleoprotein and a single strand of siRNA or miRNA that acts as a template of the mRNA complement [58, 59]. In plants, this naturally occurs to regulate gene expression as well as to defend the plant against viral pathogens, transposons, and foreign genetic material [58].

According to Nahid et al. [58], RNAi is now widely explored to confer resistance in legumes against viral pathogens, although in some families of viruses, i.e., *Geminivirida*e which are pathogens of various higher plants in temperate areas, its efficacy remains questionable. However, Ahmad and Mukhtar [60] suggest that the same viruses are currently being explored as vectors for virus-induced gene silencing (VIGS) as well as for studies of viral gene function and replication in plants. An example of RNAi-induced gene silencing has been exhibited in *M. truncatula* using the protocol by Floss et al. [59]. It can also be exemplified by the silencing of the p34 protein, which is a major allergen in soybean [61].

Legume	Explant tissues	Transgenes	Technique of transformation	Transformation response	Reference
Grain legumes					
Glycine max (L.) Merril	Callus tissue from cotyledonary nodes	*Cry8*-like gene from *Bacillus thuringiensis* (*Bt*)	*Agrobacterium*-mediated gene transfer	Stable integration of the gene was confirmed by Southern hybridisation, indicating a 92% higher survival rate in transgenic plantlets when exposed to the pest *Holotrichia parallela*. Increased mortality rate, deformed larvae and growth inhibition of the pest were also reported	Qin et al. [42]
	Half-seed explants	*Gus* and *aadA* selectable marker genes	*Agrobacterium*-mediated gene transfer	Transformation efficiency was 3.8% and the transgene was confirmed in the T1 progeny using phenotypic analysis and Southern blotting	Paz et al. [43]
	Protoplasts isolated from juvenile leaf tissue	E1-GFP-encoding gene (p2GWF7-E1 gene construct)	Protoplast-mediated gene transfer	Relatively high transformation efficacy	Wu and Hanzawa [44]
	Cotyledonary node tissue	GsWRKY20 gene from *G. soja* and glufosinate selectable marker gene	*Agrobacterium*-mediated gene transfer	Glufosinate selection and RT-qPCR were used to confirm positive gene integration. When the transformants were exposed to drought conditions in the field they exhibited enhanced drought tolerance	Ning et al. [45]
Phaseolus vulgaris L.	Leaf primordia	*Gus* reporter, *bar* selectable marker and *HVA1* drought tolerance genes	Particle bombardment	Putative transformants were confirmed using PCR and Northern hybridisation. Transformation efficiency was variable for each cultivar but highest on day 15 after the bombardment at >80%	Kwapata et al. [46]
Vicia faba L.	Leaf tissue	Genes encoding green fluorescent protein (GFP) and necrosis- and ethylene-inducing peptide (Nep1)-like protein (NLP)	*In planta Agrobacterium* infiltration-mediated gene transfer (*Agro*-infiltration)	Transient expression of GFP was confirmed using confocal microscopy and found to be high.	Debler et al. [47]

Legume	Explant tissues	Transgenes	Technique of transformation	Transformation response	Reference
Vigna anguiculata (L.) Walp	Embryo tissue explants	*AtUBQ3pro:ZsGreen* reporter gene	*In planta Agrobacterium* infiltration-mediated gene transfer (*Agro*-infiltration)	Transformation efficiency was 3.9% but no reports on the transfer of the transgene to the progeny	Citadin et al. [48]
	Cotyledonary node segment	α-amylase inhibitor-1 gene	*Agrobacterium*-mediated gene transfer	Transgene transmitted to progeny with 1.67% transformation efficiency	Citadin et al. [48]
	Root tissue	*CRISPR-Cas9* gene construct	Genome editing using *A. rhizogenes*	Hairy root induction was induced at approximately 67% efficiency and the transformants were confirmed using fluorescence under a light microscope and PCR quantification	Ji et al. [49]
	Shoot apical meristems	*Gus* reporter gene	Biolistics method (Gene gun)	0.9% transformation with confirmed transgenic progeny	Citadin et al. [48]
Lens culinaris Medik.	Cotyledon with embryo axis	*Gus* reporter and *hpt* selectable marker genes	*Agrobacterium*-mediated gene transfer	Putatively transformed shoots confirmed by *gus* analysis, transgenes confirmed by PCR	Tavallaie et al. [50]
Pisum sativum L.	Leaf tissue	Genes encoding green fluorescent protein (GFP) and necrosis- and ethylene-inducing peptide (Nep1)-like protein (NLP)	*In planta Agrobacterium* infiltration-mediated gene transfer (*Agro*-infiltration)	Transient expression of GFP was confirmed using confocal microscopy at high efficiency. The irregularly shaped epidermal cells were shown to be more amenable to transformation	Debler et al. [47]
Cicer arietinum	Single cotyledonary node explants	*pOpt-EBX 35S::uidA 35S::NPT II* gene construct	*Agrobacterium*-mediated gene transfer	PCR screening confirmed putative transformants, with the transformation and regeneration efficiencies being highest when the explants are subjected to micro-injury and grown under LED light	Bhowmick et al. [51]
Arachis hypogaea	De-embryonated cotyledon (half-seed explant)	*Gus* reporter and *hptII* selectable marker genes	*Agrobacterium*-mediated gene transfer	85% transformation efficiency with vigorous regeneration in putatively transformed plantlets. Confirmation of putative transformants was done using PCR, RT-PCR, Southern hybridisation and GUS histochemical analysis	Tiwari et al. [52]

Legume	Explant tissues	Transgenes	Technique of transformation	Transformation response	Reference
Forage legumes					
Stylosanthes guianensis (Aubl.) Sw.	Cotyledon protoplasts	*hpt II* selectable marker gene, GUS reporter gene (*uidA*) and *mgfp5* (green fluorescent protein, GFP)	Electroporation-mediated gene transfer	Transformation efficiency was higher when a higher electric charge was applied on the protoplast explants. For the reporter gene, stronger electric pulses induced membrane damage while less intense charge could not enhance reporter gene expression	Quecini et al. [53]
Model legumes					
Medicago truncatula	Root protoplasts	35S::SYMRK-GFP and 35S::ERN1-GFP gene constructs	Protoplast-mediated gene transfer	Protoplast viability was relatively high, and the transformation efficiency was 62.4% on average.	Jia et al. [54]
Lotus japonicus	Root protoplasts	35S::SYMRK-GFP and 35S::ERN1-GFP gene constructs	Protoplast-mediated gene transfer	Localised GFP expression was confirmed in the cytoplasm and the nucleus of the root protoplasts. Also, the SYMRK and ERN1 genes were detected in the plasma membrane and nuclei of root protoplasts, respectively. Transformation efficiency was 63.3% on average	Jia et al. [54]
	Somatic embryogenic callus	*Hyg* selective maker gene	*Agrobacterium*-mediated gene transfer	TDZ-induced somatic embryos reported as highly regenerable and through a repetition of somatic embryogenesis transformation cycles, the production of chimeras was reduced	Barbulova et al. [55]
	Callus tissue from root and shoot segments	Carotenoid cleavage dioxygenase 7 (LjCCD7) silencing gene	RNA interference (RNAi)	RT-qPCR was used for protein quantification and confirmed decreased expression of the gene construct following transformation. The transformants further showed varied phenotypic responses as compared to non-transformed hosts, i.e. height reduction, increased biomass, elongated primary roots and increased branching	Liu et al. [56]

Table 1.
Transgenic properties introduced by molecular breeding in major legumes.

3.2 Mutation breeding

Mutation breeding is defined as an induced change in the nucleotide sequence of plants for genetic improvement purposes, especially in self-pollinating plants [62]. It can be induced through the use of chemical, physical or biological mutagens to confer disease resistance as well as to improve yield and morphophysiological properties in agronomically important legumes [63]. Ionising radiations, such as gamma and X-rays, are the most preferred physical mutagenic agents as they yield reproducible, easily applicable, and high mutation properties, although ultraviolet (UV) radiation has previously been used as well [63, 64]. The most commonly used chemical mutagens include base analogues, antibiotics, alkylating agents, hydroxylamine, and nitrous acids, for example, ethyl methane sulphonate (EMS), diethyl sulphate (DES), and methyl nitrosourea (MNU), amongst others [62, 64, 65].

Although it is an inexpensive procedure that has high efficacy and yield, acquiring the desired mutation from a mutagenesis event can be difficult to achieve sometimes [62]. This is potentially attributed to the use of physical and chemical mutagens, which as explained by Wang et al. [57], typically results in '...genome-wide random DNA alterations'. However, it has been widely used to develop important cultivars and varieties of legumes mainly in Asia which accounts for 60% of the total legume mutant production, Europe (30%), and North America (6%) [63]. Progress in legume mutation breeding is discussed in detail by Suresh and Kumar [63] and Kumar et al. [65] for induced mutagenesis in chickpea.

Another way in which mutations can be induced in legumes is through transposon-based mutagenesis [57]. This is achieved by incorporating a transposable sequence into a binary vector, which is then introduced into the genome of a legume host using *Agrobacterium*-mediated genetic transformation. The method was investigated in barrel medic, *L. japonicus* as well as in soybean and was reported as successful [57].

3.3 Genome editing

Genome editing is a technique of molecular breeding that involves targeting and using exogenously applied restriction enzymes, known as endonucleases, to alter specific genetic sequences of the plant genome [66]. The technique involves three widely applied nucleases, i.e., zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeat CRISPR-associated protein 9 nuclease (CRISPR/Cas9). The latter two are mostly used and regarded as the most versatile during application. The endonucleases recognise specific domains in the genome sequence and use that as the cleavage site [49].

The model legumes, soybean, and vetch (*Aeschynomene evenia*) are amongst plants that have been successfully transformed using this method [57]. Wang et al. [57] and Kankanala et al. [67] mention that the first application of the CRISPR/Cas9 technology was done using *Agrobacterium rhizogenes*, which resulted in the successful editing of both exogenous and endogenous DNA sequences. Explants that have been used for this purpose include callus tissue, leaf discs, flower tissue, and protoplasts, all of which are said to enable inheritance of the edited genome by the progeny, i.e., lead to stable transformation [66]. Another example of legume genome editing was reported by Ji et al. [49], where cowpea was effectively mutated at approximately 67% efficiency using the CRISPR/Cas9 and *A. rhizogenes* method.

3.4 Direct gene transfer methods

3.4.1 Particle bombardment

Amongst the methods which are used to transfer transgenes between organisms is particle bombardment which was initially used to develop the first transgenic soybean. It is also referred to as biolistics (short for biological ballistics) and involves the direct transfer of DNA-coated particles into semi-permeabilised host cells using high-speed propulsion [68]. It was used over 2 decades ago to develop the first transgenic crop called Roundup Ready and has continuously been used to transform various other plants [69]. Unlike *Agrobacterium*-mediated genetic transformation, this technique can be used to transfer transgenes to various host tissues, to transform the chloroplast genome and is said to have a broader host range [60]. However, it employs very expensive equipment and has limited efficacy.

3.4.2 Protoplast-mediated gene transfer

Gene transfer using protoplasts has also been explored to source explant tissues competent for DNA transfer [68]. This method employs the transfer of naked DNA treated using either polyethylene glycol (PEG) or electric current as the fusogenic agent. The use of electrofusion-mediated gene transfer remains preferred over polyethylene glycol treatment of protoplasts because of the higher success rates obtained in the former [70]. Although several chemical agents have been utilised during the procedure, the combination of PEG and divalent cations at alkaline pH has been extensively employed. This enables plasma membrane destabilisation and subsequently, DNA uptake which will further be incorporated into the host legume genome.

One of the major determinants of a successful gene transfer procedure is the availability of an efficient selection system [71]. Therefore, to select and identify transgenic hybrid cell lines generated from protoplast transformation, several methods have been employed. Selectable markers, such as antibiotic and herbicide resistance marker genes, growth morphology, vital staining using fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) as well as the molecular marker-based selection, are amongst the known selection systems used when working with somatic hybridisation of protoplasts [70].

However, there are several disadvantages associated with the protoplast method. Protoplasts are difficult to handle, the recovery of viable plantlets is poor in certain species of plants, the success of DNA integration is limited by rearrangement, and requires careful optimisation of culture media and culture conditions [68, 70]. Also, the rate of somaclonal variations generated from protoplast-mediated genetic transformation is highly increased.

3.4.3 Electroporation-mediated gene transfer and silicon carbide fibres

Another miscellaneous method used in the direct transfer of DNA to plants is electroporation-mediated genetic transformation, which employs the uptake of DNA through a semi-permeable plasma membrane by plant cells and protoplasts using an electric pulse [70]. Another method, silicon carbide fibres also known as whiskers, involves the treatment of explant material in a buffer solution that consists of DNA and silicon carbide fibres [68, 69]. Although it requires no complex or expensive equipment, the use of this method carries a danger posed by the fibres on human health, and thus requires careful handling by experienced personnel [69]. These approaches have provided some insights for modern biotechnology, i.e.,

elucidating gene function, gene over-expression and silencing, transposon-based mutagenesis, and other molecular-based studies [3].

3.5 *Agrobacterium*-mediated gene transfer

Agrobacterium-mediated gene transfer is now the mostly used procedure for genetic transformation in soybean, groundnut, common bean, and various other legumes [10, 41]. This technique capitalises on the pathogenicity of *Agrobacterium tumefaciens (*also known as *Rhizobium radiobacter),* which involves a complex system of virulence (*vir)* gene operons and virulence proteins (VirA-VirJ) that work synergistically to cause crown gall disease to the infected plant, to transfer desired genes into host tissues. The transgene of interest is incorporated into the T-DNA region of *A. tumefaciens* tumour-inducing plasmid, known as the Ti-plasmid, whose oncogenes (*auxA, auxB,* and *ipt*, encoding tryptophan monooxygenase, indole acetamide hydrolase, and isopentenyl transferase) have been deactivated [60, 68]. Another species of *Agrobacterium*, *A. rhizogenes* which causes hairy roots in dicotyledonous plants, has been used in transformation studies as well, mainly for functional genomic studies [60].

The global use of the *Agrobacterium* method is exemplified by the identification of molecular markers responsible for abiotic stressors, such as manganese toxicity, salinity stress, waterlogging, and phosphorus deficiency in soybean [72, 73]. Soybean crops have been improved to confer disease resistance such as bean pod mottle virus (BPMV) where the resistant cultivar expresses the capsid polyprotein from BPMV, *Sclerotinia sclerotiorum*, where the resistant cultivar expresses germin (gf-2.8) from wheat and *Heterodera glycines*, whose soybean resistant cultivar also expresses the chitinase gene from *Manduca sexta* [72].

Gene transfer mediated by *A. tumefaciens* can be done in *vitro,* where the explant tissues are imbibed in an infection inoculum containing the bacterium, followed by co-cultivation or *in vivo,* where the explant tissues become infiltrated with the infection inoculum (*Agro*-infiltration) [69]. Thus far, both methods have been extensively explored (**Table 1**), albeit with the respective challenges that come with each procedure. Although this technique has exhibited higher success rates in contrast to direct gene transfer methods of genetic modification, it also faces several challenges, which are discussed below.

4. Challenges encountered during gene transfer

While some methods are very effective and promising, there are shortcomings associated with each of the techniques. Direct gene transfer methods face a risk of transgene silencing as a result of spontaneous rearrangement that occurs during transfer. Moreover, the increased number of transgene copies in the host, which may be recognised as foreign genes by the plant may lead to transgene instability which results in low rates of transformation [68, 74]. Furthermore, Kohli et al. [75] and Tiwari et al. [52] highlight that the vector backbone may be incorporated into the host cells' genome along with the T-DNA, referred to as 'co-transfer of vector backbone sequences', which was previously only observed in microprojectile bombardment. This occurs as a result of ineffective backbone cleavage and may be encountered at very high rates [75]. In some instances, histochemical assays only confirm a low efficiency of transgene integration within the host plant, which ultimately limits the success of the method.

Molecular breeding employs various technological tools, some of which may be costly, time-consuming, and require complex equipment [5]. Because the

techniques used are artificially induced, the plants being transformed may exhibit unpredictable responses, such as the occurrence of somaclonal variations [76]. Such variations may be of physiological, genetic, or biochemical nature and although some may become interesting to a plant breeder, their occurrence is mostly unwanted and is therefore considered problematic.

The efficacy of *Agrobacterium*-mediated gene transfer is limited by the host-range restrictions of the bacterium towards a few specific genotypes [60]. It is further described that this host range limitation results in the method only being amenable to transform the nuclear genome, unlike in biolistics. The recalcitrant nature of various legumes and their narrow gene pool, such as in soybean greatly affects transformation and regeneration rates in specific genotypes, thus limiting the success of the technique.

Perhaps the most significant of these problems is the concern expressed by the general public regarding the safety of genetically modified (GM) crops, which not only negatively influences crop acceptance but eventually affects rapport between the co-farmers who produce them as well [5, 40]. The consumers are both concerned about the safety of consuming GM crops on their health and the environment. As a result, the use of crops with genetic modifications, especially through genetic transformation, continues to be challenged.

5. Optimising the techniques used in legume improvement programmes

In light of the problems facing genetic transformation procedures, it became imperative for plant biotechnologists to devise strategies of gradually improving the techniques, from which consistent, reproducible, and efficient protocols can be developed. This is continuously being explored through optimising the factors that affect each method of transformation, such as culture media supplements, *Agrobacterium* density and strains, the source and age of explant tissues, and ambient culture conditions [61, 69]. Thus far, there have been considerable improvements and it is evident that the constraints of legume genetic transformation can be greatly minimised and ultimately abated [71].

Atif et al. [77] and Christou [1] have reported that optimising conditions affecting the growth and development of soybean during *Agrobacterium*-mediated gene transfer has led to increases in transformation frequencies by about 16%. Systems, such as sonication-assisted *Agrobacterium* transformation (SAAT), have recently been introduced and are gaining popularity as methods of enhancing genetic transformation in legumes.

5.1 Refinement of culture media additives

Supplements included in culture media, for example, phytohormones, antioxidants, and antibiotics, play a vital role in the success of *in vitro* regenerated plants. According to Atif et al. [77] and Somers et al. [71], the inclusion of antioxidants, i.e., ascorbate, α-tocopherol, and glutathione, in co-cultivation media improves efficiencies of transformation by protecting the infected tissues from oxidative stress. Plant phenolics such as acetosyringone may be added to the infection inoculum to enhance *Agrobacterium* signalling to the wound site. Iron and copper chelators, as well as enzyme inhibitors, are also amongst the supplements which Newell-McGloughlin et al. [61] suggest including in culture media.

Co-cultivation is amongst the factors that have been emphasised to play a key role in genetic transformation experiments of various crops. Several studies have reported improved transformation efficiencies when co-cultivation was optimised.

These include studies by Liu et al. [78], Paz et al. [43] and Tiwari et al. [52] which optimised the concentrations of antioxidants, thiol compounds, and antibiotics included in co-cultivation culture medium. However, further optimisations conducted in other studies suggested that some constituents of the co-cultivation medium may play an inhibitory role on *in vitro* plant regeneration when applied at higher concentrations, for example, L-cysteine [2] and antibiotics [79]. Furthermore, Paz et al. [43] reported improved shoot formation irrespective of the inclusion of L-cysteine and dithiothreitol (DTT) in culture media. In a study by Zia et al. [80], infection efficiency was improved when the explants were imbibed in an *Agrobacterium* suspension for an hour, followed by a 5-day co-cultivation period while optimising antibiotic concentrations for each specific culture medium.

5.2 Optimisation of explants

The regenerability of explant tissues used for gene transfer greatly depends on the type of explant used and the physiological conditioning of the explant in time of culture, which subsequently influences the organogenic capability of the explants. In a review by Mariashibu et al. [37], different types of explant tissues utilised in the genetic transformation of soybean are discussed. This study elicits advances in the methods of regeneration that have been utilised since the production of the first transgenic soybean whose protocols primarily involve either shoot organogenesis or somatic embryogenesis. Although there are certain limitations, there has been a considerable improvement regarding the innovation of culture systems used in transformation studies.

Immature embryos, epicotyls, hypocotyls, primary leaf, stem-node, and cotyledonary node segments have all been used as explants of enhanced regenerability due to their totipotent nature [37]. Amongst them, cotyledonary nodes were found to be more efficient, in terms of the duration of growth, organogenesis, and response to the exogenous application of phytohormones [8, 43]. However, this regeneration system still requires the optimization of several growth parameters which influence the regeneration process so that the low frequencies may be overcome.

Zia et al. [80] investigated the use of half-seed explants while optimising the duration of co-cultivation and washing of infected explants. Additionally, the study explored various cultivars and the response of each to *Agro*-infection as well as the concentrations of antibiotics used during soybean transformation. This is mainly because antibiotics have been reported to negatively affect the organogenic capability of explants when used at supra-optimal concentrations [81]. Several studies have also reported on the efficiency of pre-priming treatments to enhance the physiological competence of the plant to *in vitro* regeneration, such as osmopriming, hydro- and halo-priming [82, 83], phytohormone pre-treatment [69, 84], and thermal treatment [82].

5.3 Increasing the affinity of host-pathogen interactions

The bacterial infection inoculum is another important factor when optimising genetic transformation. Newell-McGloughlin [61] suggested that *Agrobacterium* T-DNA delivery may be facilitated by eliminating factors that inhibit host-pathogen interactions after infecting the explants with *Agrobacterium*. However, it is imperative that the duration of explant exposure to conditions that enhance such interactions, be optimised so as to limit overgrowth of the bacterium and the eventual death of explants. Several studies have reported that using hypervirulent *A. tumefaciens* strains enhanced both T-DNA delivery and transformation efficiency [37, 71, 81].

In a study by Li et al. [85], a 96% infection rate and an 18% increase in the regeneration of successfully transformed soybean explants were reported in comparison with the frequencies recorded in the existing cotyledonary node protocol by Paz et al. [43] when bacterial density, bacterial suspension culture and the duration of co-cultivation were optimised.

5.4 Optimising selection and protein quantification systems

As Somers et al. [71] describe, an efficient selection system is necessary when conducting transformation because it enables a precise and reliable prediction of putatively transformed plantlets. In this way, the erroneous selection of escapes and chimeric plants can be avoided so that the transformation and regeneration efficiencies are predicted with accuracy. Newell-McGloughlin [61] also emphasise this fact and mention that this optimisation led to the increased number of transgenic plants and reduced the time in culture. Selectable marker genes encoding selective agents, such as hygromycin and glufosinate, are the most commonly used to enhance the recovery of transformants. The correlation between the efficiency of selection systems and transformation rates strongly suggests that there is an interaction between the system of selecting putative transformants, the type of culture, and the genotype of the plant in question [42].

6. Conclusion and recommendations

6.1 Conclusion

Legumes form part of a large number of globally cultivated plants that have been used for several years as staple foods in underdeveloped countries. From their use as food crops to being employed as sources of various legume derivatives in the industrial sector, leguminous plants are rich sources of proteins, oil, essential amino acids, micronutrients, and phytoestrogens. All these nutraceutical compounds play essential roles in human and animal health, by preventing, reducing, or completely alleviating certain diseases. Additionally, they play an imperative role in the environment and the agronomic sector, providing additional nitrogen by fixing atmospheric nitrogen into usable forms, increasing the balance of micronutrients in the soil through various cropping systems, and acting as the sink for phytoremediation. These properties and benefits conferred by legumes have invaluable potential in eradicating food insecurity, and thus make it possible to believe in a future where malnutrition, undernourishment, and poverty are greatly minimised.

However, it is still important to understand that legume propagation is not without challenges. In fact, there is an increase in the problems faced by both conventional and biotechnological improvement of these crops, with the increasing demand. Climate change, anthropological effects, and biological infestations are the major hurdles that lead to crop losses and decreased productivity in crop breeding. Additionally, the recombinant techniques, which are continuously gaining popularity in crop production, also face challenges, albeit with significant improvements achieved thus far. There are various ongoing optimisation investigations, whose goal is to ultimately counteract any of these challenges faced either during genetic transformation or regeneration, especially under tissue culture conditions. All of these studies target different areas of transformation that have significant effects on the processes involved during gene transfer and plantlet development to provide optimum conditions required by the explants for successful improvement.

6.2 Recommendations

There are promising target areas that may either provide insight or lead to breakthroughs in the ongoing optimisations. The duration of co-cultivation and its supplements can be further investigated since various studies have reported different findings in this regard. Although antibiotics play a pivotal role in controlling contamination in culture, it is necessary to investigate whether or not excluding them from culture media is an amenable option. Explant types and their physiological conditioning have been reported to improve explant survival rates during regeneration, which makes it a potential target area to be optimised, especially for legume plants that are reluctant to grow *in vitro*. It is only when such promising optimisation are extensively explored that stable genetic improvement protocols can be devised, and until then, it seems there is much work to be done. Nonetheless, it remains evident from the many ground-breaking breakthroughs achieved thus far, that the future of genetic transformation, especially in food crops will be unparalleled.

Conflict of interest

The author declares no conflict of interest for this manuscript.

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Chapter 4

Legume Breeding: From Conventional Method to Modern Technique

Parastoo Majidian

Abstract

Legume species have various applications in organism's nutrition, medical, and conversion industries because of their high oil, high protein, and high value materials. These crops can prevent soil erosion and increase soil nitrogen for further crop cultivation by bacteria symbiosis as well. Concerning the benefits of these crops, there is a need for more breeding attempts to gain genetic achievements. Accelerated higher genetic gains are required to meet the demand of ever-increasing global population. In recent years, speedy developments have been witnessed in legume genomics due to advancements in next-generation sequencing (NGS) and high-throughput genotyping technologies. A fundamental change in current conventional breeding programs, combined with modern techniques, is of great importance. Thus, a combination of modern and conventional breeding techniques may conduct our goals to reach great achievement on legume breeding regarding industrial and medical uses, human and livestock nutrition faster.

Keywords: legume, classical and molecular breeding

1. Introduction

Legumes are of great importance as nutritional and economic values that form part of the diet of millions of people worldwide. Legume seeds include an important source of proteins and peptides (double or triple of most cereals), carbohydrates and dietary fibers, and a good source of some micronutrients such as vitamins, fatty acids, folic acid, and minerals that have significant health benefits [1]. The leguminosae or fabaceae family consists of about 12,000 species distributed throughout the world and adapted to a great variety of habitats [2].

In addition, numerous significant plant species belong to leguminosae family such as beans, faba beans, chickpea, cowpea, clover, pea, peanut, pigeon pea, alfalfa, sweet lupin, and lentil which have various applications for human and livestock nutrition, medical industry, and other conversion industries. In addition, some species are used as ornamental crops and as sources of timber and fuel, especially in tropical regions.

One of the significant criteria of legumes is the capacity to produce symbiotic interactions with bacteria called rhizobia that fix atmospheric nitrogen (N) benefiting the plant, which in turn delivers carbon to the bacteria [3]. This symbiosis

reduces the production costs and the risk of environmental pollution due to the use of synthetic N fertilizer. It is estimated that a total of 50–70 MT of N are fixed biologically in agricultural systems annually, 16.4 MT in soybean, and 12–25 MT in pasture and fodder legumes [4].

Legumes crops can be used as an alternative for feeding the global population and contribute to developing sustainable agriculture, taking into account their nutritional, economic, and environmental benefits. However, there is not enough data for these crops than cereals [5]. During the last 50 years, legume production is exposed to the negative effect of biotic and abiotic stresses, which cause a reduction in its yield [6, 7].

The other difficulty in legume production except soybean is the limited availability of genetic resources of legume crops in developing countries [8]. In addition, legume breeding has hindered by the lack of robust doubled haploid protocols for legumes species compared to cereal and oilseed crops [9].

Several studies have been investigated by researchers regarding leguminosae genetic data resources such as DNA chips, databases of Targeting Induced Local Lesions In Genomes (TILLING), Bacterial Artificial Chromosome (BAC) libraries, and several bioinformatics tools as "The Legume Information System" (http://legumeinfo.org/) [10].

Thus, the objective of this chapter is to express and compare classical breeding methods in legume crops as well as modern technologies including marker-assisted selection (MAS), quantitative trait loci (QTLs) mapping, and biotechnology.

2. Classical breeding methods

2.1 Accessions and genetic variation

Evaluation of crop genotypes and cultivars by phenotypic and genetic traits is basic research in breeding programs in order to group accessions based on their genetics, to make knowledge of their genetic background, and select the parental lines for further crossing breeding projects [11]. In this regard, the characterization of germplasm banks of legume crops worldwide has been crucial for the development of agriculture because they are the reservoirs of genetic diversity [12].

To recognize the core collection of legume species and to distinguish various groups of parental lines for crossing programs, the genetic diversity of this family crop has been expressed in this chapter [13]. Utilization of molecular markers is one of the simple techniques to identify genetic diversity of legume species such as SSR (single sequence repeat), AFLP (amplified fragment length polymorphism), RAPD (random amplification of polymorphic DNA). Due to being highly self-pollination as well as low and very low outcrossing rate value in legume germplasm, most of them has genetically similar values and show low to moderate genetic diversity criteria such as allele by locus, heterozygosity, and polymorphism information content (PIC) at intra-population and intragroup levels. While what is important that the genetic variability among population and group of accessions for further breeding programs. In previous studies, researchers reported on the data obtained from genetic variability parameters including (observed heterozygosity of 2–32%), (alleles by locus of 1.5–19), (PIC of 1–66%) in landraces of common bean, soybean, chickpea, lentil and pea, and varieties of these crops from America, Europe, and Africa [14–16]. In contrast, faba bean collections have shown considerably higher observed heterozygosity (20–36.3%), expected heterozygosity (27%), and PIC values (28.7%) than other legume species [17].

2.2 Phenotypic inherited traits

Some morphological and phenological properties such as growth habit, plant height, pod cross-section, number of pods in plant, pod curvature, hypocotyl color, flower color, days to flowering, node numbers, seed number, seed number per pod, number of flower buds, and 100-seed weight, biological yield display significant differences in most of legume germplasm which is relevant to crop yield and appropriate index for breeding purposes [18]. Morpho-physiological and reproductive traits are consistent in different species of legumes [19–21].

Monogenic traits such as color, shape, texture, presence/absence of certain characters are successfully controlled by conventional breeding approaches. While, multigenic traits (quantitative traits) such as plant yield, resistance to abiotic stresses, and so on are highly affected by environment and by genetic × environment interactions which are time-consuming and less precise in breeding techniques [22].

To quantify the proportion of phenotypic variance among individuals in a population, plant breeders utilize heritability as additive genetic effects in the narrow sense (NSH) [23]. The sum of additive, dominance, and epistasis effects is defined as heritability in a broad sense (BSH). Quantitative genetics as heritability determine the responses of selection and depends on selection method (i.e., mass, pure line, pedigree, bulk, backcrossing, etc.) and the type of selection [23]. In soybean, high heritability values have been estimated for plant height, number of clusters per plant, number of primary branches per plant, seed yield per plant, and number of pods per plant [24].

In common bean, it was determined that high values of BSH, ranging from 0.55 to 0.91 for seven phenological and morphological traits [25]. In other previous studies, it was pointed out the results showed that the BSH values for yield and the yield components ranged from 0.115 to 0.642 higher than BSH for a number of days until flowering [26]. Because of the narrow genetic base of chickpea, it takes time to produce high-yielding cultivars, for example, resistance to *Ascochyta* blight in this crop resulted from eight parental di-allele crosses and their F2 [27].

In lentils, heritability values of various traits have been estimated using traditional genetic improvement. In the last study, some morphological properties including total dry matter per plant, seed yield per plant, number of pods per plant, and number of seeds per plant showed low heritability, while days to 50% flowering, days to maturity, and seed weight indicated higher heritability value of 80% [28]. In another study, other seed quality traits have also been studied. For example, raffinose-family oligosaccharides and sucrose levels were highly heritable (BSH values $\geq$0.85) [29]. Regarding abiotic stresses, cold tolerance heritability was assessed based on NSH values varied from 0.31 to 0.71 under field conditions and peaked at 1.0 under controlled conditions. Based on the results, additive genes controlled cold tolerance under controlled conditions, while field conditions had a negative effect on cold tolerance and made it sensitive [30].

Regarding pea, BSH as well as NSH values for resistance to two fungal diseases (*Erysiphe pisi* and *Mycosphaerella pinodes*) was estimated as high BSH (0.62–0.81) and moderate NSH (0.43–0.57) values, respectively [31]. Also, high BSH values as about 0.62 were gained for the heritability for days to maturity, plant height, pod length, and 100-seed weight, whereas, moderate heritability values were indicated for plant height, pod length, and 100-seed weight [32].

In faba bean, the least affected agronomic and yield-related traits across the environment were the seed weight and the days to flowering, and the number of pods per plant, while, the strong environmental effects were detected on seed yields and the number of stems per plant [33]. In another study, an important trait for conventional breeding as frost tolerance in faba bean was indicated high heritability

after hardening [34]. Generally, the main objective of breeding programs is to genetically evaluation of legume germplasm in order to select superior lines aiming at improving genetic diversity in their progenies and detect heritability of different traits which are seeking by breeders.

3. Bioengineering

The first plant species that its entire genome sequenced was Arabidopsis thaliana regarding to *Arabiodopsis* Genome Initiative Project 2000. This achievement led to further advances in the field of sequencing technologies by the release of the genome sequence of more than 50 species consisting of rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*), and so on [35]. The Arabidopsis plant model has allowed the study of physiological and metabolic processes during plant growth and in responses to abiotic and biotic stress through genome-wide gene expression analysis [36]. This type of analysis has also enabled the identification of the genes responsible for certain traits such as drought and salinity tolerance [37].

Genomics has made available the use of DNA-based molecular markers for the development of MAS in plant breeding programs [38], which uses genotypic selection instead of phenotypic selection employed in conventional breeding. MAS integrates two main systems such as QTL mapping and candidate gene or major gene localization [39]. These methods are based on analyses of association, in which the traits are studied in a large and diverse population and through linkage disequilibrium (LD), where a segregating progeny of parental lines that contrast in certain traits are studied [40].

In recent years, six legume species from the leguminosae family were thoroughly sequenced such as *Cajanus cajan*, *Cicer arietinum*, *Glycine max*, *Lotus japonicas*, *Medicago truncatula*, and *Phaseolus vulgaris* with the genome length of 833, 738, 1112, 472, 373, and 588 Mb, respectively, which their number of genes and transcripts varied from 28,269–48,680 and 25,640–243,067, respectively.

In addition, other legume species including *Pisum sativum* (4450 Mb), *Lupinus angustifolius* (924 Mb), *Trifolium pratense* (440 Mb), and *Arachis hypogaea* (2800 Mb) were entirely sequenced which were significant for omics studies explaining their genes, proteins, transcription factors, metabolites as well as physiological processes. For example, omics studies on *L. japonicas* resulted in Rhizobium infection and nodulation and salt acclimatization processes based on different techniques including Serial Analysis of Gene Expression, cDNAarray of 18,144 non-redundant ESTs isolated from *L. japonicus*, an Affymetrix GeneChip® with 50,000 probe-sets and real-time RT-PCR, a Microarray profiling using the Lotus Genechip® [41]. In parallel, the first version of the completely common bean genome sequence was recently released [42], and also the genome sequence of chickpea is also available in "The Cool Season Food Legume Genome Database" [43]. Legume genome references have also enabled the application of the RNA sequencing (RNA-seq) approach to conduct global transcriptomic profile studies and to discover new genes and ESTs [44, 45]. Overall, thousands of EST, uni-gene, SSRs, and SNPs have been published for lentils [46], groundnut [47], pigeon pea [48], and pea [49].

Great efforts have been made to compare the genomes between models plant species and crop legumes for an accurate translation of the information gained [50]. It was documented that the genome of lentil species such as *L. ervoides* and *Lens culinaris* has high similarity with *M. truncatula* using comparative genomics which identifies a few major translocations and transfer EST-SSR/SSR sequences from the model *M. truncatula* to enrich an intraspecific lentil genetic map [51]. In pea, it

was reported the construction of a high-density pea SNP map, and the validation of syntenic relationships between pea and other legumes species [52]. In faba bean, there is synteny between its region related to days to flowering with other legumes such as medicago, lotus, pea, lupine, and chickpea. Moreover, QTL mapping studies exhibited the similarity between pod length and a number of seeds per pod of faba bean and L. *japonicas* [53].

4. State of the art fabaceae species breeding methods

Achievement in genomics field such as Quantitative trait loci mapping (QTL), marker-assisted selection (MAS) led to improve our data in legume breeding as 1) cultivar identity/assessment of "purity", 2) evaluation of genetic diversity and parental selection, 3) study of heterosis, 4) identification of genomic regions under selection, 5) marker-assisted backcrossing (MABC), 6) marker-assisted pyramiding, 7) early generation MAS, 8) combined MAS, and 9) multi-parents advanced generation intercrossing [54]. Several techniques, as well as strategies for mapping quantitative traits for the identification of quantitative character genes, have been developed in this century which have accelerated and optimized the cultivar development process [55, 56]. Moreover, relevant technical advances have been accomplished to accelerate the breeding of legumes, such as the increased speed of single seed descent by shorter generation cycles through flowering and fruit set in vitro [57].

Important advances in genomic resources have been made in legumes, encompassing a large number of QTLs and genes mapped for different characters, including agronomic, yield-related, or resistance to biotic or abiotic factors traits. Chickpea, common bean, and soybean are three fabaceae species that have been improved through MAS, showing clear and significant progress in the last years. In lentil and faba bean.

Regarding disease resistance-related genes/QTL, achievements obtained were obtained MAS in breeding lines and cultivars.

Although, the classical breeding techniques can transfer these traits and their useful alleles to the breeding line, the introgression by MAS save time selecting for resistant lines [58]. Also, advanced lines or cultivars of common and snap beans with quantitative traits for certain diseases have been produced using MAS [59]. MAS also allows the use of pyramiding approaches, which has become an important method permitting the introgression of several genes and QTLs on a single line [60]. Fewer achievements on other quantitative traits (i.e., yield) have been reported in the literature. Efforts have been made to successfully introgress QTLs for yield-enhancing traits in soybean [61], and drought tolerance-related traits in chickpea [62]. The advantage of MAS in legumes is to successful translation of quantitative traits of interest (major genes/QTLs that control those characters) in commercial lines regardless of being slow incorporation of QTL using MAS selection. High-quality genome sequence of white lupine (*Lupinus albus* L.) was obtained based on long-read sequencing technologies in order to increase and stabilize lupine yield [63].

5. Genetically modified legumes

Great progress in the regeneration and genetic transformation of certain legumes has been made. Global water scarcity and soil salinization have boosted the research for genetic engineering water stress and/or salt tolerance-related

genes in legume crops such as alfalfa [64], chickpea [65], *M. truncatula* [66], and pigeonpea [67], among others. In soybean, several genes controlling traits, such as soybean cyst nematode resistance [68], seed oil [69] and methionine [70] content, drought resistance [71], among others, have been genetically modified (GM). The most successful case of public knowledge is glyphosate-resistant transgenic soybean, which has been commercialized for over 20 years, and it is undoubtedly the most important genetic modification in soybeans [72]. Other legume species, such as narrow-leaf lupine (*L. angustifolius* L.), have also been successfully genetically transformed to develop glyphosate-resistant lines [73]. Glyphosate is a low-cost, foliar-applied, broad-spectrum herbicide that has molecular targets in essential amino acid biosynthetic pathways, which kill the plant [74]. The activity of this herbicide is to block the shikimate pathway by specific inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [75]. By inhibition of EPSPS, biosynthesis of aromatic amino acids impairs misregulated the shikimate pathway, affecting plant growth. The development of glyphosate-resistant crops (GRCs) utilized the CP4 gene from Agrobacterium spp., which encodes a glyphosate-resistant form of EPSPS, initially introduced in soybean [76]. The vast majority of the commercial GRCs on the market contain the CP4 EPSPS gene that confers glyphosate resistance [77]. GRCs have simplified weed management practices, reduced crop production costs, and have had positive effects on the environment [78]. While, the potential improvement of weeds resistant to glyphosate cause big concerns due to its high utilization and its genes potential introgression from GM crops into wild relatives (i.e., gene flow) and its high risks of environmental impacts [79]. Although gene flow is a legitimate concern of GM soybean, transgenes frequently represent a gain of function, which might release wild relatives from constraints that limit their fitness [80]. In parallel, several glyphosate resistance management strategies have been proposed by weed specialists to slow down the appearance of weed resistance biotypes to this herbicide [81]. One technology that has been well documented in the development of transgenically stacked-herbicide resistance traits (glyphosate + glufosinate + dicamba) in which the appearance of weeds resistant to any of these herbicides would be greatly diminished [82]. In Latin America, the bean golden mosaic virus (BGMV) from infection of whitefly is a major constraint to bean cultivation. This results in the creation of GM common bean resistance to bean golden mosaic virus (BGMV) by silencing the replication-associated protein gene (rep) [83].

6. Conclusions

Legume breeding includes different aspects starting from genetic diversity identification and evaluation and improving genetically traits by classical and modern breeding methods. Achievement in legume breeding was gained in fields of phenotypic inherited traits identification, bioengineering, and genetically modified legumes which result in improvement of various traits in legumes such as tolerance to different biotic, abiotic stress, and increase yield. Furthermore, great efforts have been performed to identify and conserve genetic resources of legumes such as wild species, landraces, old cultivars, research materials, breeding lines, and advanced cultivars through classical and state of the art breeding approaches.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

Phenotypic Analysis of Pigeon Pea Reveal Genotypic Variability under Different Environmental Interaction

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Abstract

Pigeon pea is one of the most important leguminous crop globally. However it is a neglected pulse crops in South Africa in terms of research and production. Most farmers grow local landraces with low yields and there is lack of diverse material. The objective of the study was to determine the presence of genetic diversity among the pigeon pea genotypes using quantitative and qualitative phenotypic traits. The trials were conducted in Mafikeng and Nelspruit in South Africa. The trials were laid out in randomised complete block designs replicated three times. The quantitative and qualitative phenotypic data were recorded according to pigeon pea descriptor list. The phenotypic data were analysed using analysis of variance, Pearson's correlations, principal component analysis, and biplots constructed using principal coordinate analysis, Shannon weaver diversity indices and frequencies. The results showed highly significant differences among the genotypes based on plant height, pod bearing and seed number per pod meaning there was vast genetic diversity among the genotypes. Seed yield was positively correlated with seed number per pod, seed number per plant and pod weight whereas pod bearing was negatively associated with hundred seed weight meaning improving seed yield will automatically improve other positively correlated traits. Principal component analysis showed five most important PCs contributing to a total variation of 84.7%. The traits that contributed to the most variation to the total variation observed were plant height, pod length, seed yield, pod bearing and days to flowering. The Shannon weaver indices ranged between 0.98 and 1.00 showing the presence of variation among the qualitative traits measured. The clustering grouped genotypes into three clusters with Tumia and ICEAP 00540 being the most diverse. The diverse genotypes can be used as parents for hybridization and development of transgressive segregants in breeding programmes. There was vast presence of genetic diversity among the pigeon pea genotypes evaluated.

Keywords: agro-morphology, characterisation, genetic diversity, PCA, pigeon pea

1. Introduction

Pigeon pea (*Cajunus cajan*), a diploid legume crop species (2n = 2x = 22) [1]. This crop is considered as underutilised plant species despite its importance. The crop is a

perennial legume crop, that can be considered as multipurpose crop due to its use for livestock feed, and food for humans. It also improves the fertility of the soil through atmospheric nitrogen fixation [2, 3]. The crop can be intercropped with other crop species. The crop plays an important role in food and nutritional security [4]. Pigeon pea is a good source of mineral elements and vitamins [5]. This crop has high potential to cope with climate change and providing nutritional and food security. It has the ability to survive and give good economic benefits when planted under dryland farming conditions, when there is limitation of rainfall and sustain the livelihood of poor rural populations in tropical and sub-tropical regions of the African continent. Furthermore, the crop helps in protecting the environment from soil erosion, towards enhancing productivity of marginal agricultural lands. The seed of the crop can be eaten as a green vegetable and dry pulse and is an important source of nutritional components [3]. The green pods and foliage of the plant are mainly used as livestock feed [6]. It is climate change crop including heat and tolerate drought [7]. The crop is cultivated by the resources poor small scale farmers with the low input agriculture. Despite the important of pigeon pea for food security and income generation, the cultivation of this crop is neglected in Southern Africa due to unavailability of improved cultivars. Hence, genetic improvement of this crop is important to increase production and productivity of the crop in Southern Africa.

For an efficient evaluation and utilisation of the genetic materials, detailed knowledge about genetic diversity, and information on collection and classification are important and the basis for crop improvement programs [8, 9], which is elucidated through different marker systems such as agro-morphological, biochemical and molecular markers. Among these, agro-morphological characterisation is considered as the initial step for designing breeding programs [10, 11] although influenced by environment unlike with DNA-based markers. The assessment of genetic diversity using agro-morphological traits is still of paramount importance to plant breeders and curators because they will be able to select potential parents based on yield and its components, and farmer preferred agronomic traits. Yohane et al. [12] assessed eighty one pigeon pea accessions for presence of genetic diversity using agro-morphological traits. Assessing genetic diversity helps to study heterosis [13], selection of transgressive segregants and genes of novelty, and has a role in collection and conservation of germplasm for crop improvement [14]. In order to have all these done, sound statistical tools are required for data analysis for assessment of genetic divergence [15]. In order to reduce the volume of data and identify a few key or minimum descriptors that effectively account for the majority of the diversity observed, saving time and effort for future characterisation efforts the data must be subjected to multivariate analysis [16].

Multivariate analytic tools have proved to be vital in crop improvement [17]. The tools include principal component analysis and cluster analysis among others. These tools are currently effective for studying the variability and relationships between accessions [18, 19]. The principal component analysis (PCA) includes the total variance of variables, explains maximum of variance within a data set, and is a function of primary variables. PCA shows which of the traits are decisive in genotype differentiation [20]. It enables easier understanding of impacts and connections among different traits by finding and explaining them [16]. Cluster analysis identifies and classifies objects individuals or variables on the basis of the similarity of the characteristics they possess, so the degree of association will be strong between members of the same cluster and weak between members of different clusters. It aims to allocate a set of individuals to a set of mutually exclusive, exhaustive groups such that the individuals within a particular group are similar to one another while the individuals in the different groups are dissimilar. It is also helpful for parental selection in the breeding program and crop modelling [16]. PCA and cluster analysis are preferred tools for morphological

characterisation of genotypes and their grouping on similarity basis [21, 22]. Combination of these two approaches gives comprehensive information of characters which are critically contributing for genetic variability in crops [23]. The knowledge of different landraces and their evaluation are necessary for improvement strategy development in any crop [24], as these traditional landraces are the potential donor parents for improved varieties [25]. Hence, the aim of the study was to determine the presence of agro-morphological diversity using quantitative and qualitative traits.

2. Materials and methods

2.1 Plant material and experimental sites

Nineteen pigeon pea genotypes were obtained from ICRISAT in Kenya and Tanzania (**Table 1**). The trials consisting of 19 pigeon pea genotypes were planted in Mafikeng (t 25° 48′S, 45° 38′E; 1012 m.a.s.l.) and Nelspruit (−25.451496 S, 30.969084 E; 670 m.a.s.l.) in 2019/20 growing season in North West and Mpumalanga Provinces of South Africa. Mafikeng is located eight kilometres from the city of Mafikeng towards the border between Botswana and South Africa. It falls within a semi-arid tropical savannah region and receives a summer rainfall, with an annual mean of 571 mm [26]. The rainfall on site is erratic which makes the prospects for crop cultivation highly vulnerable. Approximately, 68% of the annual precipitation in this area falls between November and January in a few relatively heavy downpours, with a pronounced dry season from April to September. The mean maximum temperature is 37°C, while the mean minimum temperature ranges from 7–11°C. The field in Nelspruit was characterised by sandy loam soil with mean temperature of 19.8°C and an annual precipitation of about 796 mm. Nelspruit is the capital city of Mpumalanga province which neighbours Mozambique.

2.2 Trial design and management

The trials were laid out in a randomised complete block design replicated three times with a plot consisting of two rows of 4 m length in each site. The spacing between the rows was 90 cm and the spacing between the plants was 60 cm. The insect pests that were prevalent were aphids and pod borers and were controlled by insecticides used on legumes. Plants were irrigated thrice a week. Weeding was done manually using hand hoes.

2.3 Data collection

Data were recorded according to standard descriptor list of pigeon pea [27]. The quantitative data recorded included plant height (PHT), days to 50% flowering (DFF), pod bearing (PDB), leaf length (LFL), leaf width (LFW), pod length (PDL), pod width (PDW), pod weight (PWT), stem diameter (STD), number of branches (BRN), seed number per pod (SNT), number of seeds per plant (SNP), hundred seed weight (HSW) and seed yield (SYD). The qualitative data included base flower colour, second flower colour, vigour at 50% flowering, pod form, seed colour pattern, seed shape, and pattern of streaks.

2.4 Statistical data analysis

The recorded quantitative data were analysed using analysis of variance, principal component analysis, and Pearson correlations. The qualitative data were analysed

Number	Genotype Name	Origin/source
1	ICEAP 01147	ICRISAT
2	ICEAP 01154–2	ICRISAT
3	ICEAP 01150–1	ICRISAT
4	ICEAP 01179	ICRISAT
5	ICEAP 00979–1	ICRISAT
6	ICEAP 01172–2-4	ICRISAT
7	ICEAP 01159	ICRISAT
8	ICEAP 01544–2	ICRISAT
9	ICEAP 00540	ICRISAT
10	ICEAP 00554	ICRISAT
11	ICEAP 00557	ICRISAT
12	ICEAP 00850	ICRISAT
13	Ilonga 14-M1	Tanzania
14	Mali	Tanzania
15	Ilonga 14-M2	Tanzania
16	Karatu-1	Tanzania
17	Kiboko	Tanzania
18	Komboa	Tanzania
19	Tumia	Tanzania

Table 1.
A list of pigeon pea germplasm used in the study.

using frequencies, spearman correlations, and Shannon weaver diversity index. The biplots were constructed using principal coordinate analysis in SAS version 9.6. A dendrogram was constructed using R-Studio in R software version 3.4.

3. Results

3.1 Genotype by environment interaction

Significant differences were observed on site, genotype and genotype x site interaction on **Table 2**. There were highly significant differences for sites based on days to flowering, plant height, branch number, stem diameter, pod bearing, pod length, pod weight and significant differences for seed number per pod. There were highly significant differences on genotype based on pod length and pod weight. There was a site x genotype interaction based on plant height, pod bearing and seed number per pod.

3.2 Pearson's correlations

Correlations of 14 quantitative traits measured in the study are shown in **Table 3**. Days to flowering was highly significantly and positively correlated with plant height, branch number, stem diameter, and hundred seed weight. Also significantly and positively correlated with pod weight and negatively correlated with pod bearing. Plant height was highly significant and positively correlated with

Source of variation	DF	DFF	PHT	BRN	STD	LLT	LWT	PDB	100SW	PDL	PDW	SNP	PWT	SEP	SYD
Site	1	85323.8964***	175005.3755***	1141.9065***	11556.661***	0.20771930	722.546528	41198.177***	83.365845	1957.2698***	10.92122415	138.27418*	142.23962***	1.3447577	45.9124209
Genotype	18	76.16079	1908.7439	15.609855	13.50758	1.86169956	327.193243	1417.31874	163.285802	507.819808***	3.70017595	56.996765	46.0183087**	8.3367554	20.0367551
Site x genotype	18	72.88342	2867.4964**	16.660853	14.71866	2.04927369	325.352283	2733.83027***	165.910418	322.152094	4.03877894	61.196266*	30.7783374	8.8655005	17.9394124

** = Significant at 0.05 significance level, ** = Significant at 0.25 significance level, *** = Significant at 0.01 significance level.*

Table 2.
Combined analysis of variance for MD.

Variable	DFF	PHT	BRN	STD	LLT	LWT	PDB	100SW	PDL	PDW	SNP	PWT	SEP	SYD
DFF	**1**													
PHT	**0,701*****	**1**												
BRN	**0,625*****	**0,751*****	**1**											
STD	**−0,900*****	**−0,667*****	**−0,492*****	**1**										
LLT	−0,089	0,017	0,040	**0,241**	**1**									
LWT	−0,034	−0,019	0,075	0,169	**0,672*****	**1**								
PDB	**−0,498*****	**−0,405*****	**−0,341*****	**0,504**	**0,190***	−0,056	**1**							
100SW	**0,525*****	**0,431*****	**0,296****	**−0,574**	−0,053	−0,003	**−0,353*****	**1**						
PDL	0,183	0,046	−0,011	−0,136	0,117	0,095	−0,010	0,159	**1**					
PDW	0,063	0,083	0,114	−0,024	0,003	−0,110	0,014	−0,060	0,018	**1**				
SNP	0,086	0,133	0,089	−0,076	−0,085	**−0,202***	0,020	−0,063	**0,436*****	0,135	**1**			
PWT	**0,189***	0,068	0,013	−0,139	0,102	0,055	−0,006	0,135	**0,986*****	0,161	**0,526*****	**1**		
SEP	0,183	0,107	0,064	−0,130	0,060	−0,037	0,005	0,072	**0,858*****	**0,453*****	**0,669*****	**0,932*****	**1**	
SYD	0,183	0,096	0,042	−0,136	0,065	−0,013	0,001	0,092	**0,928*****	**0,248*****	**0,694*****	**0,974*****	**0,976*****	**1**

DFF = Days to 50% flowering, PHT = plant height, BRN = Branch number, LLT = Leaf length, LWT = Leaf width, PDB = Pod bearing, 100SW = hundred seed weight, PDL = Pod length, PDW = Pod width, SNP Seed number per pod, PWT = Pod weight, SEP = Seed number per plant, STD = Stem diameter, SYD = seed weight per plant. The bold values are significant, hence shown with the asterisks.

Table 3.
Pearson correlations of the quantitative traits measured on MD pigeon pea.

branch number per plant, stem diameter, and hundred seed weight, and negatively associated with pod bearing. Branch number had a negative association with stem diameter and pod bearing, and a positive correlations with hundred seed weight. Stem diameter had a positive correlation with leaf length, pod bearing and a negative association with hundred seed weight. Leaf length showed a positive correlation with leaf width and pod bearing. Leaf width had a negative association with seed number per pod. Pod bearing had a highly significant negative correlation with hundred seed weight. Pod length showed a positive association with seed number per pod, pod weight, seed number per plant, seed yield. Pod width showed a positive and highly significant correlations with seed number per plant and seed yield. Seed number per pod was positively correlated with pod weight, seed number per plant, and seed yield. Pod weight had positive correlations with seed number per plant and seed yield. Seed number per plant was highly significant and positively correlated with seed yield.

3.3 Principal component analysis

Five most important PCs were identified contributing 32.9%, 24.9%, 12.7%, 8.3% and 5.9%, to the total variation of 84.7%, respectively (**Table 4**). The first PC had pod length, pod weight, seed number per plant and seed yield contributing the most variation. In the second Pc, days to flowering, plant height, branch number, stem diameter contributed the most variation. Leaf length, and leaf width contributed the most variation in third PC. In the fourth PC, pod width was the most

Traits	F1	F2	F3	F4	F5
DFF	0,572	**−0,719**	0,047	−0,018	0,014
PHT	0,465	**−0,702**	0,123	0,237	−0,248
BRN	0,372	**−0,639**	0,194	0,370	−0,313
STD	−0,522	**0,735**	0,119	0,125	−0,068
LLT	−0,002	0,207	**0,873**	0,176	−0,043
LLW	−0,041	0,076	**0,907**	−0,024	0,070
PDB	−0,272	0,584	−0,007	0,167	−0,224
100SW	0,372	−0,536	0,092	−0,351	0,316
PDL	**0,820**	0,416	0,127	−0,300	0,034
PDW	0,267	0,107	−0,151	**0,781**	**0,530**
SNP	0,615	0,310	−0,262	0,151	**−0,452**
PWT	**0,866**	0,436	0,074	−0,166	0,057
SEP	**0,882**	0,435	−0,048	0,141	0,100
SYD	**0,893**	0,447	−0,016	−0,037	−0,020
Eigenvalue	4,616	3,495	1,777	1,163	0,822
Variability (%)	32,968	24,962	12,694	8,307	5,869
Cumulative %	32,968	57,931	70,625	78,932	84,801

DFF = Days to 50% flowering, PHT = plant height, BRN = Branch number, LLT = Leaf length, LWT = Leaf width, PDB = Pod bearing, 100SW = hundred seed weight, PDL = Pod length, PDW = Pod width, SNP Seed number per pod, PWT = Pod weight, SEP = Seed number per plant, STD = Stem diameter, SYD = seed weight per plant.

Table 4.
Factor loadings of the most import PCs of the MD short duration pigeon pea.

contributor to variation whereas in the fifth PC, pod width and seed number per pod were the traits that contributed the most variation.

3.4 Principal coordinate analysis

The principal component biplot of the quantitative traits, F_1 had 32.97% and F_2 had 24.96% (**Figure 1**). Stem diameter and pod bearing were negatively correlated with plant height, branch number, seed yield, and 100 seed weight. Seed number per pod, pod length, pod width, pod weight, seed yield, and seed number per plant were positively correlated with hundred seed weight, branch number and plant height. The same traits were also correlated with stem diameter, pod bearing, leaf width and leaf length.

The biplot for the qualitative traits, the F1 showed 37.97% and F2 had 20.5% (**Figure 2**). The first quadrant showed base flower colour, flowering pattern, vigour at 50% flowering, second flower colour. The second quadrant had pod form and seed colour pattern. These traits were positively correlated with one another in both quadrants. The third quadrant consisted of pattern of streaks which was positively correlated to vigour at flowering, seed colour pattern, pod form, and seed shape. The fourth quadrant consists of seed shape which is also correlated with pattern of streaks, flowering pattern, base flower colour, and second flower colour and negatively correlated with seed colour pattern.

3.5 Frequencies of qualitative traits

The frequencies of eleven qualitative traits measured are shown in **Table 5**. Vigorousness at flowering was high with 71.4% of plants being vigorous and intermediate was 23.2%. The base flower colour was dominated by yellow flowers followed by orange-yellow. The second flower colour was predominantly composed of red flowers (71.4%). The pattern of streaks was dominated by sparse streaks

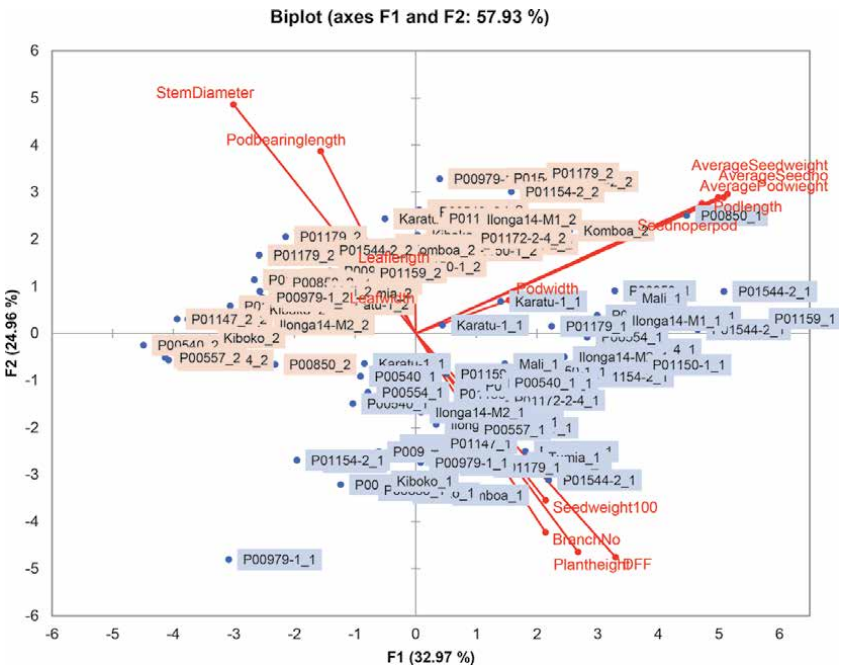

Figure 1.
PCA biplot for quantitative traits of medium duration (MD) pigeon pea.

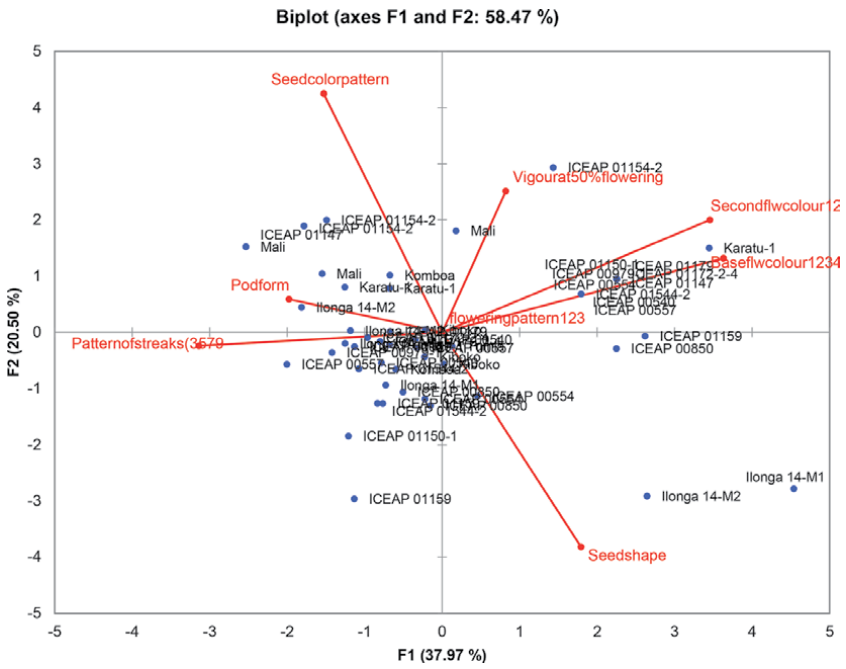

Figure 2.
PCA biplot of qualitative traits for MD pigeon pea.

(35.1%), followed by uniform coverage of second colour and dense streaks. Flowering patter was hundred percent determinate for all genotypes. All plants of various genotypes had 100% stems thicker than 13 mm with green stems dominating (63.2%). The growth habit was predominantly composed of spreading types (75.4%) followed by erect and compact at 22.8%. The genotypes were dominated by cylindrical pods 96.40 with speckled seed colour pattern at 71.4% followed by mottled and speckled at 17.9%. The shape of the seed was predominantly globular (64.3%) with oval shape being 21.4%.

3.6 Shannon weaver diversity

Shannon weaver diversity indices are shown in **Table 5**. The diversity indices ranges from 0.96 (second flower colour) to 1.00 (flowering pattern and stem thickness). All traits showed significant variation except for flowering pattern and stem thickness (**Figure 3**).

3.7 Hierarchical clustering

A dendrogram was constructed using hierarchical clustering in GenStat version 20. The dendrogram grouped genotypes into three clusters. The first cluster was composed of one genotype, Tumia. The second cluster was composed of two sub clusters that were divided into sub-sub clusters. The cluster consisted of seventeen genotypes as shown in the dendrogram. The third cluster consisted only ICEAP00540. The genotype Tumia and ICEAP00540 were far distantly related with the rest of the genotypes, and the other seventeen genotypes were significantly related as were grouped together. Tumia and ICEAP00540 has tallest plants and matures later than other genotypes, but the latter has small seed size and highest pod bearing whereas the former has big seed size. The rest of the plants are intermediate.

Trait	Score	Frequency (%)	Cumulative (%)	Shannon Weaver (H′)
Vigour at 50% flowering	Low	5,36	5,36	0.99
	Intermediate	23,21	28,57	
	High	71,43	100	
Base flower colour	Light yellow	19,65	19,65	0.97
	Yellow	51,78	71,43	
	Orange-yellow	28,57	100	
Second flower colour	Red	71,43	71,43	0.96
	Purple	28,57	100	
Pattern of streaks	Sparse	35,09	35,09	0.97
	Medium amount	15,79	50,88	
	Dense	22,81	73,68	
	Uniform coverage of second colour	26,32	100	
Flowering pattern	Determinate	100	100	1.00
Stem Thickness rating	Thick (>13 mm)	100	100	1.00
Growth habit	Erect and compact	22,81	22,81	0.98
	Semi spreading	1,75	24,56	
	Spreading	75,44	100	
Stem colour	Green	63,16	63,16	0.98
	Sun Red	36,84	100	
Pod form	Flat	3,64	3,64	0.99
	Cylindrical	96,36	100	
Seed colour pattern	Plain	3,57	3,57	0.99
	Mottled	7,14	10,71	
	Speckled	71,43	82,14	
	Mottled and speckled	17,86	100	
Seed shape	Oval	21,43	21,43	0.98
	Globular	64,29	85,71	
	Square	14,29	100	

Table 5.
Frequency percentages of qualitative traits for MD pigeon peas.

4. Discussion

The knowledge of genetic variation for a trait and trait correlations are important components of any breeding objective. There are highly significant differences for sites based on days to flowering, plant height, branch number, stem diameter, pod bearing, pod length, pod weight and significant differences for seed number per pod. This indicates that the expression of the significant traits varied with the environments were tested on. Their performance were not stable across sites. There were highly significant differences on genotype based on pod length and pod

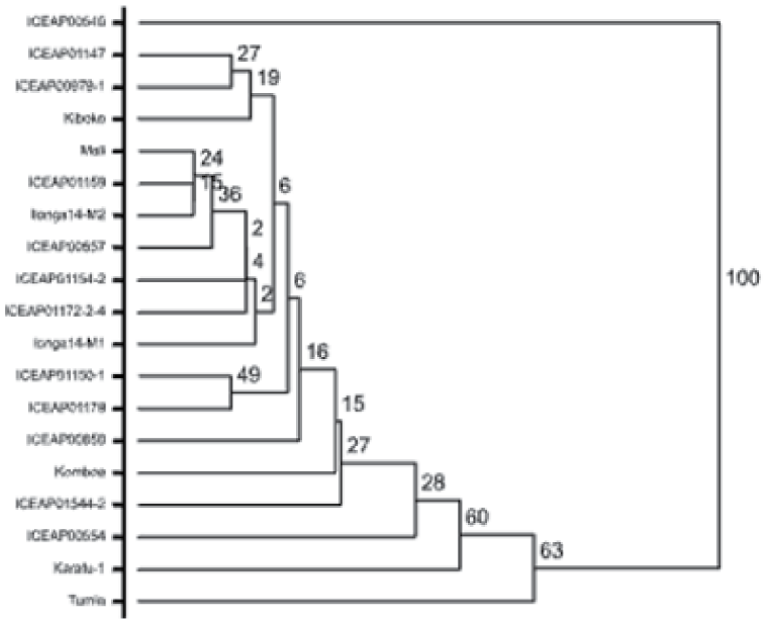

Figure 3.
A dendogram of fourteen quantitative traits constructed using hierarchical clustering.

weight. This highlights the presence of genotypic variation among the genotypes evaluated based on the two traits which can be exploited for cultivar improvement in future breeding programmes. Additionally, there was also a site x genotype interaction based on plant height, pod bearing and seed number per pod. The significant differences on genotype x site interaction could be attributed to the different reactions of the accessions to sites or due to differences between the sites. In each environment, phenotypic manifestation is the result of the action of the genotype under the influence of the environment. However, when considering a series of environments, in addition to the genetic and environmental effects, an additional effect can be detected from their interaction [28, 29]. Ssignificant genotype × environment interaction on yield and yield components of in this study concur with the results by Kimaro [30] as well as in other legume crops such as dry bean and cowpea [31, 32].

The positive correlations exhibited by most secondary traits show that multiple trait selection would be possible and the weak correlations among the traits would result in an inefficient selection or low genetic gains [12]. In this study seed yield was positively correlated with seed number per pod, seed number per plant and pod weight whereas pod bearing was negatively associated with hundred seed weight. The positive correlations of various traits in this study shows the usefulness of the traits for selection in crop improvement and they can further be used for improvement of seed yield [33, 34]. Similar trends were reported by Sodavadiya et al. [35] and Linge et al. [36] and Prasad et al. [37] in pigeon pea studies. Furthermore, Yohane et al. [12] reported a significant positive correlation between grain yield and a hundred seed weight, Kinhoégbè et al. [38] reported positive correlation with pod length, pods per plant, branches per plant and number of seeds per pod which concurs with the results in this study. This findings suggests the usefulness of this trait for selection. The results are in accordance with the correlations in this study.

The Principal component reveal five most important PCs with pod length, pod weight, seed number per plant, seed yield, leaf length, leaf width, days to flowering,

plant height, and stem diameter being the most contributing traits to the total variation observed. This suggests that these traits are useful for selection. Other reports indicated that trait contribution to different PCs varies with genetic diversity within the tested germplasm and the number of traits evaluated [25]. The biplot also showed the different grouping of pigeon pea genotypes based on specific traits. These findings suggested that both qualitative variables and quantitative variables data can reveal diversity providing different but complementary information.

Majority of pigeon pea landraces showed a strong tendency to spreading growth habit, yellow based flower colour, with red second flower colour, sparse pattern of streaks, green stems, with globular and speckled seed colour pattern. The results are in contrast with the results of Kinhoégbè et al. [38] where the authors reported genotypes with semi-spreading growth habit, lanceolate leaflet shape, light yellow base flower colour, and plain seed colour pattern. Similar results have already been reported in the morphological variability of Tanzanian pigeon pea germplasm [39] and world-wide collection [40]. Shannon weaver indices also confirmed the presence of genetic diversity based on qualitative traits. Thus, in spite of the influence of environmental factors, qualitative variables can be used to characterise pigeon pea genetic resources.

The pigeon pea genotypes were clustered into three major groups, indicating that there genotypes in the three groups are distantly related. The ones in the same cluster they are closely related and they maybe of the same source or origin. Selection of genotypes from these cluster may not be desirable to get higher yield benefits and transgressive segregants [40, 41]. Therefore, for any hybridization programs, the choice of suitable diverse parents based on genetic divergence analysis would be more fruitful than the choice based on the geographical distances. ICEAP 00540 and Tumia would be the ideal genotypes for use as a parents in any pigeon pea breeding programme for agronomic improvement. The identified genotypes in different clusters show that their interrelationship may be due to free exchange of materials that may have overlapped in the previous diversity distribution pattern of the domesticated species [42, 43]. Niranjana et al. [44] also reported three clusters in their findings on pegion pea. Reddy and Jayamani [45] reported seven major groups of the sixteen pigeon pea genotypes studied for genetic diversity using multivariate analysis. Qutadah et al. [46] also reported seven clusters in their pigeon pea genetic diversity study. Other cluster groups were revealed by various researchers [38, 47].

In conclusion, the study revealed the presence of genetic diversity among the pigeon pea genotypes studied based on the analysis of variance and multivariate tools used for analyses. The results indicated that the higher level of genetic diversity observed within the acquired genotypes from ICRISAT and Malawi will enable efficient utilisation and pigeon pea improvement in breeding programs in South Africa and other countries. The variability among the genotypes will also help to select the parents for hybridization. The selection combined yield related traits will reduce the more breeding work therefore suggested that yield correlated traits selection with respective genotypes. Further characterisation using molecular techniques as well as conservation attention for these germplasms should be conducted.

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Disclosure statement

The authors have not declared any conflict of interests.

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Chapter 6

Genetic Improvement of Minor Crop Legumes: Prospects of *De Novo* Domestication

Ochar Kingsley, Yu Lili, Su Bo-hong, Zhou Ming-ming, Liu Zhang-Xiong, Gao Hua-wei, Sobhi F. Lamlom and Qiu Li-juan

Abstract

Minor crop species and their wild relatives are resilient to multiple environmental stressors and are a great potential resource for promoting global food and nutritional security. However, since many of these species are deficient in a few or several desirable domestication traits which reduce their agronomic value, further work on their trait improvement is required in order to fully exploit their food benefits. Thus, to some extent, a minor crop may be regarded as semi-domesticated species based on the extent to which it is deficient in a number of agronomically significant domestication traits. Quite recently, research has revealed prospects of creating new crops out of wild plant species via *de novo* domestication. Minor crops deficient in desirable domestication traits as well as their wild relatives can possibly be subjected to such a systematic process of redomestication and *de novo* domestication in order to increase their food, nutritional, or raw material utilization value. This review discusses the feasibility of employing CRISPR/Cas-mediated genome editing techniques for the genetic enhancement of minor legumes and *de novo* domestication of their wild relatives.

Keywords: CRISPR, *De novo d*omestication, genome editing, legume, minor crops, ortholog

1. Introduction

The yield productivity of many major crop species, including those in the grain legume category, is hampered by unpredictable environmental conditions. This phenomenon has triggered the need to generate new crop species with prospects as good complements or alternatives to the major food crops [1, 2]. The new crops are not only expected to be endowed with better adaptation potential against one or multiple environmental threats, but also, they must exhibit preferred agronomic and nutritional composition attributes to satisfy growers, breeders, and consumers' claims. Efforts have therefore been made over the past few years in the collection of crop wild relatives in order to exploit their essential alleles for genetic improvement of elite crop species. Minor crop species or neglected and underutilized crop species (NUCs) and their wild relatives are nutritionally important, just as the known crop wild relatives/progenitors of major crops [1, 3, 4]. Consequently, minor species have in recent times

gained research recognition for their potential value for agricultural sustainability and for safeguarding against food insecurity [3, 5, 6]. These crops, which are members of the family of Leguminosae or Fabaceae have been considered as one of the most valuable species with numerous prospects for food in many parts of the world. As Leguminous species, these crops contain food nutrients that are essential for building a healthy human body [7, 8]. Also, they form a key component of many processed food products and animal feeds [9]. Legume species have over the years played significant roles in cropping systems for soil nutrient improvement, weed control, reclamation of wastelands and consequently contributed towards promoting ecological sustainability [1, 5]. Of the more than 20,000 plants species classified as legumes and distributed across some 800 genera [9–11] only a smaller number are fully explored and utilized for food, feed, and other agricultural and human required purposes [12].

Currently, genetic enhancement by *de novo* domestication of minor crops and their wild relatives using a genome editing system has been confirmed as a useful approach to explore and expand food crop resources for agricultural, food, and nutritional sustainability [13–15]. The technique offers prospects for developing new crops for today and future usage. However, scientific exposition in terms of the possibility of conducting *de novo* domestication schemes to convert semi-domesticated minor crops and their wild relatives to fully domesticated crop species is less reported. Now following advances in molecular biological technology, which provides powerful tools for genetic study, it has become more convenient to apply genomics to the study of minor crops which further paves the way for conducting *de novo* domestication experiments [16, 17]. Therefore, the current review discusses the feasibility of applying *de novo* domestication for the creation of new crop species from minor legumes and their wild relatives. Some suggested requirements for conducting a successful *de novo* domestication of a named minor legume which is also applicable in other non-leguminous minor crop species have been provided.

2. The need to explore minor legumes as alternative crop species

While there is much commitment to guarantee adequate food production, availability, and supply to people of all areas across the globe, agricultural productivity is still being confronted with several human-induced and exogenous environmental conditions (**Figure 1**) [1]. This forms an integral component of the reasons for the uncertainties in any attempts to safeguard against human food and nutritional insecurity [18, 19]. The current human population statistics reveal a predicted burgeoning trend across all continents and that from the present estimated 7.5 billion people, the global population is predicted to hit 8.5 billion by 2030, 9.7 billion by 2050, and beyond 11 billion by 2100 [20–22]. This phenomenon calls for increased agricultural productivity or increased food crop yields through the application of advanced agricultural technology and enhanced diversity of plant resources [6, 19]. Conversely, in many parts of the global agricultural productivity is low and this has partly been attributed to factors including low adoption of improved technology exacerbated by fluctuations in climatic conditions [18, 23, 24]. Rising urbanization with accompanying suite of developmental projects as well as indiscriminate exploitation of natural resources in some parts of the world is deteriorating and claiming vast areas of arable lands [15, 22, 23, 25, 26]. There is also increasing depletion and wilting of water bodies in some parts of the world [23]. The reality is that these factors in concomitant with human selectivity behavior for specific foods and food products will increasingly impose a great burden on food production, food quality, and supply systems in both the present and the future [25]. In addition, the major food crops feeding the world today are also a few with some of them cultivated outside of their historically originated and domesticated environments where they are

Figure 1.
Why there is a need for alternative crop species.

probably better adapted to thrive well [6, 22, 27, 28]. Even those which are still being produced in their centers of origin, edaphic conditions, and altered climatic variables have become major limitations to their maximum growth and development resulting in a dwindling yield output [29].

In the midst of the above global worries [18, 20, 22–24, 26] food production must not only increase but also worldwide availability and supply must be guaranteed. So, there is increasing interest to discover new opportunities and means required to increase the human food resources base. The cultivation, utilization, breeding, and preservation of leguminous crop species are well discussed, and they represent one of the food resources extensively consumed across the globe [30, 31]. Therefore, it is imperative to explore and exploit these new crop species as an alternative or supplement food resources endowed with economically valuable traits such as resiliency to environmental threats and adaptation to different production conditions. In this way, minor crop legumes will thrive well in their niches and consequently attain their maximum growth, development, and consequently, improved yield output [32].

3. Legumes and minor legumes: an overview

Generally, grain legume species are angiosperms and members of the family of Fabaceae with a characteristic high protein composition in their grains. They are specifically grown or harvested for human consumption needs mainly as food, unlike forage or pasture legumes which are used for animal feed. Legume species

are regarded either as major or minor by virtue of the extent of their consumption utilization, economic value, as well as research and breeding commitment, received. The major legume crops are known for their full domestication, a high and broad range of consumption, extensive cultivation, efficient utilization in research and genetic improvement as well as their popularly exchanged status across wider geographical regions. These features make major legumes distinct from minor ones.

Minor legumes constitute one of the most attractive categories of legume species identified and though spread across the world, and existing as both cool-season and warm-season legumes [33], they are predominantly endemic to tropical regions [3, 24, 31]. The significance of these species has in recent times been well expounded. They are a source of food security crops for rural farmers during lean cropping seasons and also possess valuable traits which can be exploited for modern crop breeding programs. They are endowed with agriculturally significant attributes such as resiliency to multiple biotic and abiotic conditions, thus making them essentially significant for incorporation into cropping and food systems [31, 34, 35]. However, most of these species may be vulnerable to extermination under unprotected agro-biodiversity fields with the few surviving not being fully exploited in terms of their incorporation into crop production and breeding programs. As a result of the increasing global demand for grain legumes and their products, there is now extensive research commitment by various governments and institutions to expand crop germplasm resource base including minor crops and their wild relatives, and consequently improve upon their economically significant traits.

4. Minor legumes as food and nutritional security crops

Leaders and diverse communities across the world endorse the fundamental rights of all persons irrespective of the location to have adequate food and thus stay out of persistent hunger or food deprivation [36, 37]. However, over 800 million people especially in less privileged locations of the world still require a great effort in order to meet their food requirements [38, 39]. Malnutrition is still a worry in various parts of the world and one out of every nine people across the globe suffers from persistent hunger [40]. This phenomenon appears to raise major concerns globally, especially by policymakers and international communities such as the United Nations and FAO who are tasked with specific roles of promoting adequate food supply to all persons [36, 37]. Across the globe now, matters of food insecurity have become a concern, and strategic modalities to assuage a likely worsening occurrence are extensively been discussed [41–43]. To help tone down food and nutritional insecurity, there must be an increasing effort to search and utilize alternative opportunities especially by promoting diversity within crop species [6, 24, 44]. Therefore, advancing sustainable agricultural productivity has become one of the keys focuses to realize this goal. Grain legumes are noted for their potential in the maintenance of food and nutritional security since they contain numerous nutritional requirements and most of these species are resilient against diverse environmental conditions responsible for general crop yield reduction [5, 45, 46]. At present, there is enough evidence that minor crop species could help in addressing these challenges especially in less developed economies and arid regions which are more vulnerable to food shortages [18, 24]. To bridge food and nutritional requirement gaps, it is estimated that some additional boost of 70% of food must significantly be produced within this period up to 2050 during which the world's human population is predicted to grow from 7.5 to 9.7 billion [47, 48]. It is obvious that many developing countries with burgeoning population growth rates but fewer agricultural technology applications will be most seriously hit. Given this occurrence, minor crop species which are

more endemic in these regions have become one of the best alternative approaches to help avoid food insecurity [3, 24].

5. Genomics and genome sequencing of legume resources

In recent times, following the emergence of next-generation sequencing technology many cultivated crop species have had their genomes sequenced [19, 49]. With the increasing number of molecular databases and computational analyses tools, genome information of such species has been stored making these crops now more amenable to crop improvement by molecular and genomic techniques [49, 50]. The molecular databases have particularly paved the way for mapping and identifying causal mutations, candidate genes, or QTLs associated with diverse traits of domesticated legumes [51–53]. Therefore, in modern crop improvement systems, the application of genomics as a complement to conventional breeding schemes has become a common practice [54]. Through genomic-based techniques, traits that are deficient in the major legume cultivars can now be introduced from other plant species. A few years ago, research about minor crop species was very much focused on their collection, morphological characterization as well as other information required for their documentation such as degree of consumption, production, nutritional value, market value, medicinal value, and, ethnobotanical descriptions [39]. Now it has become more convenient to apply genomics to minor crops and develop databases for storing and making available detailed information about their genome sequences [55–57]. Thus, there are some minor crop species that have now entered a genomic epoch (**Figure 2**) with research efforts ongoing to convert several of these species into genomic resource-rich crops [3, 31, 56, 97]. In this way, the beneficial

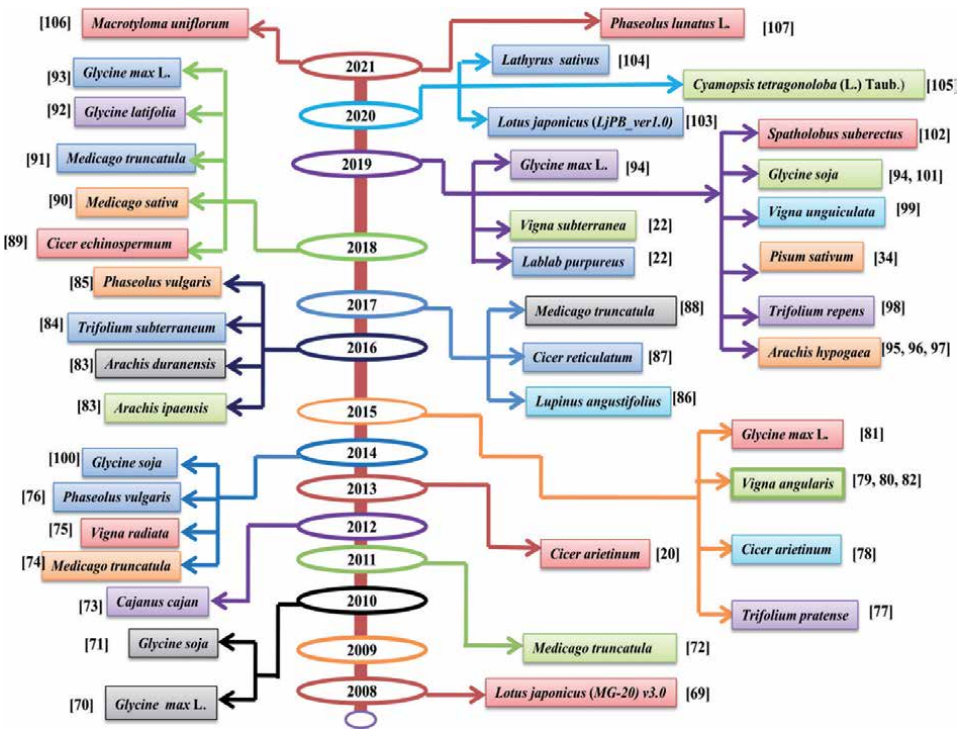

Figure 2.
Some genomic-rich legume species [58–96].

traits or gene constituents of these species can fully be exploited and incorporated into the basket of major legume crops feeding the world today. Additionally, there are minor crops that have desirable traits and are good model species for research. Thus, vital information such as minor crop legume evolution dynamics can easily be delineated and promote effective breeding [49].

Besides, the application of functional gene cloning and marker-assisted selection in crop breeding has gained momentum and is currently being extended from sole application in major crop species to minor crop species. Following the release of the genome sequences of some minor legume crops, further molecular genetics and genomic studies have been conducted to delineate causal mutations, genes, and QTLs underlying specific traits [49]. Whole-genome resequencing, genome-wide association studies, whole-genome organization analysis of genes of interest, and genomic selection techniques have been employed to study domesticated traits in some minor legume crops [16, 17, 98–105]. The increasing understanding of the genomics of major-minor crop legume species is accelerating the process of their genetic enhancement and paving the way for the domestication of their wild relatives by *de novo* approaches [106, 107]. Intuitively, a minor crop with available reference genome sequence information will easily be amenable to the genetic enhancement and *de novo* domestication of its wild relative than the one without a reference genome sequence. With the presence of a reference genome sequence, variations in phenotypic and genotypic attributes of the model and target minor crop species are easily compared, discerned and the requisite information elucidated. The genome sequences of many plant species including cultivated crops, and model plants, are currently available [4] and such known genomic information can be translated to other closely related species [105].

6. Crop domestication

Since the time of Vavilov's concept on the origin of domestication of cultivated crops [108] the topic of crop domestication and evolution of agriculture has received in-depth research interest [15] with much enthusiasm from diverse disciplines including genetics, history, archeology, paleobotany, and anthropology [109, 110]. Generally, hunting of wild animals and gathering of crop species for human sustenance was a stage in the history of early humans that preceded the Neolithic revolution age [111]. The Neolithic revolution marked the era of major agricultural innovations and inventions. Perhaps one of the foremost events which occurred during this period of human existence was the shift from hunting and gathering to the culture of sedentary living. It was during this period that crop domestication commenced. The domestication events generally proceeded as a gradual trait transformation process where plant species were unconsciously made adapted for agriculture and hence the two events, domestication and agriculture can be postulated to have occurred concurrently. The Neolithic era form of domestication involved the selection of specific plant species by virtue of human desired traits and over a longer period of continuous selection, the selected species were attuned to human cultivation and management practices. The Neolithic humans selected plant species endowed with preferred phenotypic attributes or traits such as better yield, taste, storability, increased seed size, less dormancy, and adaptability to management tools [112–114] thus leaving the rest (the largest chunk) as wild in their natural settings. This phenomenon differentiated crops domesticated from their wild relatives and progenitor species. As a result of few plant species selected for cultivation, the domesticated ones were positioned to have a narrowed biological diversity relative to their wild relatives and progenitors [115–117]. Over a longer

period of continuous cultivation, genetic and genome alterations possibly occurred, and sometimes created new genotypic and phenotypic variants [118]. Therefore, genes of the domesticated crops became fixed and linked to specific plant phenotypes or traits [119, 120]. These selected genes are called domesticated genes and their underlying traits are domesticated traits [118]. Intuitively, the selection of a given plant species as a domesticated crop based on its phenotype also meant an indirect selection of certain mutations and genes which remained unknown until plant breeding began. A further crop genome alteration in the post-domestication period can largely be attributed to the emergence of classical plant breeding [15].

The process of domestication also led to today's concept of genetic bottlenecks, and domestication syndrome, the suite of traits that confers a distinguishing characteristic on domesticated crops relative to their progenitors [111]. Domestication syndrome serves as an important cursor to facilitate discovering, mining and utilizing unexploited, underutilized, and neglected genes in crop wild relatives and minor crops [121, 122]. Generally, the combined effects of domestication and plant breeding are the result of altered crop phenotypic and genotypic architecture. Insight into crop domestication syndrome is particularly a prerequisite for effective and efficient minor crop domestication in this era of genomics and genome editing in crop breeding [123].

6.1 Crop redomestication for genetic enhancement of minor legumes

The major global agricultural challenges have already been mentioned previously in this work, and now there are uncertainties with regard to meeting the food and raw material needs of the ever-burgeoning human population. To tackle this occurrence with forethought, there is a need to take expedient actions that can facilitate expanding the human food resources base. This condition has been necessitated based on the established reality that only a negligible number out of the earth's endowed thousands of known plant species have been fully domesticated and currently been used as human food, animal feed, and raw material resources [124]. These major crops are losing their conferred natural adaptation potential such that their products will require human manipulation, especially where these crops are to be cultivated outside their original environmental niches [15]. The numerous species left in the wild as crop wild relatives and progenitors possess a wider genetic diversity and offer an ideal opportunity to be exploited for crop genetic enhancement. Now, as a result of the adoption of the major crop species many crops which are endowed with value for food have been less utilized or neglected though they have received some amount of domestication (semi-domesticated). These categories of crop species especially, legumes harbor essential alleles which can be exploited for improving upon the traits of cultivated ones. There is now an idea to revisit minor species and crop wild relatives which have either received some amount of domestication or none at all for their incorporation into major food crop resources. These semi-domesticated species including minor crops which are deficient in one or more desirable domestication traits can then be redomesticated using modern molecular breeding techniques. For minor crop species, their collection and further usage for experimental studies are being conducted along with their wild relatives.

By and large, plant breeding has become the surest way to adapt or develop new crops for major cultivation in unfamiliar environments. The utilization of alleles from crop wild relatives and progenitors for the genetic enhancement of domesticated or semi-domesticated traits of crop species is generally referred to as redomestication. This holds immense prospects to attune cultivated species to the prevailing environmental stresses. Another way, the traditional approach to achieve

redomestication of lost or neglected crop species is perhaps, to encourage their extensive cultivation in their inhabiting niches. Crop redomestication is an opportunity to circumvent agricultural challenges arising from climate change, reduce crop diversity, and consequently help promote agricultural sustainability. Genome editing has become the most convenient and fastest technique for achieving precise and targeted genome modification in crops and can be used for genetic enhancement or redomestication of economically useful domesticated minor legumes.

6.2 *De novo* domestication

Extensive effort in crop varietal development with the intent to raise crop yield productivity must be carried out along with exploration of new opportunities. As a way to enhance increased crop productivity, the application of biological science, technology, and innovations have been advanced to facilitate efforts in discovering new and suitable alternative techniques for raising food crop productivity and quality [125]. Among others, exploiting the benefits of crop germplasm resources is suggested and currently, there is extensive work in progress towards identifying, characterizing and utilizing new genes and QTLs of crop wild relatives and progenitors for crop improvement projects. As a way of incorporating alternative strategies, the concept of crop domestication has been revisited in order to domesticate new crops for increasing the human food resource base [106]. A major goal of domesticating new crops is to attune them to thrive well under human management and manipulative control [126]. So, those plant species which to date are not very much amenable to human cultivation and management environment but possess valuable properties for food, feed, and raw materials can be subjected to a new form of domestication. Domesticating new crop species will increase crop diversity and resiliency of agriculture for crop improvement [15]. However, the Neolithic era form of crop domestication takes many years or generations to select for desired crop species with conferred modified phenotypic characteristics (acquisition of domestication traits and thus be an adaptation for cultivation) [117]. Based on the current genome engineering techniques combined with OMICs technology there is now a possibility to domesticate new crops on a fast-track approach [117, 127].

Minor crop legumes, though are promising genetic resources required for advancing effective crop breeding, their utilization has been limited by virtue of certain undesirable traits associated with them. Therefore, minor crop species are also regarded as semi-domesticated species, lacking vital domestication traits [128]. Genome editing as a breeding tool is of immense prospects in the quest to increase crop productivity, in particular the interest in breeding minor crops. Many important traits associated with crop wild relatives and minor crop species can now be exploited to enhance crop productivity [129]. However, these traits are controlled by polygenic inheritance patterns. The polygenic genes of these categories of species are somewhat difficult to be manipulated for incorporation into cultivated crop genetic backgrounds [13]. Consequently, in order to take full advantage of their beneficial traits, a genome editing approach can be used to edit target loci in minor crops and their wild relative species in order to confer on them desired domestication traits [127]. This form of domestication is recommended and it is commonly referred to as "*de novo* crop domestication". By definition, *de novo* domestication is an innovative strategy proposed for breeding new crop species where domestication genes are introduced into non-domesticated and semi-domesticated plants [15]. In this approach, crop wild relatives or semi-wild plants, or non-domesticated species are made to acquire desirable domestication traits [106, 130] while their inherent desired phenotypes such as resilience to biotic and abiotic conditions are

maintained [54, 128]. The possibility to successfully perform *de novo* domestication of crop wild relatives has been ascertained based on recent successful experimental studies reported [14, 118, 126, 128]. These achievements provide a solid prospect for addressing a number of conditions that are constraints in general crop production such as reduction in crop diversity. For instance, considering that most crop wild relatives are endowed with special adaptation potential to numerous environmental stresses [131], *de novo* domestication offers a possibility to expand agricultural production to land areas that perhaps are considered unproductive and marginal lands for crop cultivation. The technique presents a unique opportunity and prospects to incorporate several crop species into the list of crops feeding the current global population. Still, in addition to their inherent desired properties such as climate resilience, the new crops are anticipated to be conferred with beneficial domestication traits and therefore produce breeders, processors, and consumers' desired traits. Among others, such beneficial traits will include improved performance of agronomic traits, increased edible yields, and improved quality attributes. Perhaps, what is more, intriguing about *de novo* domestication to the crop breeder is that the new crop domesticates will potentially address the current declining nature of crop diversity [3].

6.3 Genome editing as a tool for *de novo* crop domestication

Generally, crop species are endowed with a plethora of phenotypic traits which play major roles in determining the overall yield productivity. Nonetheless, some species may have certain inherent characteristics which rather place a limitation on their growth, development, and yield productivity. In crop breeding, desirable traits are maintained in the host or transferred to other species for the genetic improvement of their traits via isolated genes. Though more often, many undesirable characteristics may be associated with undomesticated, semi-domesticated, or wild/weedy forms of plant species, these species are endowed with key genes which are worth exploiting for achieving specific breeding goals. In crop improvement, various genes underlying desirable traits of wild relatives have been introgressed into the genetic backgrounds of elite cultivars [132]. Both conventional and molecular approaches are amenable for accomplishing this goal. Conversely, genes can also be isolated and transferred from domesticated crop species into that of wild-type plant species genomic backgrounds. The resulting newly created crop species is made to acquire an ideal domestication trait. Though both conventional and molecular techniques are applicable, the conventional approaches are less speedy, and sometimes unintended and undesirable traits or genes are incorporated [133]. In this instance, molecular techniques are found more versatile.

In recent times, creating new crop species from wild crop relatives on a fast-track approach has been possible through genome sequencing technology which has made available to the public, information on the genome sequences of several crop species [24]. The availability of genome sequences has further enabled the identification of genes and QTLs of several domestication genes and their underlying traits. Stacking domesticated genes in the genetic backgrounds of targeted crop wild relatives holds a possibility to develop new crop species/varieties by *de novo* [13]. The emergence of genome editing technology has added much impetus to crop improvement programs [118, 134, 135] where many genes can be targeted simultaneously to confer multiple traits on undomesticated or semi-domesticated species (**Figure 3**). Genome editing is considered the most cogent way to create new crop species from wild relatives in the process of *de novo* domestication [24, 136] especially traits that are monogenically inherited [118].

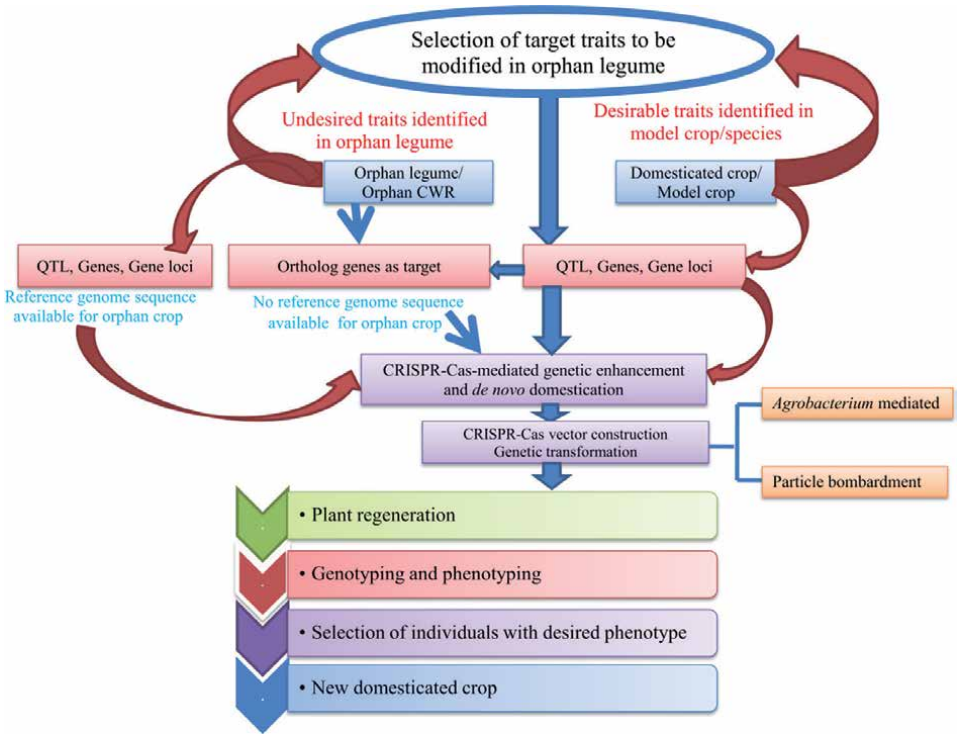

Figure 3.
The process of de novo *domestication of minor crops and their wild relatives.*

6.4 CRISPR-Cas-mediated approach to *de novo* domestication

Extensive effort in crop varietal development with the intent to raise crop yield productivity must be carried out along with exploration of new opportunities. As a way to enhance increased crop productivity, the application of biological science, technology, and innovations have been advanced to facilitate efforts in discovering new and suitable alternative techniques for raising food crop productivity and quality [125]. Among others, exploiting the benefits of crop germplasm resources is suggested and currently, there is extensive work in progress towards identifying, characterizing and utilizing new genes and QTLs of crop wild relatives and progenitors for crop improvement projects. As a way of incorporating alternative strategies, the concept of crop domestication has been revisited in order to domesticate new crops for increasing the human food resource base [106]. A major goal of domesticating new crops is to attune them to thrive well under human management and manipulative control [126]. So, those plant species which to date are not very much amenable to human cultivation and management environment but possess valuable properties for food, feed, and raw materials can be subjected to a new form of domestication. Domesticating new crop species will increase crop diversity and resiliency of agriculture for crop improvement [15]. However, the Neolithic era form of crop domestication takes many years or generations to select for desired crop species with conferred modified phenotypic characteristics (acquisition of domestication traits and thus be an adaptation for cultivation) [117]. Based on the current genome engineering techniques combined with OMICs technology there is now a possibility to domesticate new crops on a fast track approach [117, 127].

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certain undesirable traits associated with them. Therefore, minor crop species are also regarded as semi-domesticated species, lacking vital domestication traits [128]. Genome editing as a breeding tool is of immense prospects in the quest to increase crop productivity, in particular the interest in breeding minor crops. Many important traits associated with crop wild relatives and minor crop species can now be exploited to enhance crop productivity [129]. However, these traits are controlled by polygenic inheritance patterns. The Polygenic genes of these categories of species are somewhat difficult to be manipulated for incorporation into cultivated crop genetic backgrounds [13]. Consequently, in order to take full advantage of their beneficial traits, a genome editing approach can be used to edit target loci in minor crops and their wild relative species in order to confer on them desired domestication traits [127]. This form of domestication is recommended and it is commonly referred to as "*de novo* crop domestication". By definition, *de novo* domestication is an innovative strategy proposed for breeding new crop species where domestication genes are introduced into non-domesticated and semi-domesticated plants [15]. In this approach, crop wild relatives or semi-wild plants, or non-domesticated species are made to acquire desirable domestication traits [106, 130] while their inherent desired phenotypes such as resilience to biotic and abiotic conditions are maintained [54, 128]. The possibility to successfully perform *de novo* domestication of crop wild relatives has been ascertained based on recent successful experimental studies reported [14, 118, 126, 128]. These achievements provide a solid prospect for addressing a number of conditions that are constraints in general crop production such as reduction in crop diversity. For instance, considering that most crop wild relatives are endowed with special adaptation potential to numerous environmental stresses [131], *de novo* domestication offers a possibility to expand agricultural production to land areas that perhaps are considered unproductive and marginal lands for crop cultivation. The technique presents a unique opportunity and prospects to incorporate several crop species into the list of crops feeding the current global population. Still, in addition to their inherent desired properties such as climate resilience, the new crops are anticipated to be conferred with beneficial domestication traits and therefore produce breeders, processors, and consumers' desired traits. Among others, such beneficial traits will include improved performance of agronomic traits, increased edible yields, and improved quality attributes. Perhaps, what is more, intriguing about *de novo* domestication to the crop breeder is that the new crop domesticates will potentially address the current declining nature of crop diversity [3].

7. Requirements for a successful *de novo* domestication experiment

7.1 Orthologous genes for *de novo* crop domestication

Functional conservation of gene orthologs within and across species facilitates the improvement of traits associated with undomesticated species and hence the development of new crops out of minor species [49]. Many previous reports on orthologous gene analysis are available in diverse crop species including legumes [137]. In a situation where a minor legume has no available reference genome sequence, ortholog genes of a related species or a relevant model species such as genes identified in Arabidopsis thaliana can be used. This idea is based on the premise that two or more crop species that share close characteristics for their phenotypes will likely share the same underlying genes and thus the same mechanism of genetic architecture for the traits in question. If this condition is justified, then the genes implicated for the occurrence of similar phenotypes in different crops or species

are orthologous and will likely share sequence similarities and often many similar functional roles [6]. Many functional gene orthologs and their significant roles in phenotypic variations in diverse crops have been previously reported [138–141]. For instance, several gene orthologs of A. thaliana are known in other domesticated crops for which reason knowledge about orthologous genes of domesticated species or a model species can be transferred for genetic enhancement and domestication of minor crops and their wild relatives. An orthologous gene with high sequence affinity to the phenotype common among different domesticated crop species is an indicator of its potential and significance to be mined and utilized for minor crop improvement [6, 49]. Domestication gene orthologs that are functionally characterized can be targeted for CRISPR-Cas9/sgRNA-mediated gene knock-out, knock-in, activation, or inactivation in minor legumes, thereby generating new species conferred with domestication phenotypes. Silencing a gene reduces the gene's molecular function and, in this case, a genome sequence of the target minor legume is required in order to identify gene orthologs associated with domestication traits in a related or model species [106]. The success of mutating target genes to create new phenotypes in-ground cherry was based on knowledge about orthologous genes via the study of domesticated tomatoes [128]. Details of the possibility of employing gene orthology for domesticating new crops (*de novo* domestication) or improvement of minor crops which have already undergone some degree of domestication are explicitly explained in previous review work by Dawson et al. [6].

7.2 A prior knowledge of domestication traits and gene loci

By applying the techniques of genome editing, many domestication syndrome traits can be integrated into minor legumes [128, 142]. Therefore, the conversion of undomesticated, semi-domesticated, wild relatives or minor crop species to full beneficial domestication crop species implies the incorporation of the desired gene from a model species or editing of targeted domestication genes or gene loci [13]. Here, the expression of the modified gene or genes will intuitively confer domestication traits on the intended minor crop species. Consequently, prior knowledge about domestication traits and their controlling gene loci (domestication genes) (**Figure 3**) in the major legume crops is a prerequisite for accomplishing *de novo* crop domestication [6, 31, 143]. Besides, the current scientific research using molecular approaches has equipped us with a deeper insight into several of the mutations which occurred during the era of domestication, the affected gene loci, as well as implicated biological pathways. The type and nature of the mutations have also been well elucidated in many cultivars. The emergence of genomic technology and bioinformatics has further made it easier to isolate these genes for further analysis and utilization in crop breeding [55, 144]. Now, based on the understanding of the causal mutations association with plant phenotypes as well as the genes involved, it has become much more convenient to edit targeted genomic loci by the process of genome editing technology. Consequently, when *de novo* domestication is mentioned, genome editing becomes the focus as it represents the most vigorous molecular-based technique applied in the development of new crops out of the wild and minor species [118]. A clear understanding of domestication traits and their associated genes which are needed for effective and efficient genome editing is obligatorily [6, 111].

7.3 Well established efficient transformation protocol

One of the most important conditions required to achieve a successful goal in *de novo* domestication of a named minor legume is an experimentally established

efficient genetic transformation protocol for the target species [50, 106, 145]. During transformation, especially via plant tissue culture system, a number of factors influence both success and efficiency and must be well established for the target species. This includes selection and optimization of vector construct, Vector constructs and delivery, transgene expression, selection of appropriate explants, and an assessment of overall transformation efficiency [13, 146, 147]. Since this knowledge may not be readily available for many minor crop species, research commitment in optimizing ideal transformation protocol will be practically essential to facilitate *de novo* domestication of undomesticated and semi-domesticated plant species [13, 135, 148, 149]. Whether or not the transformation system involves Agrobacterium tumefaciens- or A. rhizogene-mediated system or by particle bombardment approach, prior knowledge of an experimentally proven protocol is highly ideal to facilitate accomplishing a speedy and desirable result (**Figure 3**).

7.4 Genome sequencing information of the target minor crop legume

To have an available genome sequence of an organism is a fundamentally significant requirement for conducting molecular-based analysis including the identification and isolation of desirable genes for trait improvement programs [132]. The availability of genome sequence information of a target species offers a great opportunity to conduct a successful and resourceful experiment in the breeding of minor species. So far there has been much impetus in sequencing the genomes of some minor legume species. Similarly, interest and commitment to advance genome sequencing projects of several other species have also been reported [150]. For instance, the African Orphan Crops Consortium seeks to embark on genome sequencing projects by targeting over 100 minor plant species including minor legumes [49]. At present, the complete or drafted genome sequences of a number of minor legumes have been released [20, 22, 62, 151]. This facilitates the mapping of quantitative trait loci (QTLs), functional gene isolation, marker-assisted selection as well as genome engineering [152]. Among others, the economic value for nutrition and general food security, raw material, and desirable agronomic traits will likely form part of the major considerations in selecting a minor crop for whole genome sequencing or genome assembly [22]. For minor legumes in which there are readily available reference genome sequences, further genome resequencing experiments have already been conducted to identify target genes [17, 33, 99, 86, 153, 154]. Such available information makes these crops more amenable for genome engineering experiments to improve upon specific traits and for conducting *de novo* domestication of their wild relatives.

7.5 Available reference genome sequence of related species

Knowledge gained by studying domestication events in model crops can be translated into the breeding of related crop wild species as well as minor crops and their wild relatives. This process requires detailed knowledge of the genome features of the model and target plant. That is when a minor legume targeted for *de novo* domestication has no available reference genome sequence but is closely related to a model species at the level of family, genera, or species, knowledge about the model species becomes easier and more applicable for manipulating the minor crop genome by editing targeted genes [155]. Knowledge about genes and genomic features underlying domesticated crop phenotypes are a rich resource for identifying their orthologous genes which must be targeted for *de novo* domestication of minor legume, their wild relatives, or minor crop species as a whole). In this case, ortholog forms of genes known to be associated with the domestication

traits become the target to generate *de novo* genome-edited minor crop (**Figure 3**). For instance, in their experiment, Lemmon et al. [128] studied the wild relative of tomato (Physalis pruinosa) which belongs to the Solanaceae family as the cultivated tomato with many conspicuous phenotypes of P. pruinosa akin to the S. pinpinellifolium. Here, mutating ortholog genes of domesticated tomatoes in the wild relative as possible. The success reports of previous works in *de novo* domestication experiments involving crop wild relatives were in part due to background knowledge about their model species and domestication traits. Therefore, genome editing technology holds immense prospects for creating new crops out of minor crop species.

8. Conclusion and future perspectives

Certainly, the current food crops were domesticated thousands of years ago by the early humans during the period historically termed as the Neolithic era. Following crop domestication, are the various strategies of crop genetic improvement including phenotypic selection, hybridization, mutagenesis, biotechnology, and the most recent tool, genome editing. However, in the past, the application of crop improvement techniques focused mainly on trait enhancement of major crop species resulting in research neglect of minor crop species. This phenomenon caused a majority of crop species to be tagged as minor, orphan, or neglected and under-utilized species. The minor crop species are huge in number and widely spread across the globe. Following the rapid growth rate of the human population, climate change, the continuous reduction of arable lands, and food and nutritional security concerns, there is a need to look back to minor crops and their wild relatives. The recognition of minor crops is based largely on their numerous economic values including adaptation to biotic and abiotic conditions, medicinal endowment, presence of desirable alleles, and potential as model species. Of the 2000 legume species known, only a few including soybean, peanut, have been fully explored and utilized for food, feed, and other agricultural purposes. Efforts have been made in the collection of legume crop wild relatives in order to exploit essential alleles that they harbor for the purpose of genetic improvement of crop species. Legume species play significant roles in the cropping systems for soil nutrient improvement, weed control, and reclamation of wastelands for arable crop production. Minor legumes are specifically important in the quest to meet the protein requirements of all people, especially in parts of the globe where vegetable proteins have become the key source of human protein requirements in diets. To increase the diverse utilization value of minor crops such as for food, feed for livestock, and their potential for soil nutrient management, there is the need for further trait improvement or genetic enhancement of their traits. Current research findings have underscored the prospects in creating new crops out of minor crop species which are deficient in economically desired agronomic traits. One of the recent approaches in the genetic enhancement of minor crop species is de novo crop domestication. While de novo crop domestication approaches are diverse, genetic engineering (or transgenesis) and genome editing are the most rapid ways to generate new crop species. However, over the years, the production and consumption of transgenic crops have always been debated about and hence do not have full acceptance by the general republic. The emergence of genome editing as a molecular breeding tool presents a solution to the limitations associated with transgenic crop production. For instance, using genome editing tools, a loss-of-function mutation occurring within a domesticated gene could potentially confer a desired domesticated phenotype on the target species (redomestication). Similarly, mutations occurring in non-domesticated

genes can give rise to new individuals conferred with domesticated traits (de novo domestication). The multiplex editing potential of the CRISPR-Cas system has been used to achieve simultaneous editing of multiple loci and thus create new edited individuals endowed with a range of complex traits. De novo domestication has previously been successfully applied in genetic modification to increase the trait values of tomato and rice. Considering that minor grain legumes are important food and nutritional security commodities in many parts of the world, and thus the need to increase their food value, the de novo domestication technique holds huge prospects in the genetic enhancement of these underutilized legumes and their wild relatives. The different mechanisms of CRISPR-Cas applications can be employed for genetic enhancement or de novo domestication in minor legume species and their wild relatives to generate new alternative crops. The current challenges that need to be addressed include a boost of public confidence in research findings and the need for openness in the application of modern molecular technology. De novo domestication offers prospects for developing new crops for today and the future in a relatively smart fashion and thus, safeguards food security and agricultural sustainability. While over the years many research efforts have been seen in promoting the utilization of minor crop legume species, there is yet more work to be done in areas including (1) documentation and conservation of minor crop species in gene banks, (2) comprehensive characterization of minor crop species at both phenotypic and molecular levels, (3) collaborative research among domestic and international researchers and institution in identifying and promoting the utility value of minor crops and their wild relatives, (4) research partnership in a multidisciplinary and inter-institutional approach aimed at converting many minor crop species into genome-rich resources and (5) training to increase research expertise in genetic enhancement and domestication of minor crop species and their wild relatives. Overall, it must be reemphasized that genome sequencing of minor crop species is fundamentally requisite in the quest to develop alternative crops since the identification of target gene loci and QTLs are central to achieving successful genome editing experiments and consequently de novo crop domestication. However, it must be noted that de novo domestication via genome editing system may not necessarily be a universal approach to extract the full benefits preserved in minor crop legumes and their wild relatives, instead regional or sub-regional specific approaches should be considered paramount.

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Chapter 7

Legume Genetic Resource Security as Main Requirement for Future Challenges

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Abstract

Evaluating the genetic diversity of landraces has exposed us to the diverse relevance of wild line contributory to a wide range of systems ranging from morphology, physiology, biochemistry, anatomy, toxicity, etc., allowing to their genetic constituent. Today, the world is facing many global challenges. This has put the world in disarray and poses a threat *via* its impact leading to non-promising food security for a rapidly growing population, an increase in the production and release of greenhouse gases as a consequence of anthropogenic activity, and an increase in the level of pollutants in the environment. A well-characterized crop genetic resource is a precondition for effective breeding and genetic conservation in the world of legume security. There is a need to collect, study and conserve legume genetic resource to tackle future challenges. This will help project latent benefits of undescribed leguminous lines of various crop species.

Keywords: genetic diversity, genetic resource, legume security, genetic conservation, wild lines, environment

1. Introduction

Global climate change has contributed to the decrease in food production, increasing the demands for food by the world populace. Impedes to food production can also be owed to terrorism, poverty, natural disasters among others. To meet the global food demands, the focus should be on promoting the cultivation and utilization of other crops, which have been neglected and underexploited but have the potential to enhance food and nutrition securities, especially in the developing countries [1]. Leguminous plants are known to be second to cereals in the entire accessions of crops found in the world genetic resource. There is a need to study extensively the genetic resource of legumes and their underexploited species. Legumes are known for their nitrogen-fixing ability, a powerful tool for soil fertility retainability. Symbiotic bacteria are contained in the nodules of legumes, which help to fix nitrates and supply the host plant with nitrite in exchange for carbon metabolites. Legumes are tagged "poor farmers' crop" because of their significant role in signaling economic benefits relevant to agriculture at a low-input subsistence level.

Despite its use in crop rotation or intercropping, farmers' preference for legumes in agriculture has declined over recent decades. This can be attributed to the certain factors such as production constraints (weed, pest, disease, etc.) and consistent usage of inorganic fertilizers (nitrogen-rich) in agriculture. The practice seems good to many farmers notwithstanding the depleting impacts of nitrogen percolates. The success of future agriculture depends on bridging the gap between ecological sustainability and yield-related economic constraints. If the use of nitrogen-rich fertilizer persists, there is an obligation to proffer an economically and eco-friendly solution to protect soil quality in a sustainable manner through the instrumentality of legume-based conservation agriculture [2] and the development of improved legume varieties with effective rhizobial strains, which can be introduced to different cropping systems. The aim of this review on legumes security is to identify gaps in knowledge that should stimulate the need to prioritize areas in legume research.

2. Legume security

It is quite true that "When the purpose of a thing is not known abuse is inevitable." Interestingly abuse is not limited to wastage but also *underutilization of resources*. Legumes generally are known to have originated from different regions and domesticated in another. The world crop production has revealed farmers-consumer preferences as demand and consumption increase for some crop over another. Leguminous plant species is a typical example of an underutilized crop despite its massive constituent of economic importance. The contribution of legumes to the world food basket is not significant despite its rich sources of dietary protein to millions of people, more so in the developing countries [3]. Leguminous plant species production has been reported to be minimal because of certain production constraints that have discouraged farmers from cultivating them. Legumes are known to be greatly sensitive to the unfavorable environment resulting in unstable and inconsistent yield. This has discouraged farmers from cultivating legumes. This is a major concern whose root needs to be addressed with immediate effects else we keep losing valuable genetic resources ranging from the wilds to landraces and domesticated cultivars. It is quite unfortunate how that some species of *Phaseolus* common to the sub-Saharan African countries are no longer available in the location where they are endemic, most likely the case of other leguminous plant species in places they are known to be *native*. The exploitation of leguminous plant species that are considered to have potentials for greater use by humans, particularly for grain and fodder legumes, is increasingly threatened.

2.1 Leguminous plant genetic resource

Plant genetic resources are plant genetic materials of actual or potential value. They describe the variability within plants that comes from a human and natural selection over millennia. Their intrinsic value mainly concerns agricultural crops. Grain legumes contribute greatly to the world's overall food production. Legumes are the primary source of dietary proteins in many developing countries, where protein hunger and malnutrition are widespread. Grain legumes constitute about 15% of the 7.4 million accessions conserved globally in gene banks, of which more than half of germplasm in gene banks have not been characterized and lack evaluated data, which ultimately limit the utilization and exploitation of germplasm in legume improvement programs. Characterization of all gene bank accessions should be of prime priority for enhancing the utilization. Legumes are among

the most valuable gifts of nature to man, animals, and the environment. They are sustainable, affordable, water-efficient, and low-carbon footprint crops.

The development of core, mini-core, reference sets, and trait-specific germplasm has presented a platform for breeders to exploit gene banks for possible improvement of crops. New sources of variation were easily identified with these developed lines (genotypes), but notwithstanding there is still a need to evaluate these collections for unique and rare traits undisclosed and underutilized. Generally, crop species such as leguminous species known for their narrow genetic base get to be widened by adopting a breeding approach, simply by the utilization of crop wild relatives and new resources of legume cultivars [1]. Legumes and cereals among other crop plants played an impeccable role owing to the development of modern-day agriculture. The legume family, *Fabaceae*, is rated one of the first three largest families of flowering plants, with 946 genera and 24,505 species respectively according to hierarchical classification [2]. For most non-wild cultivars, they have proven to be incontestable in their nutritional contents and value for both humans and animals which attest to their recognition as the second most important plant source of nutrients [3]. Legumes are extensively distributed in diverse agroclimatic zones globally, from the mountain and north pole regions to the tropic and subtropics.

Specific features of legumes include taproot, trifoliate leaves, flower with corolla and petals (winged), and keel, which facilitate nitrogen fixation in the soil. The family is composed of three subfamilies, namely, *Caesalpinioideae*, *Mimosoideae*, and *Papilionoideae* [4]. Among them, the subfamily *Papilionoideae* is of great economic importance as it constitutes majorly most of the commercially known leguminous crop species. Naturally distributed among pulses are *Lathyrus* and *Vicia*, which have the largest number of the genus.

Legumes perform a significant role in meeting humans' and animals' nutritional and dietary needs. The major known grain legumes include cowpea, chickpea, lentils, dry beans, pigeon pea, green gram, fava beans, and black gram. Soybean and groundnuts are industrially utilized and known to be oil-producing legumes. The vegetable types of legumes identified include beans, yard long bean, and garden pea, consumed as immature seeds and pods. Lucerne, berseem, and grass pea serve as forage legumes inclusive of cowpea, while tuber legume consists of zombi pea, winged bean, African Yam bean (now beginning to gain attention), etc. *Abrus precatorius* possesses poisonous seeds that contain the toxin abrin. Additionally, grain legumes such as cluster bean, horse gram, moth bean, and pillipesara are underutilized promising legumes primarily grown in the Indian subcontinent, China, and Southeast Asia, and they are also equally important in ensuring food and nutritional security.

Legumes are the reservoir of protein, carbohydrate, fiber, and other minerals in trace amounts. In addition to these, legumes contain constituents that are beneficial to the health of humans and animals. Too much but a few of such constituents include low glycemic index (GI), which makes them superfood that provides long-term health benefits. The isoflavone content in legumes (soybean, chickpea, fava beans, groundnut, etc.) plays a role in plant defense [5] and improvement in human health can also be traced to root nodulation. Legumes serve as fodders (vegetative parts) for livestock. Nitrogen fixation is very peculiar to legumes through which the fertility and texture of the soil are enriched and improved for other crops to thrive adequately. Legumes also play a vital role in the intercropping system [6].

Hence, there is a need to explore sustainable improvable working strategies to develop and diversify legume production. To make progress to the exploration plan, there is a need to adopt diverse genetic resources in any crop improvement program. This can be considered a most suitable sustainable strategy among others

to conserve vital genetic resources for the future. Germplasm with a rich reserve of genetic diversity would forever remain a powerful tool in any crop improvement program. Reviews have it that globally, gene banks hold about 1 million accessions of the leguminous crop.

A vast category of genetic resources is conserved *ex situ* in gene banks, wherein a considerable amount of reserves remains underutilized in nature. Hence, it becomes a matter of concern and priority to collect the maximum amount of diverse genetic resources into the germplasm before it is lost forever. Recently on the Plateaus (in Nigeria), some lines of *Phaseolus* were discovered to be extinct and no longer available in a location within the region where it is expected to be endemic (Bokkos and Mangu). Crop wild relatives (CWR) are the reservoir of genes for breeding. To explore the potential of CWRs in today's changing climate, collection and conservation have to be of the topmost priority else we are left with no tool to improve cultivars.

For progress in the sustainability in agricultural production, "*Conservation through use*" approach is a possible way. Continuous storage of the genetic resources in gene banks will not solve the purpose until it is effectively and judiciously utilized. In handling germplasm, genetic integrity is required and should be maintained solely to the end that the variability of genetic resources would still be available for use in the future majorly in conventional breeding programs (this cannot be over-emphasized notwithstanding advances in technology). It is so unfortunate how an ample amount of genetic resources available in gene banks are without characterization and evaluated data.

Genetic resources are the fourth most essential input after water, soil, and light. It is relatable to harness legumes to solve global challenges such as population explosion, land infertility, malnutrition, and hunger. There is not much of a priority on leguminous crop plants and hence get masked by cereal production across the globe. In addition, farmers no longer find it appealing to cultivate legumes for either consumption or profit-oriented. This has led to a substantial decrease in research on legumes. Global climatic change and environmental instability have in a way to pose a strong need for research on landraces and crop wild relative of legumes in an effective manner, although still at a threshold state. Legumes have the potential to contribute significantly to the economy and ecological framework of (eco-friendly agricultural land use and sustainable forage production) a community particularly in the tropics [7].

Initially, the purpose of germplasm has been to preserve genetic resources only, but recently, attention has shifted to conservation through use. Interestingly, legume genetic resource has been harnessed to develop agro-ecological cultivars, which include zombi pea, winged bean, grass pea, etc., with new alleles, which has helped in developing biotic and abiotic stress-tolerant varieties. Making such progress for sustainability in agriculture would be labor in futility if we fail to identify various possible constraints to the utilization of germplasm tools for legume production. With the current advancement in technology, trait discovery and markers-assisted selection of traits need to be explored for possible large-scale screens to eventually help to reveal the latent genetic potential of the legumes' germplasm conserved in the gene banks.

2.2 The underutilization of germplasm is a route to legumes extinct

Germplasm is the lifeline and heart of plant breeding. It is the genetic tool used to preserve the genetic pool of crop species. There is no plant breeding program without a germplasm reserve. The management of legume genetic resources begins with germplasm collection, conservation, identification, characterization,

evaluation, and documentation. The most research institute has worked tirelessly to ensure a proper management of legume genetic resource. The CGIAR centers such as CIAT (Centro Internacional de Agricultura Tropical), ICARDA (International Center for Agricultural Research on Dryland Agriculture), ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), and IITA (International Institute for Tropical Agriculture) remain custodians of the largest germplasm collections for bean, chickpea, cowpea, faba bean, lentil, and pigeon pea, while the Australian gene bank (ATFCC, Australian Tropical Crops & Forage Genetic Resources Center) has the largest collection of pea germplasm. It is of interest to know that from the known gene banks, legumes constitute about 15% of the whole accessions [8].

Based on plant utilization and conservation, legumes are categorized into mostly and less cultivated species. Legumes categorized as mostly cultivated are popular and common with the well-established domestication, agronomic practices, utilization, and conservation. Examples include broad bean (*Vicia faba*), chicken pea (*Cicer arietinum* L.), cowpea (*Vigna unguiculata* L.), groundnut (*Arachis hypogaea* L.), pea (*Pisum sativum* L.), common beans (*Phaseolus vulgaris* L.), soybean (*Glycine max* L.), among others. The less cultivated legumes are scarcely known, less exploited, neglected, and considered underutilized. Several species in this category include rice bean (*Vigna angularis* L.), Hyacinth bean (*Lablab purpureus* L.), winged bean (*Psophocarpus tetragonolobus* L.), jack and sword bean (*Canavalia sp*.), pigeon pea (*Cajanus cajan* L.), lima bean (*Phaseolus lunatus* L.), mung bean (*Vigna mungo* L.), bambara groundnut (*Vigna subterranea* L.), African yam bean (*Sphenostylis stenocarpa* H.), marama bean (*Tylosema esculentum* L.), and Hausa groundnut (*Macrotyloma geocarpa* H). The wild species of the less cultivated grain legumes include Hausa groundnut (*Kerstingiella geocarpa* H), marama bean (*T. esculentum*), and the wild Vigna species such as *V. ambacensis*, *V. vexillata*, *V. luteola*, *V. oblongifolia*, and *V. racemosa*, among others. As stated above, several of these species are natives of sub-Saharan African countries and could be explored for food, medicine, agriculture (as to cover crops and fodder), and more importantly for genetic improvement of cowpea (possible sister lines) and related species [9, 10].

Detailed germplasm study (collection, identification, characterization, conservation, evaluation documentation, and cataloging) on leguminous plant species would not be attainable as attention is focused on specific cultivars over others. Beyond the *ex situ* and *in situ*, there is a need to (i) evaluate the morphological and biochemical traits in wild lines of minor legumes, (ii) do better cataloging that would communicate the characterized feature desired by a breeder to initiating a breeding program, and (iii) reduce the selective pressure on major legumes to avoid genetic erosion of certain of this species. Most legumes do not have improved cultivars developed from breeding initiatives. This is true of many underutilized plant species that mostly exist as landraces with many potential genetic bottlenecks and constraints on both the available genetic diversity and its distribution within and between landraces [11].

2.2.1 Plant domestication: a step forward to germplasm utilization

Genetic erosion due to the collection of new strains from known populations and domestication deficits are the two main characteristics of cultivated crops. Domestication disorder is defined as the modification in the physiology and morphology of cultivated crops that make them different from their wild ancestors, enabling them to adapt to deliberate cultivation by a man called agriculture [12, 13]. Some of these include loss of germination inhibition, changes in growth habits, seed dispersal mechanisms, etc. Different regions of the earth have contributed

to the modification in cultivars independently [14]. A technical overview is long designed to separate known domestication with crop varietal traits, that is, between short incidences and ancient processes such as cultivation [15]. It is worthy of note that paleoethnobotany has also been categorized into various forms of domestication in relation to leguminous and nonleguminous crop plants (grain and forage). Regarding the seed size, grain legumes do not show evidence of seed size increase with domestication whereas forage legumes do (or ards) [16]. Others have proposed that the sowing depth by humans might have contributed in instances to increase the biomass of the seed, but this did not seem to have a firm premise after testing [17]. This explains how agriculture adopts both art and science to function together on the available genetic diversity of plants. Plant domestication is incomplete without a discourse on selection. Provided the practice of cultivation and managements are known to being strong selection pressures during the domestication of crops, and it is expedient to study the preference and decision of humans [18]. There is an increasing indication suggesting that humans have actively changed certain ecosystems to increase the availability of certain plant resources centuries before the appearance of the pointers of domestication [19]. Notwithstanding the recent happenings, it is promising to evaluate the advance and prospects of the trends in domestication 0f germplasm [20]. Finally, it is important to bring to mind the recent occurrences of plant collection and domestication process [21] as it is not only an old practice. There is still great potential yet with domestications of germplasm with the unprecedented development of conservation tools that would allow us to produce higher and improved strains for quality food for consumers globally.

2.3 Ecological significance of legume

The nitrogen-fixing ability of leguminous plants is of crucial importance in agriculture. Prior to the use of fertilizer supplements in the developing countries of the world, the cultivation of crop plants aside from rice was dependent on legumes and waste from plants and animals for nitrogen fertilization. Crop rotation is a common practice usually carried out by alternating an economic crop such as corn (maize) with a legume, often alfalfa (*Medicago sativa*) as seen in the temperate world. Legumes are also known for their usage as animal forage (hay or silage). Pastures or other grazing areas must have legume forages, such as alfalfa (*M. sativa*), Clover (*Trifolium repens*), Gliricidia (*Gliricidia sepium*), Hyacinth bean (*L. purpureus*). Meanwhile, most of the vegetation of forests, grasslands, and deserts of the world are primarily dependent on forage legumes and could not exist without them. Ecosystems with few legume species have alternate biological means for fixing nitrogen. Too much but a few of the biological means include a symbiotic association between woody species other than legumes, actinomycetes, or bacteria and are limited mostly to boreal evergreen forests and certain coastal areas. Nitrogen fixation by free-living cyanobacteria seems to be important in aquatic ecosystems. However, irrespective of the alternative mechanisms for nitrogen fixation, they are relatively secondary to legumes.

2.4 Supplementary functions of rhizobia relationship

Legumes have the ability to form a symbiotic relationship with rhizobia (a nitrogen-fixing bacteria). A specialized organ in legumes called nodules embeds the bacteria, wherein the concentration of oxygen is very low, allowing the enzyme nitrogenase to fix atmospheric nitrogen gas. Studies on *Medicago truncatula* (Clover) have shown that nitrogenase iron-molybdenum cofactor and nitrogenase activity are synthesized by *M. truncatula* molybdate transporter (MtMOT).

The identification and characterization of regulatory components contributing to nodulation can make an offset of genetic targets and polymorphic markers to enhance the selection of superior legumes cultivars and rhizobia strains that promote food security and agricultural sustainability [22, 23].

Communications through chemical signals are the initial steps that define plant-microbe interactions, especially when between considerable inter-species. The initial recognition in the rhizosphere requires the release of some plant metabolites including flavonoids, strigolactones, and N-acetylglucosamine as well as microbial nod factors, which are lipochitooligo saccharides creating the obnoxious environment for pathogens. The legume host maintains and manages the number of nodules; it forms in association with the nitrogen-fixing rhizobial partner. This enables the plant to balance its need to acquire nitrogen with its ability to expend resources developing and maintaining nodules. Molecular mechanisms are involved in the said process [24].

The interactions between legumes and different symbiotic partners are not mutually exclusive. Moreover, reports have it that tripartite associations between legumes, rhizobia, and mycorrhiza are beneficial [25], which explored carbon allocation and the availability of resources in *M. truncatula*. Such tripartite interactions led to synergistic growth responses and stimulated the phosphate and nitrogen uptake of the plants, which allocated more carbon to rhizobia under nitrogen demand, but more carbon to the fungal partner when nitrogen was available [25]. The changes in carbon allocation were accompanied by changes in the expression of sucrose transporters, providing insights into how the host plant controls carbon allocation to different root symbionts to maximize its symbiotic benefits. A study on the effects of arbuscular mycorrhiza on plant growth and gene expression was illustrated. Twenty (20) geographically diverse *M. truncatula* accessions inoculated with the AMF *Funneliformis mosseae*, a diverse range of responses in plant physiology and gene expression, were observed among the accessions [26]. Physiological and genetic responses from the legume-rhizobia symbiotic relationship have opened up possible prospects in controlling pathogens beyond the nitrogen fixation.

Consequently, there is minimal knowledge on the resistance mechanisms against soil-borne pathogens in grain legumes, providing evidence for genetic variation of rhizosphere-related traits. The role played by root exudation in microbes-mediated disease resistance is considered together with how such characters can be introduced into legumes breeding programs [27]. There is a strong need to adopt the collection, characterization, and domestication of closely related wild lines of *M. truncatula* or other possible cultivars that serve the same functions, to be holobiont in future breeding strategies seeking to improve complex defense mechanisms in leguminous crop plants *via* nodulations as described above.

2.5 Prospects for legume production in Africa

Legumes have culturally played a key role in African agriculture on the basis of the provision of natural fertilization to the soil for small-scale farmers and have also been a cheap source of protein to African consumers. Current data from the Food and Agriculture Organization (FAO) of the United Nations legume crops in Africa were modeled [28]. FAOstat reported in 2020 that about 21,303,488 tonnes of legumes are produced in Africa, a prospect for the future of leguminous crop plants in Africa. The upscale in cultivation, production, and processing of underutilized leguminous crop plants may serve to reduce dependence on oil-producing legumes. This will generate economic opportunities as well as the ecological refurbishment through the development of legumes-based supply chains across different

producers, consumers, and regions. However, the consistent production of legumes across Africa will require not only an intensive research and development effort but also the backing of active extension services accompanied by the food chain and marketing assurance and government policy incentives.

The deficient in microelements is among the most common and disturbing global nutritional problems, posing serious health challenges within the African population. Micronutrient deficiencies have increased in recent decades due to a decrease in the quality of the diets, in both developing and developed countries. The problem is further aggravated by widespread poverty, where access to the more expensive but nutrient-rich products are difficult. Meanwhile, supplements are available to easy-to-reach consumer groups at a relatively low cost. This strategy is not sustainable in a long run and it does not build consistency in a population. Moreover, supplementation requires an intricate distribution network as it often escapes the vulnerable groups and the rural poor supplementation strategies that have therefore only achieved modest success, even in African countries that have responsive legislation and processing capacities. Legumes are major sources of dietary protein, particularly in agriculture, a developing sector of the economy. These dietary proteins are significant for nutritional trait improvement in crop breeding programs. Bio-fortified legumes would offer a diversity of micronutrients and amino acids [29], necessary to complement the comprehensive evaluation of the challenges in breeding approaches that are being used for the nutritional enhancements of leguminous crop plants. The potential of the legume microbiome in the agronomic trait improvement is also an important prospect in agricultural research.

3. Conclusion

There is a need to make rich the advance in plant genetic resource *via* the careful handling and management of germplasm to avoid genetic erosion of leguminous plants of economic importance. Underutilized legume species should be characterized, evaluated (morphologically and biochemically), and well cataloged for subsequent use by breeders for genetic gain and advance. Legumes in general are used to revive nutrient-depleted soils, especially for abandoned agricultural and grazing lands. Generally speaking, native legumes are common in these habitats because they are able to survive nitrogen-poor soils than other plants. They also produce secondary compounds such as alkaloids, flavonoids, terpenoids naturally that provide protection against predators. Some of these secondary compounds are being studied for their pharmacological potential. They are found in the leaves and fruiting parts. Owing to the future prospects in legumes there is a strong need to preserve the genetic resource *ex situ* and *in situ*.

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Conflict of interest

The authors declare that they have not known any competing financial interests or personal relationships that could have appeared to influence the work reported in this book.

Acronyms and abbreviations

AON	autoregulation of nodulation
FAO	Food and Agriculture Organization
N_2	nitrogen
AMF	arbuscular mycorrhizal fungi
MtMOT	Medicago truncatula molybdate transporter
LysM	lysin motif
AM	arbuscular mycorrhizal
RL	rhizobium-legume
RLK	receptor-like kinase
Germplasm	it is the lifeline and heart of plant breeding. It is the genetic tool used to preserve the genetic pool of crop species
Symbiotic	it is a close and long-term biological interaction between two different biological plant organisms
Mycorrhiza	it is the role of fungus in the plant's rhizosphere, its root system. The mutual symbiotic association between a fungus and a plant could also be termed mycorrhizae
Rhizobia	they are diazotrophic bacteria that fix nitrogen after becoming established inside the root nodules of legumes (Fabaceae)
Genetic	this arose out of the identification of *genes*, the fundamental units responsible for heredity
Nitrogen	it is essential to life on Earth. It is a component of all proteins, and it can be found in all living systems
Microbiome	it is the genetic material of all the microbes—bacteria, fungi, protozoa, and viruses
Agronomic	it is the science and technology of producing and using plants in agriculture for food, fuel, fiber, recreation, and land restoration
Nodulation	*Nodulation* involves the production of a special organ, the *nodule*, and also what has been called a novel organelle, the symbiosome, consisting of nitrogen-fixing bacteroids enclosed in a primarily host-derived peribacteroid membrane
Trifolium	red clover belongs to the Fabaceae family, is a legume, and has long been provided noteworthy contributions to agricultural and animal production all over the world
Kinases	it is an enzyme that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecules to specific substrates
holobiont	it is an assemblage of a host and the many other species living in or around it, which together form a discrete ecological unit, though there is controversy over this discreteness

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Section 2

Legumes Physiology

Chapter 8

Molecular and Functional Characterisation of Allergenic Non-specific Lipid Transfer Proteins of Sweet Lupin Seed Species

Maria Rodrigo-Garcia, Esther Rodriguez-de Haro, Salvador Priego-Poyato, Elena Lima-Cabello, Sonia Morales-Santana and Jose C. Jimenez-Lopez

Abstract

Non-specific lipid transfer proteins (nsLTPs) are small proteins abundant in plants, which function in transferring phospholipids and galactolipids across the membrane. nsLTPs also play a key role in plant resistance to biotic and abiotic stresses, growth and development, as well as in sexual reproduction, seed development, and germination. In addition, these proteins have previously been identified as food allergens. In the present study, we carried out a molecular and functional comparative characterisation of 25 sequences of nsLTPs of lupin legumes and other species. Extensive analysis was carried out; including comparison of databases, phylogeny, physical–chemical properties, functional properties of post-translational modifications, protein structure conservation, 2-D and 3D modelling, functional interaction analysis, and allergenicity including identification of IgE, T-cell, and B-cell binding epitopes. The results indicated that particular structural features of nsLTPs are essential to the functionality of these proteins, high level of structural stability and conservation. Information about different functional interactions between nsLTPs and ligands showed that nsLTPs can accommodate several of them with different structure; and that the relationship between structure and allergenicity was investigated through the identification of epitopes susceptible of being involved in cross-reactivity between species of the *Fabaceae* family.

Keywords: *Lupinus angustifolius*, PULSE, nsLTP, legume, seed allergenic proteins, food allergies, cross-reactivity

1. Introduction

Sweet lupin group has four lupin species currently used for food, namely, *L. angustifolius*, *L. albus*, *L. luteus*, and *L. mutabilis*. Lupin seed contain large amount of proteins ranging between 38 and 52%, depending of the species and cultivar [1]. The protein content of sweet lupin is usually higher compared to other legumes, i.e.

pea, soya, or lentil. Main protein content of lupine seed belongs to two families called globulins (80–94%) and albumins (5–15.4%) [2, 3], while other proteins (glutelins and prolamins) are in low quantities [4].

Globulins are the most abundant proteins in sweet lupin group seeds and the most polymorphic family in terms of gene and protein sequence [5]. Globulins comprise different families of seed storage proteins (SSPs): α-conglutins (legumins or 11S type globulins), β-conglutins (vicilins or type 7S globulins), γ-conglutins (basic 7S type globulins); and δ-conglutins, and others in much more less amount as 2S sulphur-rich albumins, LTPs, profilin, PRP [3, 5].

L. angustifolius and *L. albus* are particularly suitable for food because their nutritional and nutraceutical properties, help in preventing diseases such as diabetes, digestive tract and cardiovascular diseases, overweight, obesity or cancer, while reducing celiac disease problems as lupine does not contain gluten [4, 6].

Currently, products based on lupine proteins are gaining more attention in the food industry, due to their low cost, and the high demand for sustainable foods [4, 7, 8]. Besides important techno-functional (physical and chemical) properties, such as high water retention capacity and great emulsifying and foaming capacity, lupine flours or lupine protein concentrates have been used to formulate and substitute technological agents in baked, meat, and dairy products by the industry food [4].

Interestingly, and despite the great health benefits of lupin seeds, they are also a source of anti-nutritional factors such as phytic acids, saponins, phenolic compounds, enzyme inhibitors, lectins and hemagglutinins. The most problematic factors are the alkaloids because their bitter taste provided to the food [9, 10]. Fortunately, recent alkaloid content [3, 7, 11]. Some of these anti-nutritional factors an cause adverse physiological effects if they are consumed by animals while others (i.e. polyphenols and oxalates) limit the bioavailability of minerals from foods [9, 10].

Nevertheless, lupine was labelled in 2008 as an allergen in packaged foods, as recommended by the European Food Safety Authority (EFSA, http://www.efsa.europa.eu/) [7, 11]. According to the list of allergens provided in the databases of the Allergen Nomenclature Subcommittee of the World Health Organisation, the International Union of Immune Societies and Allergome (WHO; UISI, http://www.allergen.org/; http://www.allergome.org/), where the main lupine allergen is β globulins and other minor fractions such as non-specific lipid transfer proteins (nsLTP) (Lup an 3) has high relevance because their cross-reactivity [4].

Lupine allergy is normally mediated by Immunoglobulin E (IgE) and allergic reactions to lupine can occur *via* ingestion, inhalation, or occupational exposure [7]. For example, exposure to lupine *via* the respiratory tract can be considered as the primary reason for allergic sensitization in food industry workers [4, 12–14]. Co-sensitization *via* inhalation has also been proposed as a common cause of lupine and wheat allergy among bakers [4, 14]. Indirect sensitization to lupine proteins can also occur through cross-reactivity with other legumes and particularly in previous peanut allergy patients [4, 15–18]. Clinical symptoms can vary in intensity and severity, including asthma, allergic rhinitis, urticaria, nausea or gastrointestinal pain, and anaphylaxis [4, 19].

Plant nsLTPs are small extracellular proteins, which includes a significant number of allergens [20–22]. They are usually located in the outer layers of the shell of fruits and seeds and their allergenic potency can be reduced when are removed [4, 21, 23]. It has been observed that its molecular characteristics, such as its great stability against proteolysis, thermal denaturation and cross-reactivity, are linked to its allergenicity [20]. Sensitization to nsLTPs may depend on geographic differences, sensitization pathways, type of diet, and is often associated with severe symptoms [24]. In this regard, lupine β and γ conglutins may correlate with the severity of clinical reactions [4, 16], although more families may be involved.

Recently, an nsLTP was identified and included by the WHO/IUIS as an allergenic food protein in *L. angustifolius* (Lup an 3) [4].

Structural homologies of lupine allergens or commonly shared epitopes with other legume allergens lead to support cross-reactivity reactions between them [4]. The present study carries out the molecular and functional characterisation of proteins of the non-specific lipid transfer proteins (nsLTPs) family of the lupine seed (*Lupinus angustifolius* L.), compared to other of legumes and plant species as olive tree (*Olea europaea* L). For this purpose, we identified nsLTP sequences expressed in *L. angostifolius* seed, classifying and analysing phylogenetic relationships among them, the functional and the molecular processes that they are involved; we also analysed the proteins at a structural level, identifying potential motifs implicated in functional differences; and we established the potential allergenicity of the nsLTPs through identification and analysis of different epitopes involved in allergy phenomenon.

2. Material and methods

2.1 nsLTPs sequences of lupine, legumes and other plant species

Different gen and protein databases were used to search and retrieved nsLTPs from legume species and other model plants: NCBI (https://www.ncbi.nlm.nih.gov/), Uniprot (https://www.uniprot.org/), Allergome (http://www.allergome.org/index.php), and reprOlive (http://www.scbi.uma.es/olivodb/).

We retrieved 25 sequences as follow: The sequences and their access number are: *Lupinus angustifolius* (Lup an 3) Uniprot: A0A1J7GK90, *Lupinus angustifolius* (Lup an 3.0101) (Uniprot: A0A4P1RWD8), *Medicago truncatula* (Uniprot: A0A072UTH7), *Arabidopsis thaliana* (nsLTP-3) (Uniprot: Q9LLR7), *Arabidopsis thaliana* (nsLTP-5) (Uniprot: Q9XFS7), *Olea europaea* (Ole e 7) (NCBI: XP_022893508.1), *Lupinus albus* (Uniprot: A0A6A5MQ88), *Lupinus angustifolius* (Uniprot: A0A4P1RV83), *Glycine max* (Uniprot: I1J7M1), *Arachis hypogaea* (NCBI: XP_025656480.1), *Cajanus cajan* (NCBI: XP_020237462), *Phaseolus vulgaris* (Uniprot: D3W146), *Glycine soja* (Uniprot: A0A445M2F4), *Lens culinaris* (Uniprot: A0AT33), *Trifolium pratense* (Uniprot: A0A2K3M7A7), *Spatholubus suberectus* (NCBI: TKY63608.1), *Cicer arietinum* (Uniprot: O23758), *Vigna ungiculata* (Uniprot: UPI0010170F74), *Abrus precatorus* (Uniprot: UPI000F7C313B), *Arachis ipaensis* (NCBI: XP_020971907.1), *Trifolium subterraneum* (NCBI: GAU29990.1), *Prosopis alba* (NCBI: XP_028808641.1), *Vigna angularis* (NCBI: KOM57753.1), *Arachis duranensis* (NCBI: XP_015950831.1), *Pisum sativum* (NCBI: A0A158V755.1).

2.2 Multiple alignments of nsLTPs sequences of lupine and other species

We carried out multiple alignments with the 25 amino acid sequences previously obtained with the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). In addition, partial alignments with different number of sequences were also performed to be sure that reproducibility of these analysis was covered. The alignment was verified manually with Bioedit v7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and Jalview 2.11.1.4.

2.3 Phylogenetic analysis of the 25 nsLTPs sequences of lupine and other species

Different simulations of the phylogenetic analysis of the sequences were carried out with the multiple amino acid alignments, assuring accuracy and reproducibility.

It was analysed using the MEGA-X software, with the neighbour-joining method, including bootstrap defined by the software, following the Poisson model, with Uniform Rates¸ Pairwise Deletion and using 4 threads.

2.4 Physical and chemical properties analysis of nsLTPs

We used the tool Protparam (https://web.expasy.org/protparam/). We analysed isoelectric point (pI), aliphatic index (AI), and instability index (II) among others.

2.5 Functional motifs analysis

Domains and functional motifs were analysed using PfamScan (https://www.ebi.ac.uk/Tools/pfa/pfamscan/), Pfam (http://pfam.xfam.org/search#tabview=tab0), and ScanProsite (https://prosite.expasy.org/scanprosite/). The use of all these tools assured accuracy and reproducibility in the analysis.

2.6 Post-translational (functional) modifications of the nsLTPs proteins

We identified different post-translational modifications such as N-glycosylations, N-myristoylation, and phosphorylation sites for casein kinase (CK2), protein kinase C (PKC), and cAMP-dependent protein kinase (PKA) using ScanProsite (https://prosite.expasy.org/scanprosite/). We also identified post-translational modifications related to stress and REDOX regulation such as S-nitrosylation of cysteine using iSNOAAPair (http://app.aporc.org/iSNO-AAPair), and N-nitrations of tyrosine with GPS-YNO2 (http://yno2.biocuckoo.org) [25]. Carbonylation sites were identified by iCarPS (http://lin-group.cn/server/iCarPS/webServer.html). NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict phosphorylation sites. NetAcet-1.0 was used to check acetylations (https://services.healthtech.dtu.dk/service.php?NetAcet-1.0). The use of all these tools assured accuracy and reproducibility in the analysis.

2.7 Subcellular location of nsLTPs proteins

The subcellular localization was identified using pSORT (https://www.genscript.com/psort.html, http://psort1.hgc.jp/form.html), WoLF SORT (https://wolfpsort.hgc.jp/, https://www.genscript.com/wolf-psort.html) and CELLO V 2.5 (http://cello.life.nctu.edu.tw/). Subsequently, verification of the extracellular, mitochondrial, and chloroplastidial localization was made by the TargetP (http://www.cbs.dtu.dk/services/TargetP/) tool. The use of all these comparative tools assured accuracy and reproducibility in the analysis.

2.8 Secondary structure (2D) prediction of nsLTPs

The prediction of the secondary structure of nsLTPs was carried out using the PSIPRED program (http://bioinf.cs.ucl.ac.uk/psipred/.

2.9 3D structure of nsLTPs

To build the 3D structure, we used the bioinformatics tools I-TASSER (https://zhanglab.dcmb.med.umich.edu/I-TASSER/) and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The figures were drawn using the PyMOL program.

2.10 Conservational study of nsLTPs proteins in different species

The Consurf server tool (https://consurf.tau.ac.il/) was used for this purpose.

2.11 Functional interactomics analysis of nsLTPs

To carry out the interactomics analysis, the STRING tool was used to predict the interactomics analysis (https://string-db.org/cgi/input?sessionId=bwP3HkaoJSDc&input_page_show_search=on) using *Medicago truncatula* as a model species for the lupine sequences, and *Arabidopsis thaliana* for the olive sequence.

2.12 nsLTPs and multiple ligands binding analysis study

I-TASSER tool (https://zhanglab.dcmb.med.umich.edu/I-TASSER/) was used to identify the multiple ligands of nsLTPs.

2.13 Allergenicity study and identification of allergenic epitopes from nsLTPs

The selected allergen families of LTPs were obtained in the Allergome database (http://www.allergome.org/index.php). AlgPred tool (https://webs.iiitd.edu.in/raghava/algpred/submission.html) was used to carry out the study of IgE binding epitopes. It was analysed whether the protein sequences present experimentally tested IgE binding epitopes as allergen representative peptides (ARPs); if they present epitope motifs, with the MEME / MAST tool that forms matrices from sequences of known allergens; and the allergenicity potential of the 25 protein sequences was determined, based on the amino acid and dipeptide composition.

2.14 T-cell epitopes identification and analysis in nsLTPs

To carry out these T-cell binding epitope identification studies, we used the tool ProPred (https://webs.iiitd.edu.in/raghava/propred/). Identification of MHC II binding regions was carried out for the 25 amino acid sequences from lupine, olive, and other legumes using quantitative matrices. A threshold of 3% was set for the most common human HLA-DR alleles among the Caucasian population: DRB1*0101 (DR1), DRB1*0301 (DR3), DRB1*0401 (DR4), DRB1*0701 (DR7), DRB1*0801 (DR8), DRB1*1101 (DR5) and DRB1*1501 (DR2). The epitope sequences shared by three or more HLA II analysed were annotated.

2.15 B-cell epitopes identification and analysis in nsLTPs

For the identification of B-cell binding epitopes, we used the tool Bcepred (https://webs.iiitd.edu.in/raghava/bcepred/bcepred_submission.html). The 25 protein sequences of lupine, olive, and other legumes were analysed. Regarding the values for the identification of B cell epitopes, we used predetermined threshold values, being the most suitable for the study that we carried out for each of the analysed characteristics: hydrophilicity, accessibility, surface exposure, antigenic propensity, flexibility, turns, polarity, and the combination of all.

3. Results and discussion

3.1 Multiple alignments of nsLTPs proteins and phylogenetic analysis

Table 1 shows the list of nsLTPs sequences analysed with their functional domains. **Figure 1** shows the multiple alignments of 8 representative protein sequences of nsLTPs of such as Lup an 3, Lup an 3.0101, *Medicago truncatula nsLTP*, *A. thaliana* (nsLTP-3), *A. thaliana* (nsLTP-5), *L. angustifolius*, and *L. albus*. A large representative number of nsLTPs in a general alignment (**Figure A1**). The conservation of each residue in the alignment is shown with bars. Overall, the most conserved amino acids were found in the regions between the position 30 to 60 and in the N-terminal regions of the protein.

Scientific name and accession number	Number of aminoacids	*Pfam features*	Prosite (ID)
Lupinus angustifolius (Lup an 3) *(Uniprot: A0A1J7GK90)*	120	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597)
Lupinus angustifolius (Lup an 3.0101) *(Uniprot: A0A4P1RWD8)*	116	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [94-115]
Medicago truncatula (Uniprot: A0A072UTH7)	116	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [93-114]
Arabidopsis thaliana (nsLTP-3) *(Uniprot: Q9LLR7)*	115	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [93-114]
Arabidopsis thaliana (nsLTP-5) *(Uniprot: Q9XFS7)*	104	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482	PLANT_LTP (PS00597) [82-103]
Olea europea L. (Ole e 7) *(NCBI: XP_022893508.1)*	117	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [95-116]
Lupinus albus (Uniprot: A0A6A5MQ88)	132	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	—
Lupinus angustifolius (Uniprot: A0A4P1RV83)	131	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597)
Glycine max (Uniprot: I1J7M1)	117	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [95-116]
Arachis hypogaea (NCBI: XP_025656480.1)	129	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [95-116]
Cajanus cajan (NCBI: XP_020237462)	121	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [99-120]
Phaseolus vulgaris (Uniprot: D3W146)	115	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [93-114]

Scientific name and accession number	Number of aminoacids	*Pfam features*	Prosite (ID)
Glycine soja (Uniprot: A0A445M2F4)	115	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	—
Lens culinaris (Uniprot: A0AT33)	110	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [87-108]
Trifolium pratense (Uniprot: A0A2K3M7A7)	130	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	LEUCINE_ZIPPER (PS00029) [84-105]
Spatholobus suberectus (NCBI: TKY63608.1)	165	Tryp_alpha_amyl (Inhibidor proteasa/almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	LEUCINE_ZIPPER (PS00029) [6-27; 13-34]
Cicer arientinum (Uniprot: O23758)	116	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [93-114]
Vigna unguiculata (Uniprot: UPI0010170F74)	117	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [95-116]
Abrus precatorius (Uniprot: UPI000F7C313B)	124	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [102-123]
Arachis ipaensis (NCBI: XP_020971907.1)	118	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [95-116]
Trifolium subterraneum (NCBI: GAU29990.1)	123	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	—
Prosopis alba (NCBI: XP_028808641.1)	117	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [94-115]
Vigna angularis (NCBI: KOM57753.1)	122	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	—
Arachis duranensis (NCBI: XP_015950831.1)	117	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [95-116]
Pisum sativum (NCBI: A0A158V755.1)	120	Tryp_alpha_amyl (Inhibidor proteasa/ almacenamiento semillas/familia LTP) Clan: Prolamina (CL0482)	PLANT_LTP (PS00597) [97-118]

Table 1.
Domains and functional motives of nsLTPs.

nsLTPs can be considered as basic proteins with high identities among their sequences [20]. The sequences sharing high identity within the alignment are Lup an 3, *M. truncatula*, and Lup an 3.0101, as well as the sequences of nsLTPs from *L. albus* and *L. angustifolius*. The two *A. thaliana* sequences (nsLTP-3 and nsLTP-5) are also very similar each other, even though nsLTP-3 has a longer ORF. These similarities between nsLTPs are also observed in **Figure 2**, where clusters of sequences are grouped, except for Lup an 3.0101. The bootstrap values indicate the probability that the sequences are grouped by similarity. Thus, a bootstrap value of

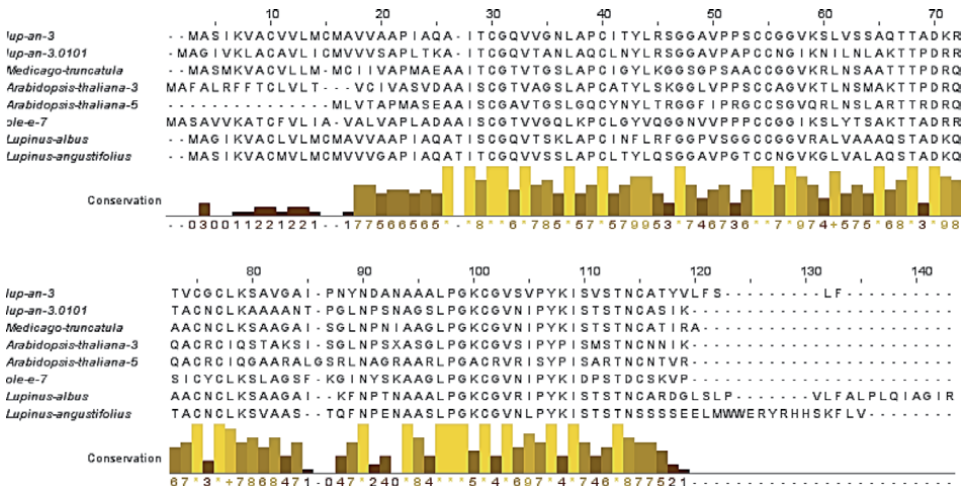

Figure 1.
Multiple alignment of nsLTPs. Eighth main nsLTP protein sequences have been aligned. The similarity index (0-10) between the aligned sequences. The conservation index is shown as yellow bars, and has values ranged from 0 to 10. Lup an 3 (Uniprot: A0A1J7GK90), Lup an 3.0101 (Uniprot: A0A4P1RWD8), Medicago truncatula *(Uniprot: A0A072UTH7),* Arabidopsis thaliana *3 (Uniprot: Q9LLR7),* Arabidopsis thaliana *5 (Uniprot: Q9XFS7), Ole e 7 (NCBI: XP_022893508.1),* Lupinus albus *(Uniprot: A0A6A5MQ88),* Lupinus angustifolius *(Uniprot: A0A4P1RV83).*

60 (in base 100) indicates that the probability that the sequences have not been randomly grouped is 60%, being the overall limit 70%.

Interestingly, it is also observed that the most related species based on nsLTPs comparisons are the species of the genus *Trifolium* (*T. pratense* and *T. subterraneum*), with a bootstrap value of 100 (**Figure 2**). Since they belong to the same genus; they appear more related to each other than to the rest of the analysed species. Similarly, the species of the genus *Arachis* (*A. ipaensis* and *A. hypogaea*), *Cajanus cajan* and *Abrus precatorius* have also grouped, with a very high bootstrap value (99.4). This similarity could be related to the original geographic regions since both arise in India and Africa and their current distributions are also very similar.

On the other hand, although Lup an 3 and Lup an 3.0101 are quite similar, in **Figure 1**, they are phylogenetically distant from each other (see **Figure 2**). In the case of Lup an 3, it has been grouped with the *L. albus* sequence, with 68.2 bootstrap values. These two species are related to *L. angustifolius*, with a bootstrap greater than 70. Lup an 3.0101, it is grouped with *Glycine max*, *Spatholobus suberectus*, *Phaseolus vulgaris*, and *Vigna angularis*, but with a very low bootstrap value. As a result, Lup an 3.0101 maybe an isoform of Lup an 3 with differences in key aminoacids since they are phylogenetically more distant.

Regarding *A. thaliana* sequences (nsLTP-3 and nsLTP-5), they appear grouped, with a high bootstrap (87.2), since they belong to the same species, and are the same kind of protein (nsLTP). Ole e 7 is grouped with *Trifolium* sequences (*T. pratense* and *T. subterraneum*) with a bootstrap value of 62.6. *Arachis duranensis* in **Figure 2** is shown as an outgroup, despite belonging to the same genus as other species included in this analysis (*A. ipaensis* and *A. hypogaea*). Although the origin of *A. hypogaea* is the hybridization of *A. duranensis* x *A. ipaensis*, the largest set of chromosomes of its karyotype comes from *A. ipaensis*, thus this protein probably comes from this set of chromosomes [26]. Furthermore, *A. duranensis* and *A. ipaensis* separated 3 million years ago [26]. Hence, *A. ipaensis* and *A. hypogaea* group have a bootstrap of 100, while *A. duranensis* has been identified as an outgroup.

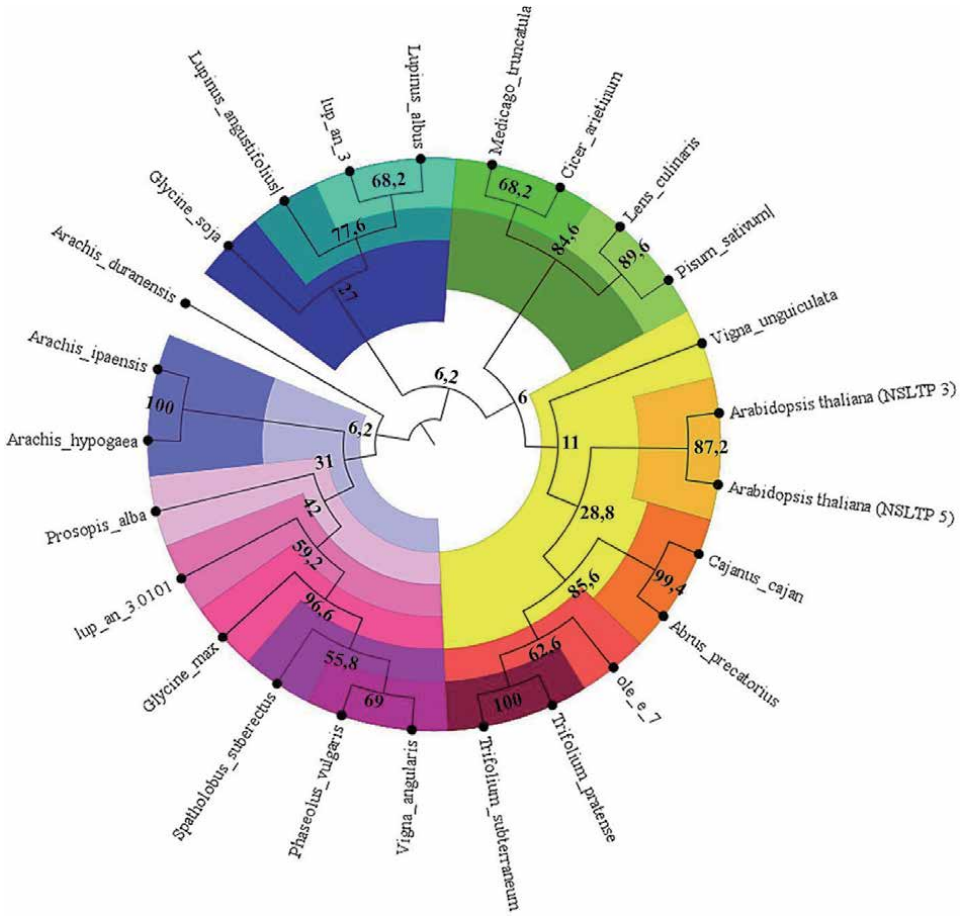

Figure 2.
Phylogenetic analysis of nsLTPs. Twenty-five representative nsLTPs sequences were used for clustering analysis. The bootstrap value (in base 100) is indicated in the nodes. The different colours indicate the groups formed.

3.2 nsLTPs physical and chemical proprieties analysis

The physical and chemical properties analysed were described in **Table 2**. The longest sequence is *Spatholobus suberectus* with 165 aa and 17354.17 Da of MW, and the shortest analysed in *A. thaliana* (nsLTP-5) with 104 aa and 10993.72 Da of MW.

Regarding Lup an 3 and Lup an 3.0101, they are 120 aas and 116 aa long, respectively, and with comparable MW such as 120. 22 kDa and 117.20 kDa, respectively.

Stability of the protein is shown as aliphatic (AI) and instability (II) indexes. II values lower than 40 proteins are stable. Most of the sequences were stable except for *A. thaliana* (nsLTP-3) (42, 46), *L. angustifolius* (42.53), *A. hypogaea* (52, 63), *T. subterraneum* (45.62) and *P. alba* (45.59). Although their values are higher than 40, they are close to the limit value. The AI has an important role in thermal stability, the higher the value of the AI, the more thermally stable a protein is. All the proteins analysed were highly stable, being the lowest value of 77.12. The stability (thermal and proteolytic) of these proteins is important at a molecular level to improve transport and defence function, as well as in their allergenic capacity even in processed and cooked foods [20].

Sequence	Number of aminoacids	MW (Da)	Ip	Aliphatic index	Stability index
Lup an 3	120	12022.25	8.88	99.17	33.21
Lup an 3.0101	116	11719.91	9.38	98.53	39.47
Medicago truncatula	116	11381.51	9.04	89.40	31.06
Arabidopsis thaliana (nsLTP-3)	115	11691.97	9.04	85.74	42.46
Arabidopsis thaliana (nsLTP-5)	104	10993.72	11.00	77.12	23.91
Ole e 7	117	11872.06	9.14	95.04	27.12
Lupinus albus	132	13209.80	9.43	102.20	34.59
Lupinus angustifolius	131	13691.95	8.62	84.89	42.53
Glycine max	117	12219.40	9.69	87.52	32.17
Arachis hypogaea	129	13284.65	8.79	92.17	52.63
Cajanus cajan	121	12733.98	9.24	98.18	21.54
Phaseolus vulgaris	115	11778.77	9.23	83.91	34.12
Glycine soja	115	11487.36	9.10	84.17	17.21
Lens culinaris	110	11024.96	8.75	86.09	30.02
Trifolium pratense	130	13792.45	9.02	108.08	39.97
Spatholobus suberectus	165	17354.17	8.84	87.70	31.69
Cicer arientinum	116	11587.65	9.07	93.53	29.50
Vigna unguiculata	117	12125.32	10.46	88.46	37.20
Abrus precatorius	124	13061.24	9.04	92.74	22.15
Arachis ipaensis	118	12009.36	9.30	97.54	37.16
Trifolium subterraneum	123	12995.34	9.28	99.19	45.62
Prosopis alba	117	11910.02	8.76	99.32	45.59
Vigna angularis	122	12744.89	9.21	81.48	36.50
Arachis duranensis	117	11777.91	9.24	90.94	15.46
Pisum sativum	120	12095.29	8.89	85.58	34.45

Table shows the protein molecular weight (MW), the isoelectric point (pI), the aliphatic index and the instability index. If the instability index value is less than 40 the protein is classified as stable, and if the value is greater than 40 it is classified as unstable.

Table 2.
Physic-chemical properties.

3.3 nsLTPs functional motifs and post-translational modification analysis

Analysis of functional motifs and post-translational modifications were carried out on 25 protein sequences of lupine, other legumes, olive trees, and model plants showed in **Table 1**, **Tables A1**–**A3**.

Table 1 shows that all the sequences have a comparable length, where the shortest sequence contains 104 aa in *A. thaliana* (nsLTP-5), while the longest contains 165 aa in case of *Spatholobus suberectus*.

Pfam functional motifs reveal that the sequences present the protease inhibitor and seed storage motif of the nsLTP family (prolamin family). The prolamin clan was integrated by trypsin-alpha amylase inhibitors, reserve proteins in seeds, and lipid transfer proteins in plants [27]. nsLTP family is a group highly conserved of

7–9 kDa proteins found in higher plant tissues, which function transfering lipids, and is divided into 2 structurally related subfamilies: LTP1 (9 kDa) and LTP2 (7 kDa).

Prosite functional motifs show that most of the sequences contain a motif belonging to the LTPs of plants as it is expected, except for *L. albus*, *G. soja*, *T. subterraneum*, and *V. angularis*.

Post-translational modifications are described in **Tables A1**–**A3**. Phosphorylations, N-myristoylation, glycosilations, N-nitrosylation (cysteine), N-nitrations, and carbonylations, were the most commonly found in the studied nsLTPs.

Phosphorylation (**Tables A1** and **A2**) is common and reversible in proteins, and generally fulfil a regulatory activity of the function of the protein (activate or inhibit its function) in processes such as growth, development of immunity, and responses to stress [28], so it regulate the nsLTPs functional roles. Furthermore, it has previously been observed that Ser and/or Thr residues in seed storage proteins are extensively phosphorylated improving the transport mechanism of these storage proteins [28].

No abundant glycosylation modifications were found while N-myristoylation are quite abundant (**Table A2**) which may indicate that snLTPs membrane location is well regulated under variable stresses conditions. N-glycosylations have also been found not abundant in nsLTPs, only found in Ole e 7, *L. angustifolius*, *T. pratense*, *V. angularis*, *and A. duranensis*. Glycosylation have been previously mentioned as markers of allergenicity and may be related to allergenic properties, due to interaction with the innate immune system [29].

Post-translational redox modifications, such as N-nitrosylation and T-nitration, and carbonylation were involved in the defence function, and coping to biotic and abiotic stresses, and redox signalling (**Tables S2** and **S3**).

3.4 nsLTPs subcellular location

The subcellular location of the 25 nsLTP proteins has been identified and the results are shown in **Table 3**.

Bioinformatic tools, CELLO and Wolf PSort, both show that all proteins are found in the extracellular environment. PSort tool also indicates that in some cases (Lup an 3.0101, *M. truncatula*, *A. thaliana* (nsLTP-3), Ole e 7, *L. culinaris*, *T. subterraneum*, and *A. duranensis*) the location of these proteins are vascular as well as extracellular. *L. angustifolius*, *G. max*, *S. suberectus*, and *P. sativum* are in the plasma membrane; and in the endoplasmic reticulum membrane as well as *P. sativum*.

Structural, biochemical, and physiological features of nsLTPs confirms that these proteins are involved in lipid transport in the vacuolar - plasma membrane secretion pathway to the extracellular space [20]. Thus, the subcellular location of the proteins analysed confirm the nsLTPs functional properties.

3.5 Secondary structure of nsLTP proteins

Secondary structures of analysed nsLTPs of lupin, Arabidopsis, Medicago and olive species are shown in **Figure 3**. α-helix structures present in the nsLTPs are shown in red, and the conserved eight cysteine motif is shown with yellow arrows, which is present in all nsLTPs [20, 22]. This conserved motif integrates four disulphide bridges making a hydrophobic environment inside the protein, where the lipids are transported, while keeping a hydrophilic external environment, maintaining the water-soluble characteristics of these proteins [20–22]. In this regard, the secondary structure of LTPs is very important to maintain the binding

Secuencia	CELLO	WOLF PSORT	PSORT
Lup an 3	Ec	Ec	Ec
Lup an 3.0101	Ec	Ec	Ec/Vac
Medicago truncatula	Ec	Ec	Ec/Vac
Arabidopsis thaliana (nsLTP-3)	Ec	Ec	Ec/Vac
Arabidopsis thaliana (nsLTP-5)	Ec	Ec	Ec
Ole e 7	Ec	Ec	Ec/Vac
Lupinus albus	Ec	Ec	Ec
Lupinus angustifolius	Ec	Ec	MP
Glycine max	Ec	Ec	MP
Arachis hypogaea	Ec	Ec	Ec
Cajanus cajan	Ec	Ec	Ec
Phaseolus vulgaris	Ec	Ec	Ec
Glycine soja	Ec	Ec	Ec
Lens culinaris	Ec	Ec	Ec/Vac
Trifolium pratense	Ec	Ec	Ec
Spatholobus suberectus	Ec	Ec	MP
Cicer arientinum	Ec	Ec	Ec
Vigna unguiculata	Ec	Ec	Ec
Abrus precatorius	Ec	Ec	Ec
Arachis ipaensis	Ec	Ec	Ec
Trifolium subterraneum	Ec	Ec	Ec/Vac
Prosopis alba	Ec	Ec	Ec
Vigna angularis	Ec	Ec	Ec
Arachis duranensis	Ec	Ec	Ec/Vac
Pisum sativum	Ec	Ec	MP/MRE

The table shows the subcelular location of each nsLTP protein assessed by the software CELLO, WOLF PSORT and PSORT described in material and methods. Ec: Extracellular; Vac: Vacuolar; MP: Plasmatic membrane; MPI: internal Plasmatic membrane; MRE: Endoplasmic reticulum membrane.

Table 3.
Subcellular location of nsLTPs.

stability of their structure to carry out their functional properties of transporting hydrophobic macromolecules [22].

Regarding the 2-D structures as α-helix, most of them are integrated by 5 α-helices and no β sheets have been found. This structure is typical in nsLTPs, and comparison with other species have shown a conserved 4 α-helices [20–22].

Interestingly, despite the low sequence identity shown in the alignment of **Figure 3**, 2D structural features among different species are conserved.

3.6 3D structure analysis of nsLTPs sequences

3D structure of 8 main nsLTP proteins analysed are shown in **Figure 4** (Lup an 3, *Lupinus albus* and Ole e 7) and **Figure A2** (*Lupinus angustigolius*, Lup an 3.0101, *Medicago truncatula*, *Arabidopsis thaliana* nsLTP-3 and *Arabidopsis thaliana* nsLTP-5).

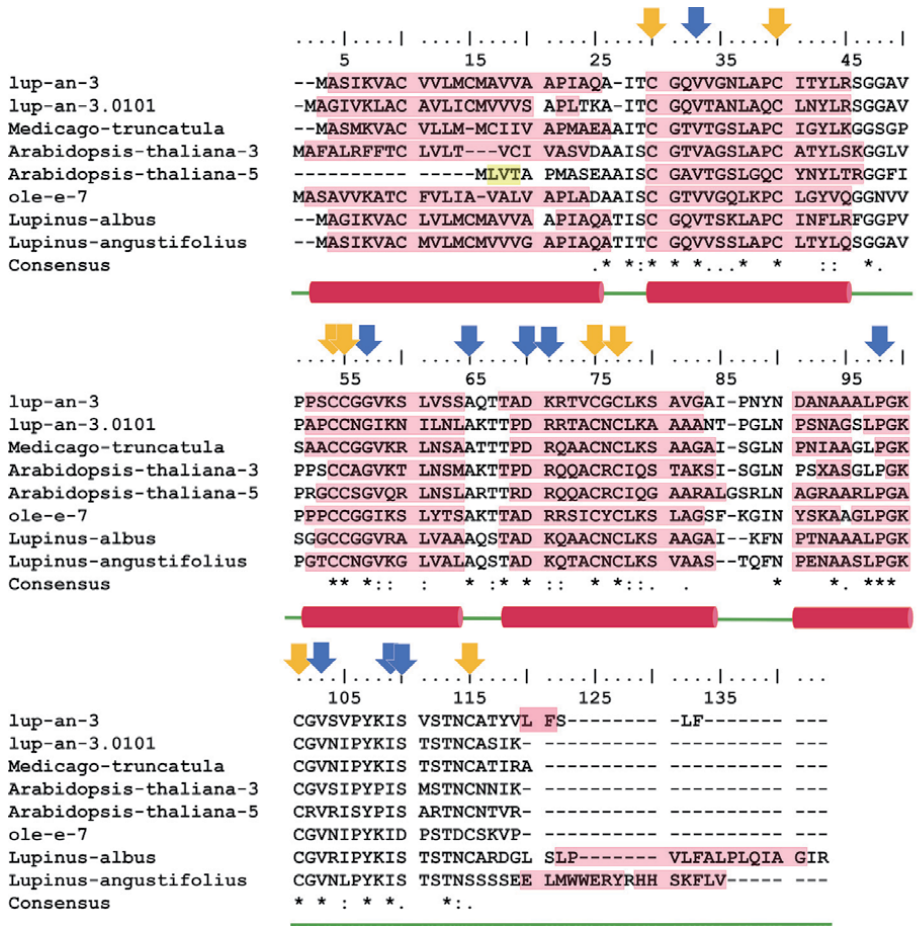

Figure 3.
Secondary structure assessment of nsLTPs. The amino acids involved in the α-helix are highlighted with red bars. Residues that are part of an α-helix and β-sheet are highlighted in red and yellow colour, respectively. The blue arrows indicate the most conserved residues (a value of 9 on the Consurf bioinformatics tool scale). The yellow arrows indicate the cysteines involved in the 8 cysteine motif (C-Xn-C-Xn-CC-Xn-CXC-Xn-C -Xn-C), where Xn is an aminoadic repeated n times.

Overall, no specific differences have been shown in the proteins modelling 3D structures. However, a detailed analysis shows differences at local level such as length of α-helices, special location of the 2-D structures. Noticeable differences in protein size as nsLTP-3 or nsLTP-5 are the smaller proteins, leading to the maintenance of a more compact structures compared to large nsLTPs such as *L. albus* or *L. angustifolius*, being more open structures to the solvent to the outside, which can affect the type of lipid they can carry.

3.7 Conservational analysis of nsLTPs

The primary and 3D structures of the nsLTPs proteins were used to analyse the conservational features of nsLTPs. The results are shown in **Figure 5** (Lup an 3) and **Figure A3** (*Lupinus angustifolius*, Lup an 3.0101, *Medicago truncatula*, *Arabidopsis thaliana* nsLTP-3, *Arabidopsis thaliana* nsLTP-5, *Lupinus albus* and Ole e 7). Most conserved residues of the proteins are found relatively close to the 8-cysteine motif. Most of the highly conserved residues are buried residues and placed around interacting locations with lipids, thus functionality is maintained over time.

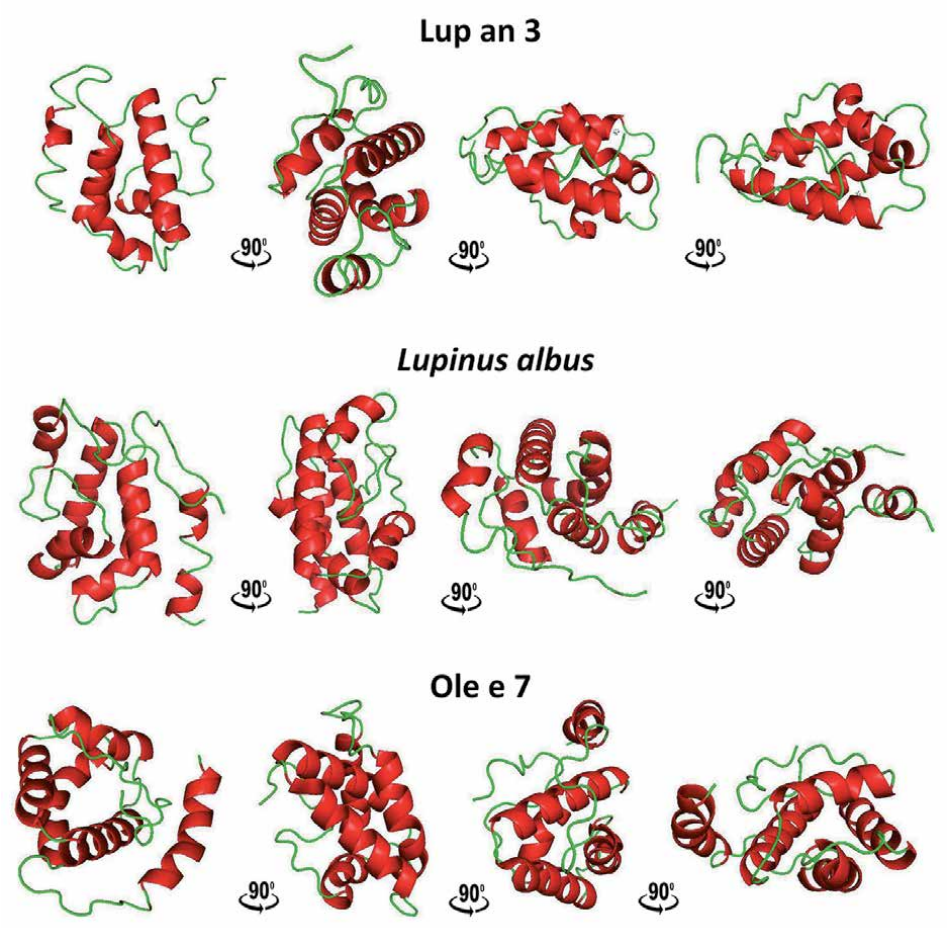

Figure 4.
3D structure of nsLTPs. 3D structures modelling of Lup an 3 (Uniprot: A0A1J7GK90), Ole e 7 (NCBI: XP_022893508.1) and Lupinus albus *(Uniprot: A0A6A5MQ88). Cartoon mode representation were build using Phymol software. A-helices are depicted in red colour.*

3.8 Functional interaction analysis of nsLTPs with their ligands

The analyses carried out using I-TASSER identified the main ligands of Lup an 3, Lup an 3.0101, *M. truncatula*, *A. thaliana* (nsLTP-3), *A. thaliana* (nsLTP-5), Ole e 7, *L. albus*, and *L. angustifolius*, they are shown in **Figure 6** and **Figure A4**. **Table A4** summarise the main ligands of lipid nature that can located in the nsLTPs cysteine functional motif, and **Table A5** summarise the functional interaction with other proteins.

Figure 6 shows the interaction of the Lup an 3 protein with stearic acid, its main ligand, and the hydrophilic environment of the nsLTP that has to maintain inside of the protein [20], fundamental for the carrying lipid function and interaction of Lup an 3 with stearic acid.

The conserved motif cysteines and disulfide bridges have considerable plasticity, allowing the ability to accommodate different ligands [20]. The plasticity of the disulphide bridge pattern can also be observed in **Figure A4**, where the *L. angustifolius* sequence maintain the hydrophobic environment only with 7 cysteines. However, the 3D and function of the protein is maintained and therefore is capable of binding to different ligands such as stearic acid or palmitic acid, among others. Notably, some of the nsLTPs can decrease specificity for ligands, which can be

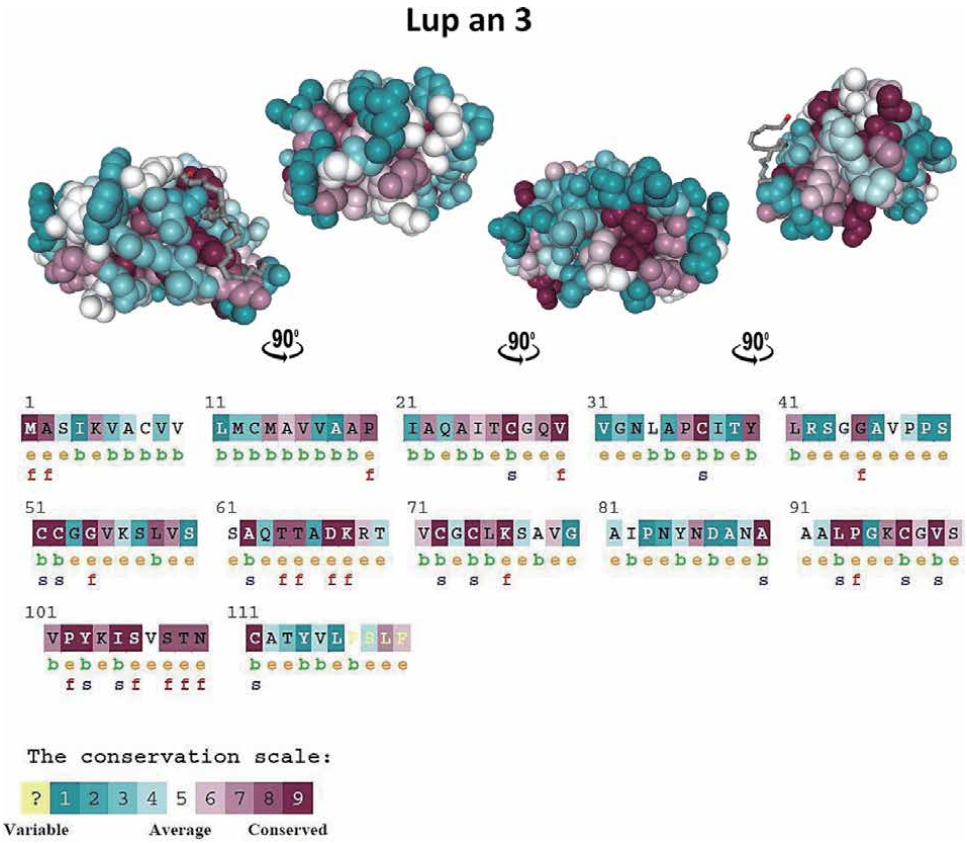

Figure 5.
Conservation analysis of nsLTPs of Lup an 3. Conservational analysis of Lup an 3 (Uniprot: A0A1J7GK90). the conservation values of Consurf was used to show the amino acids conservational index according with the colours scale (from purple – conserved to green – no conserved residues; yellow indicates no information found about this residues). Below the sequence, (e) indicates residue exposed; (b) indicated buried residue, according to the neural network algorithm in both cases; (f) highly conserved and exposed functional residue; (s) highly conserved and buried. The arrows (blue and yellow) indicate the highly conserved residues (with a value of 9) in all the sequences analysed. The yellow arrows indicate the cysteines of the conserved 8 cysteine motif and the blue arrows other representative conserved residues in the analysed sequences. Three-dimensional representation of proteins is depicted as spheres.

attributed to the flexibility of the van der Waals volume of the internal hydrophobic cavities sufficient to accommodate single or double chain lipids [30].

Table A4 shows examples of nsLTPs transport ligands of diverse nature: stearic acid (STE), 10-oxo-12-octadecenoic acid (ASY), prostaglandin B2 (E2P), 1-myristoyl-SN -glycerol-3-phosphocholine (LPC), and palmitic acid (PLM). Fatty acids are the main constituents of cellular membranes, in addition to their role as a source of energy, signalling and mediation in cellular transport. They also accumulate in the seeds of vegetables, such as palmitic acid, transported by Ole e 7, *L. angustifolius*, *G. max*, *C. cajan*, *T. pratense. A. precatorus*, *A. ipaensis*, and *T. subterraneum* (**Table A5**), which is also involved in the lipogenesis pathway. Therefore, LTPs make an important class of proteins performing membrane-associated signalling processes under different environmental stresses and an important function in lipids storage in seeds.

Prostaglandins are lipids derived from arachidonic acid that have an effect similar to gibberellins in the endosperm and maintain homeostasis and mediate pathogenesis in animals [31]. For example, prostaglandin B2, which can be

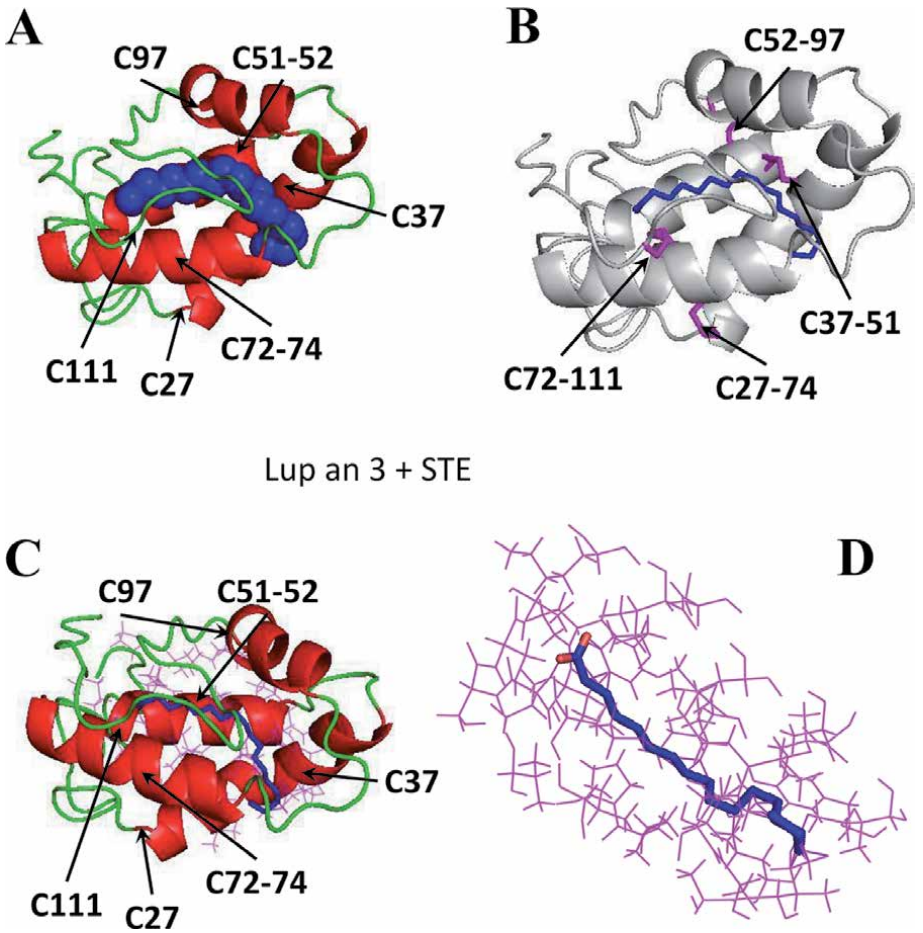

Figure 6.
Protein-ligand interaction assessment for Lup an 3. Lup an 3 (Uniprot: A0A1J7GK90) interaction with stearic acid (STE). ***A)*** *Lup an 3 protein in cartoon model with STE ligand in sphere model. Motif 8 cysteine pinpointed conserved cysteines that allow the hydrophobic environment for the lipid interaction.* ***B)*** *Lup an 3 protein interacting with STE ligand with disulphide bridges, in purple colour, created between the cysteines of the conserved 8 cysteine motif.* ***C)*** *Lup an 3 protein with STE ligand. Pink colour depicted the sites of interaction of the protein with the ligand.* ***D)*** *Interaction pocket of the STE ligand with the Lup an 3 protein.*

transported by Lup an 3.0101 and the analysed LTP of *P. vulgaris* (**Table A5**), plays a key role in the generation of the inflammatory response in animals [32], thus it could be involved in responses to allergies to LTPs.

The structural interaction between lipid ligands and nsLTP, as well as functional interaction with a plethora of proteins show the diversity of bound ligands and the heterogeneity of the binding and functionality. However, it is clear that the type and mode of lipid binding and proteins interactions with nsLTPs determine the biological function and if it affects the allergenic properties of nsLTPs.

3.9 Protein interactions study of lupine and other species nsLTPs

Potential functional pathways and molecular interactions of nsLTPs are shown in **Table A5**.

Among all proteins analysed, Lup an 3, Lup an 3.0101, *Medicago truncatula*, *L. albus*, and *L. angustifolius* interact with calmodulin-binding heat shock proteins and

with ATP-dependent DEAD-box RNA helicase (DDX1). The DDX1 family comprises enzymes that participate in RNA metabolism, and are associated with different cellular functions, including abiotic stress in plants, and regulation of cell maturation, growth, and differentiation [25]. It is observed that DDX1 interact directly with profilin present in the cytosol of all eukaryotic cells modifying the actin cytoskeleton dynamics in response to external signals or stimuli and protecting the cell from oxidative damage maintaining a redox state in the cytoplasm [33–35]. It seems that these LTPs are involved in the response to abiotic stress and cellular regulation processes, participating in the signalling pathways.

nsLTP-5 appears to interact primarily with other LTPs, such as several LTPs that belong to seed storage 2S albumin superfamily protein and are bifunctional inhibitors; or with LTPG1 protein, a glycosylphosphatidylinositol-bound LTP1 involved in the export of cuticular lipids and resistance against fungal pathogens [36]. It also interacts with the protein AT1G10770, which has an inhibitory role for pectin methyl-esterase participating in the growth of the pollen tube. Thus, it appears that nsLTP is primarily is associated with the seed storage function.

Arabidopsis thaliana (nsLTP-3), in addition to interacting with several seed storage proteins and with LTP4, also interacts with MYB96, a transcription factor that activates cuticular wax biosynthesis under drought stress, it is involved in the regulation of ABA (abscisic acid) biosynthesis, regulates seed germination and activates LTP3, or *A. thaliana* (nsLTP-3) in our case, in response to drought or frost [37]. *A. thaliana* (nsLTP-3) also interacts with ELP, a protein involved in transcriptional elongation and involved in oxidative stress signalling. In addition, LTP3 from *A. thaliana* is involved both in transport and storage in seeds, as well as in response to abiotic stresses, such as droughts or frosts, transporting lipids during the cuticle.

Ole e 7 interacts with other LTPs of seed storage 2S albumin superfamily and with LTP3. In addition, it interacts with AT3G58690, a protein kinase that may be involved in the post-translational modifications suffered by LTPs. It also interacts with an ELP, as does *A. thaliana* (nsLTP-3).

An example of an interaction network is the case of *Arabidopsis thaliana* (nsLTP-3 an nsLTP-4). The interactions with seed storage proteins and other LTPs are observed. Considering the interaction between LTP3 and LTP4 of *A. thaliana* as a model, we can observe that both proteins (LTP3 and LTP4) interact with some common proteins, which could be related to their functional roles, underlying the possibility that these proteins could transport lipids together.

Therefore, it can be concluded that nsLTPs are involved in signalling pathways in response to abiotic stress, such as drought or cool, response to pathogens such as fungi, and the storage of proteins and lipids in seeds and maintaining seed dormancy, as well as in many other functions.

3.10 Analysis of potential allergenicity nsLTPs

The nsLTPs sequences used in this study were comparatively analysed using databases such as Allergome, as described in the material and methods section. The analysis of the nsLTPs allergenicity assessment were based on primary structure of the protein, 2D and 3D, oligomerization state of proteins, functional features, as well as experimental results.

These analyses confirm the allergenic character of most of the nsLTPs sequences. These nsLTP sequences analysed are the following: All c 3 (*Allium cepa*), Ara h 17 (*Arachis hypogaea*), Aspa o 1 (*Asparagus officinalis*), Cas s 8 (*Castanea sativa*), Cit l 3 (*Citrus limonum*), Dau c 3 (*Daucus carota*), Len c 3 (*Lens culinaris*), Lup an 3 (*Lupinus angustifolius* L.), Lup an 3.0101 (*Lupinus angustifolius* L.), Mal d 3 (*Malus*

domestica), Mus a 3 (*Musa acuminata*), Ole e 7 (*Olea europaea*), Pha v 3.0201 (*Phaseolus vulgaris*), Sola l 7 (*Solanum lycopersicum*), Tri a 14 *(Triticum aestivum*) y Zea m 14 (*Zea mays*). nsLTPs have allergenic nature, which will help to continue the study of these proteins at molecular and functional level.

3.11 IgE-binding epitope assessment

Legumes contain proteins that share epitopes (full or partially), which would make possible to develop cross-reactivity between them. However, the similarity between sequences does not ensure cross-reactivity, since cases of atopic individuals have been observed occur no cross-allergenicity, even when both species share large similarity in proteins such as lupine and peanut vicilin (Ara h 1 and Lup an 1). In addition, none of the clinically studied lupine allergic individuals reacted to peanuts [4, 38, 39]. Recent studies have also shown clinically relevant cross-reactivity of lupine with other legumes, such as lentils, beans, chickpeas, peas, soybeans, and almonds [4, 15, 18, 19, 40].

The IgE results from binding epitopes analysis (**Table A6**) reveal that all the proteins analysed present Allergen Representative Peptide (ARPs) sequences highlighted in red, representing residues that share the analysed sequences and the ARPs. The SVM analysis based on amino acid and dipeptide composition show that all sequences are allergenic or potentially allergenic. Considering that all the sequences are present in seeds, the relationship between these proteins and food allergies seems to have relationship.

It can also be observed in **Table A6** that Lup and 3 and *A. ipaensis* ARPs are comparable, pointing out that their IgE binding epitopes are very similar. Furthermore, cross-reactivity between lupine and *A. ipaensis* (wild peanut) seems to have clinical importance, especially considering different cases reported of cross-reactivity between lupine and peanut [4]. Therefore, Lup an 3 appears susceptible to cross-reaction with *A. ipaensis*, based on their epitopes. The same situation occurs with the sequences Lup an 3.0101 and *P. alba; M. truncatula*, *P. vulgaris* and *G. soja; A. thaliana* (nsLTP-5), *V. unguiculata and A. duranensis; L. albus and P. sativum;* and, *L. culinaris and A. precatorus*. Members of the same protein families, in this case, nsLTPs, share IgE epitopes as depicted by our analysis, which can potentially lead to an allergic reaction due to cross-reactivity [41].

3.12 T-cell and B-cell binding epitope analysis

Hypersensitivity reactions are mediated by IgE, T- and B- cells, and these cells play important roles contributing to the pathophysiology of a wide range of allergic reactions [42]. Analysis of T- and B-cell binding epitopes (**Tables A7** and **A8**) reveals up to nine T-cell and up to six B-cell epitopes, with significant differences between species. Cross-reactivity at the T-cell level depends on homologies between amino acid sequences. Regarding the T-cell epitopes found in the analysed sequences (**Table A7**), it can be observed that epitope T1 is present in all the analysed sequences and located in the same region of the analysed proteins, also containing comparable number and sequence of residues. T2 epitope is present in most the analysed sequences with the exception of nsLTP-3, Ole e 7, and *S. suberectus*. This suggests that T2 is also highly conserved among species and is involved in cross-allergenicity among them.

Regarding the B-cell epitopes (**Table A8**), B1 and B4 epitopes are present in most of the analysed sequences, with the exception of *M. truncatula*, *L. albus*, *L. angustifolius*, *C. arientinum*, and *P. sativum* for B1 epitope; and Lup an 3,

A. thaliana (nsLTP-3), *A. thaliana* (nsLTP-5), *T. pratense* and *T. subterraneum* for B4 epitope.

It is also important to note that the T2 epitope and the B4 epitope are the same, which could be relevant when it comes to the primary sensitization process to the nsLTPs sharing these epitopes.

Furthermore, it has been observed that B5 and B6 epitopes are unique for *Lupinus angustifolius* and *Spatholobus suberectus*, respectively. Interestingly, the B3 epitope was also present in the species widely used in food worldwide such as *M. truncatula*, *G. soja* (soybean), *L. culinaris* (lentil), *T. pratense*, *C arientinum* (chickpea), and *P. sativum* (pea).

4. Conclusions

The functional analysis of nsLTPs proteins show comparable motifs in their primary sequence with prolamin storage proteins family and trypsin-alpha amylase inhibitors, involved in lipid transfer, biotic and abiotic stress response, and defence against pathogens. Differential post-translational modifications showed nsLTPs involvement in the regulation of nsLTP in multiple functional roles, beside lipid transfer. LTPs may also suffer redox-related modifications that would be related to copping different environmental stresses and signalling functions. The LTPs analysed sequences where primarily located close related to different membranes in the secretion pathway. This location is tightly related to LTPs signalling physiological functions, and the relative lipid abundance depending of the subcellular specific organelle locations.

Structural analysis and ligand interaction analysis of LTPs show the importance of the functional 8 cysteine motif (4 disulphide bridges), that are highly conserved and brings stability to nsLTPs, and maintaining the adequate hydrophobic environment for nsLTP-lipid of different nature interaction and transport, i.e. stearic acid or palmitic acid, among others.

nsLTPs has been identified as main allergens. The identification of binding IgE, T-cells, and B-cells epitopes allows us to confirm the potential allergenicity of these studied proteins such in the case of *L. angustifolius* and comparatively nsLTPs of other related and unrelated species, as well as the possibility of cross-allergenicity between some of them. This study has great application potential in the development of molecular tools for the diagnosis and allergy therapies to nsLTPs.

Acknowledgements

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Conflicts of interest

The authors have declared that no competing interests exist.

A. Appendix

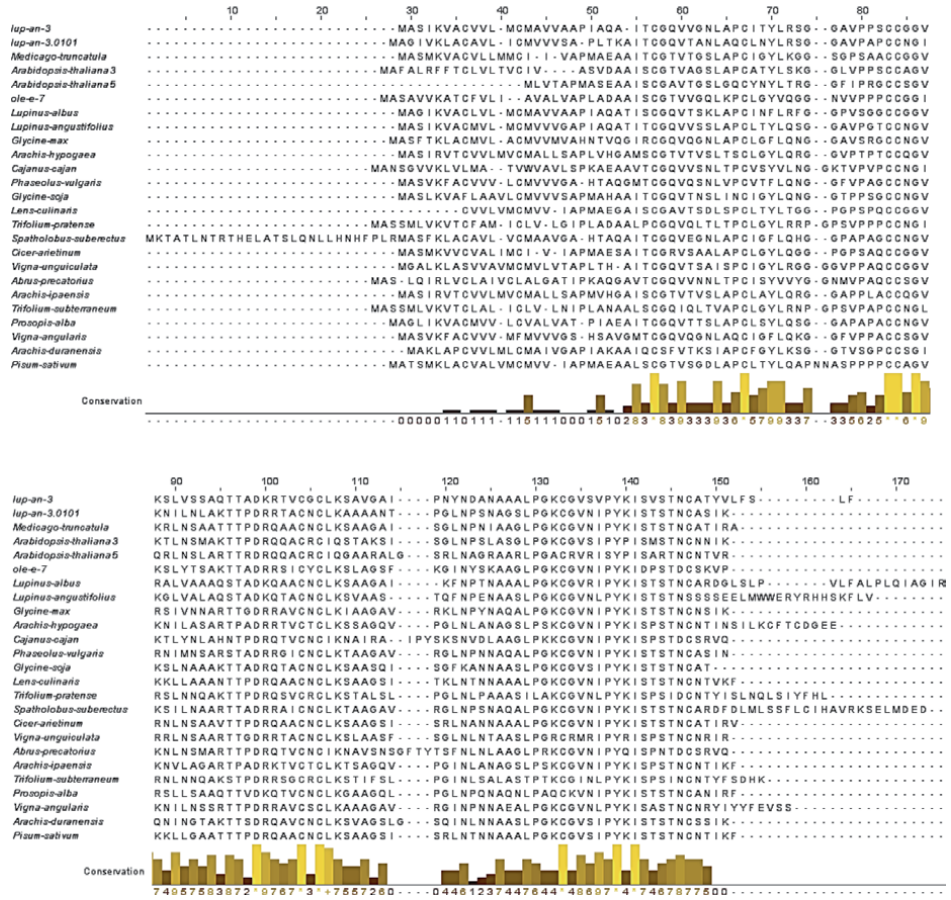


Figure A1.
Multiple alignment of nsLTPs. Eighth main nsLTP protein sequences have been aligned. The similarity index (0-10) between the aligned sequences. The conservation index is shown as yellow bars, and has values ranged from 0 to 10. Lupinus angustifolius *(Lup an 3) Uniprot: A0A1J7GK90*, Lupinus angustifolius *(Lup an 3.0101) (Uniprot: A0A4P1RWD8)*, Medicago truncatula *(Uniprot: A0A072UTH7)*, Arabidopsis thaliana *(nsLTP-3) (Uniprot: Q9LLR7)*, Arabidopsis thaliana *(nsLTP-5) (Uniprot: Q9XFS7)*, Olea *europaea (Ole e 7) (NCBI: XP_022893508.1)*, Lupinus albus *(Uniprot: A0A6A5MQ88)*, Lupinus angustifolius *(Uniprot: A0A4P1RV83)*, Glycine max *(Uniprot: I1J7M1)*, Arachis hypogaea *(NCBI: XP_025656480.1)*, Cajanus cajan *(NCBI: XP_020237462)*, Phaseolus vulgaris *(Uniprot: D3W146)*, Glycine soja *(Uniprot: A0A445M2F4)*, Lens culinaris *(Uniprot: A0AT33)*, Trifolium pratense *(Uniprot: A0A2K3M7A7)*, Spatholubus suberectus *(NCBI: TKY63608.1)*, Cicer arietinum *(Uniprot: O23758)*, Vigna unguiculata *(Uniprot: UPI0010170F74)*, Abrus precatorus *(Uniprot: UPI000F7C313B)*, Arachis ipaensis *(NCBI: XP_020971907.1)*, Trifolium subterraneum *(NCBI: GAU29990.1)*, Prosopis alba *(NCBI: XP_028808641.1)*, Vigna angularis *(NCBI: KOM57753.1)*, Arachis duranensis *(NCBI: XP_015950831.1)*, Pisum sativum *(NCBI: A0A158V755.1)*.

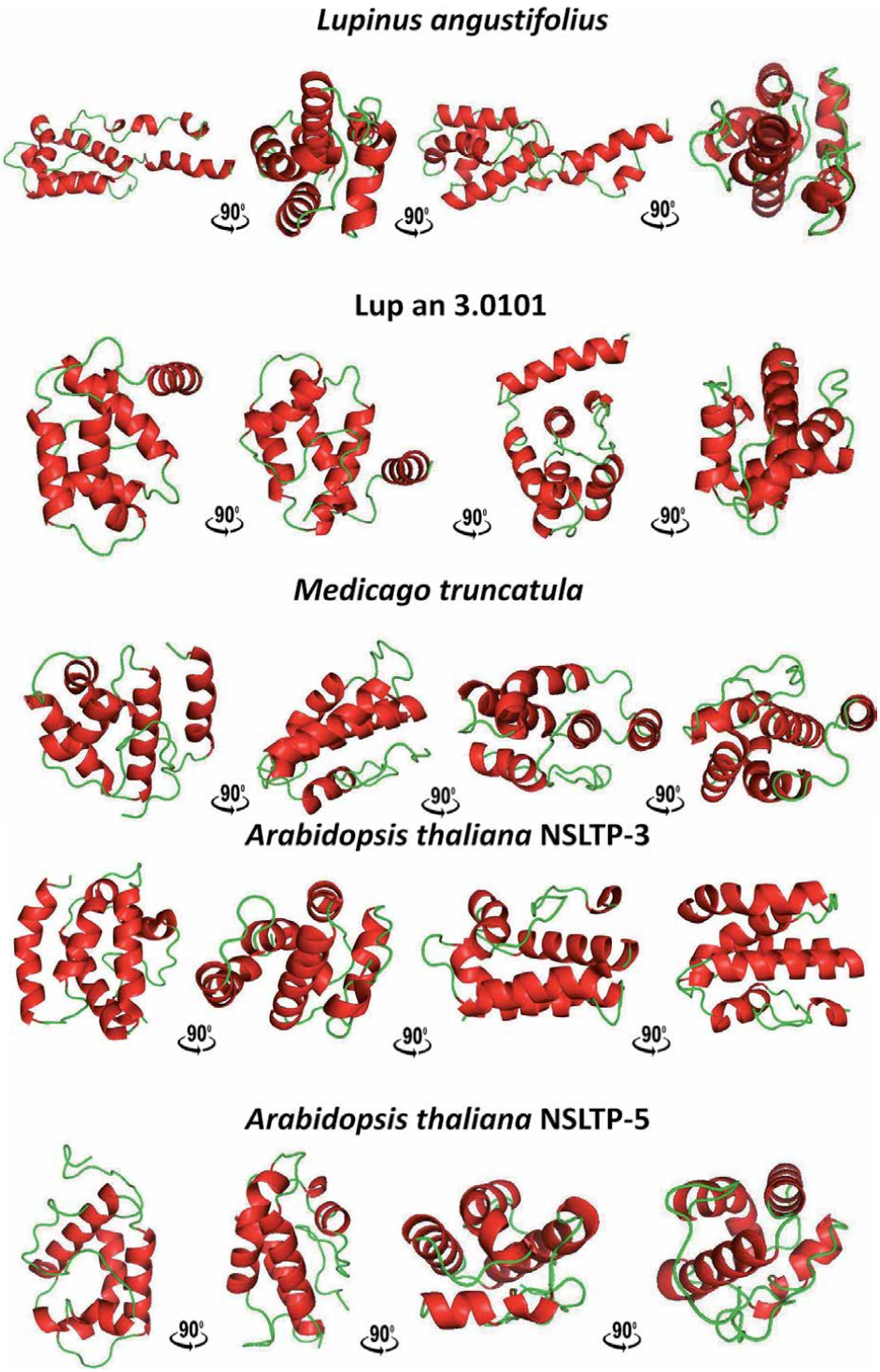
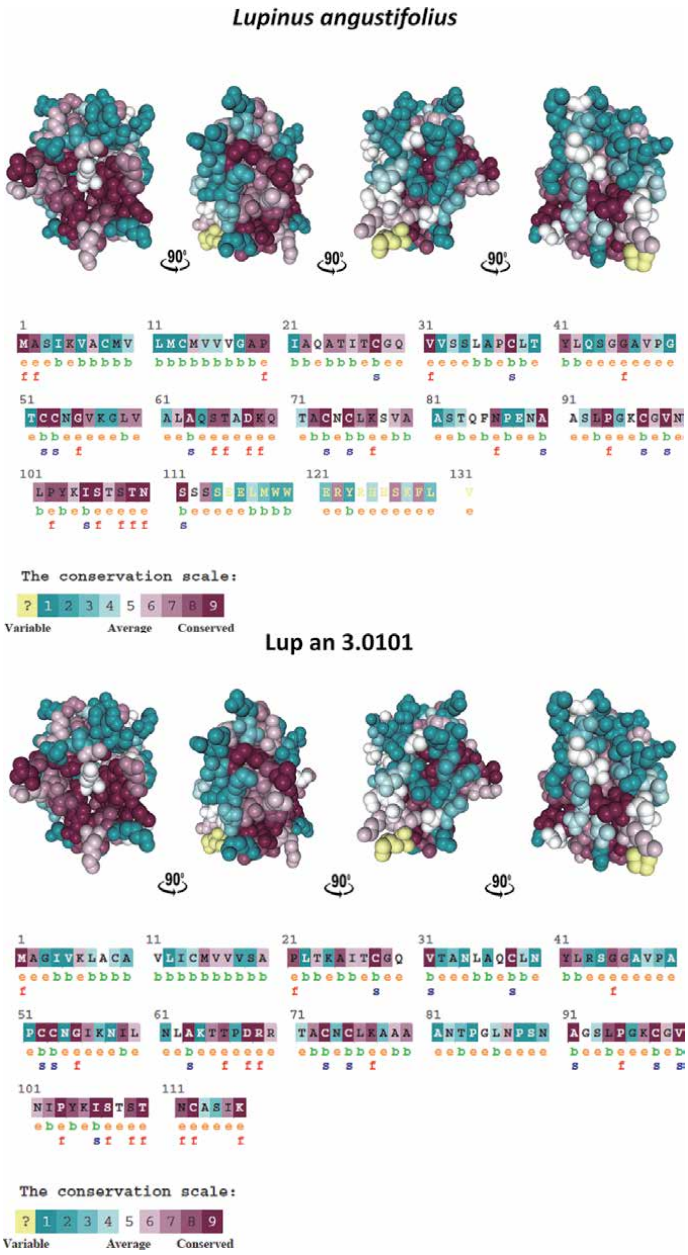


Figure A2.
3D structure of nsLTPs. 3D structures modeling of Lup an 3.0101 (Uniprot: A0A4P1RWD8), Medicago truncatula (Uniprot: A0A072UTH7), Arabidopsis thaliana 3 (Uniprot: Q9LLR7), Arabidopsis thaliana 5 (Uniprot: Q9XFS7), Lupinus angustifolius (Uniprot: A0A4P1RV83). Cartoon mode representation were build using Phymol software. A-helices are depicted in red color.


Lupinus angustifolius
90°
90°
90°
The conservation scale:
Variable
Average
Conserved
Lup an 3.0101
90°
90°
90°
The conservation scale:
Variable
Average
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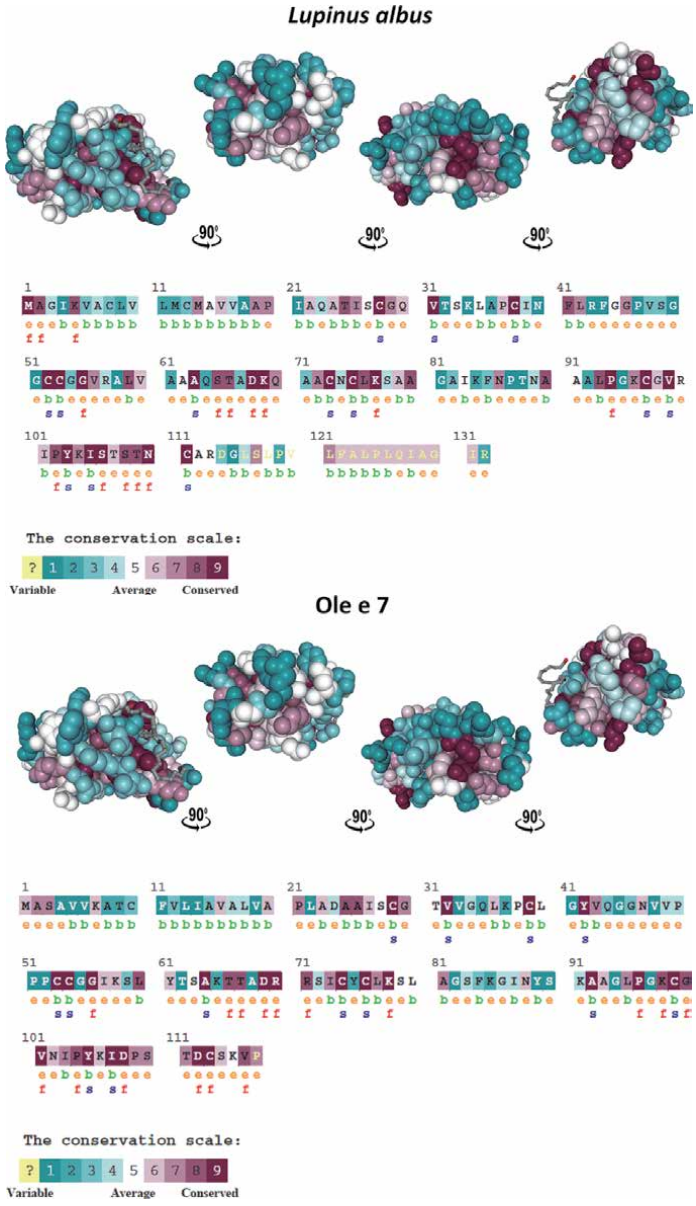
Lupinus albus
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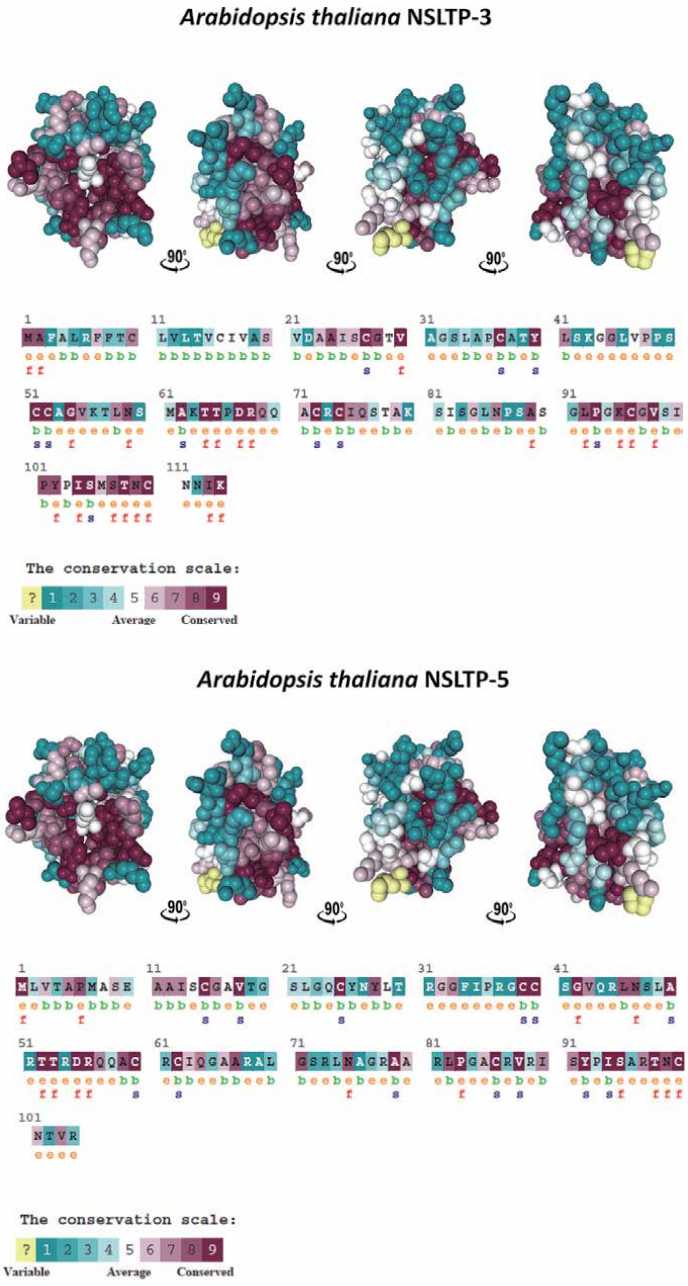
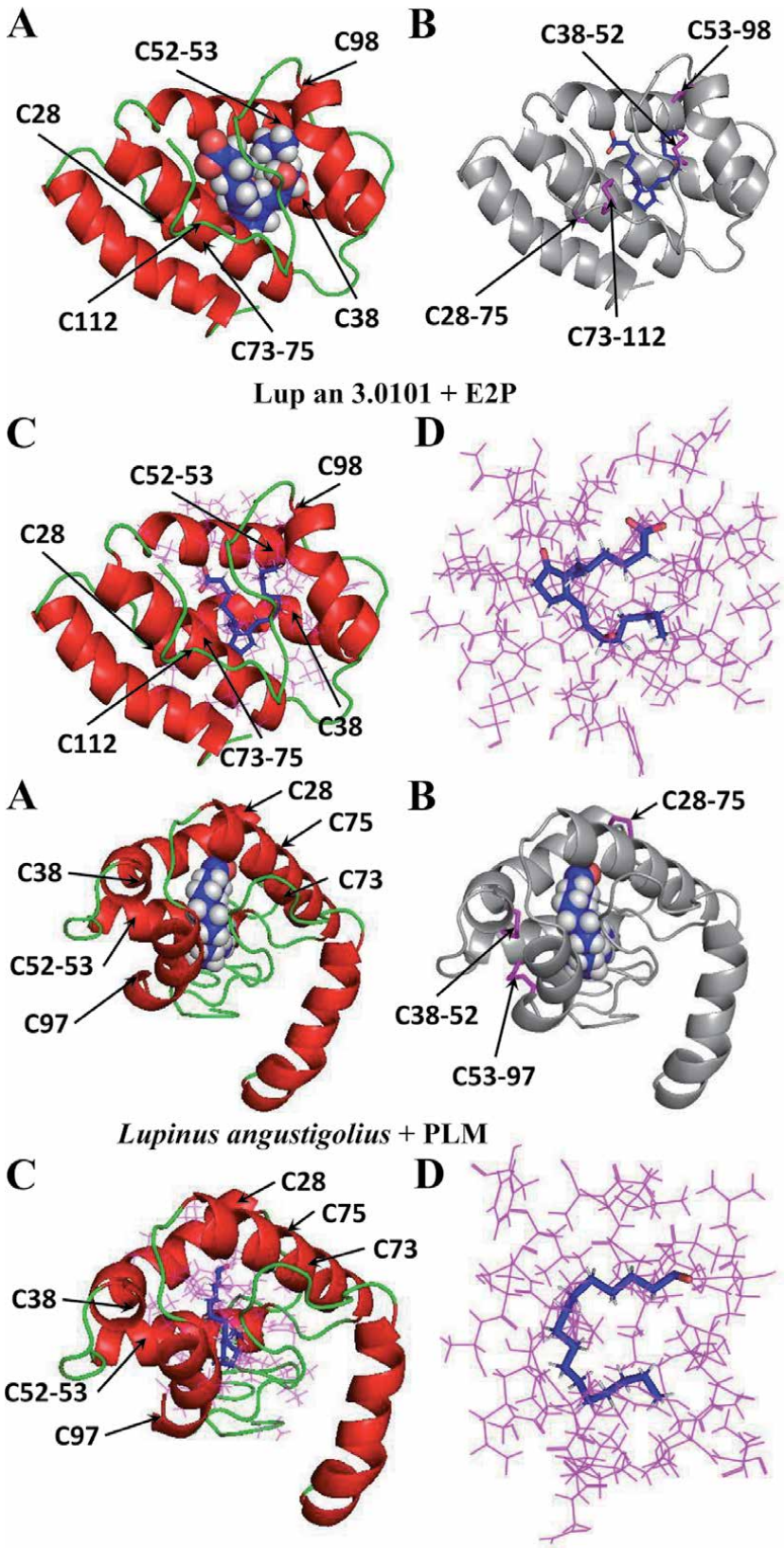


Figure A3.
Conservation analysis of nsLTPs of nsLTPs. Conservational analysis of Lup an 3.0101 (Uniprot: A0A4P1RWD8), Medicago truncatula (Uniprot: A0A072UTH7), Arabidopsis thaliana *3 (Uniprot: Q9LLR7),* Arabidopsis thaliana *5 (Uniprot: Q9XFS7),* Lupinus angustifolius *(Uniprot: A0A4P1RV83), Ole e 7 (NCBI: XP_022893508.1) and* Lupinus albus *(Uniprot: A0A6A5MQ88). The conservation values of Consurf was used to show the amino acids conservational index according with the colours scale (from purple – conserved to green – no conserved residues; yellow indicates no information found about this residues). Below the sequence, (e) indicates residue exposed; (b) indicated buried residue, according to the neural network algorithm in both cases; (f) highly conserved and exposed functional residue; (s) highly conserved and buried. The arrows (blue and yellow) indicate the highly conserved residues (with a value of 9) in all the sequences analysed. The yellow arrows indicate the cysteines of the conserved 8 cysteine motif and the blue arrows other representative conserved residues in the analysed sequences. Three-dimensional representation of proteins is depicted as spheres.*


A
C52-53
C98
C28
C112
C38
C73-75
B
C38-52
C53-98
C28-75
C73-112
Lup an 3.0101 + E2P
C
C52-53
C98
C28
C112
C38
C73-75
D
A
C28
C75
C38
C73
C52-53
C97
B
C28-75
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C28
C75
C73
C38
C52-53
C97
D

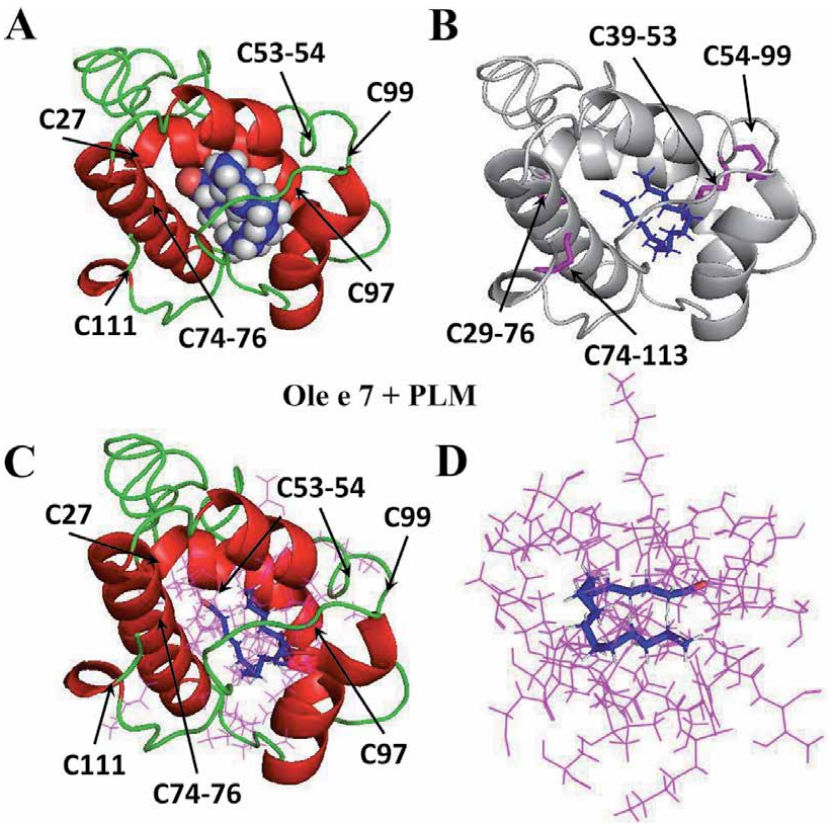

Figure A4.
Protein-ligand interaction assessment for nsLTP. Lup an 3.0101 (Uniprot: A0A4P1RWD8), Medicago truncatula (Uniprot: A0A072UTH7), Arabidopsis thaliana *3 (Uniprot: Q9LLR7),* Arabidopsis thaliana *5 (Uniprot: Q9XFS7),* Lupinus angustifolius *(Uniprot: A0A4P1RV83), Ole e 7 (NCBI: XP_022893508.1) and* Lupinus albus *(Uniprot: A0A6A5MQ88) interaction with their respective ligands. A) Lup an 3 protein in cartoon model and ligand in sphere model. Motif 8 cysteine pinpointed conserved cysteines that allow the hydrophobic environment for the lipid interaction. B) Disulphide bridges representation in purple colour, created between the cysteines of the conserved 8 cysteine motif. C) Pink colour depicted the sites of interaction of the protein with the ligand. D) Interaction pocket of the ligand with the nsLTP protein.*

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Sequence	N-Glycosilation (start-end): 'modification' (PS00001)	Phosphorilation (start-end): 'modification'				N-Myristoilation (start-end): 'modification' (PS00008)	Posttranslational modification – REDOX metabolism	
		MAPK (NethPhos)	PKC (PS00005)	CK2 (PS00006)	PKA		S-nitrosylation (C)	T-nitration (Y)
Lup an 3 (Uniprot: A0A1J7GK90)	-	-	• 3-5: SiK • 43: TYLRSGGAV • 60: KSLVSSAQT • 64: SSAQTTADK • 65: SAQTTADKR • 100: KCGVSVPYK • 108: KISVSTNCA • 109: ISVSTNCAT • 118: YVLFSLF—	• 64-67: TtaD	• 106: PYKISVSTN	• 28-33: GQvvGN • 53-58: GGvkSL • 73-78: GClkSA • 80-85: GAipNY	• 13: SIKVACVVLMCMAVVAAPIAQ • 27: VAAPIAQAITCGQVVGNLAPC	-
Lup an 3.0101 (Uniprot: A0A4P1RWD8)	-	• 66: LAKTTPDRR • 83: AAANTPGLN	• 23: SAPLTKAIT • 44: NYLRSGGAV • 109: KISTSTNCA • 110: ISTSTNCAS • 114-116: SiK	• 65-68: TtpD	• 107: PYKISTSTN	• 29-34: GQvtAN • 85-90: GLnpSN • 92-97: GSlpGK	• 28: VSAPLTKAITCGQVTANLAQC • 112: IPYKISTSTNCASIKXXXXXX	• 104: KCGVNIPYKISTSTN
Medicago truncatula (Uniprot: A0A072UTH7)	-	• 65: AATTTPDRQ	• 3-5: SmK • -29: ITCGTVTGS • 64: SAATTTPDR - 109: ISTSTNCAT • 108: KISTSTNCA • 113-115: TiR	• 64-67: TtpD	• 33: TVTGSLAPC • 45: LKGGSGPSA • 60: KRLNSAATT	• 28-33: GTvtGS • 44-49: GSgpSA • 80-85: GAisGL • 84-89: GLnpNI	• 27: VAPMAEAAITCGTVTGSLAPC • 37: CGTVTGSLAPCIGYLKGGSGP • 51: LKGGSGPSAACCGGVKRLNSA • 111: IPYKISTSTNCATIRAXXXXX	• 103: KCGVNIPYKISTSTN
Arabidopsis thaliana (nsLTP-3) (Uniprot: Q9LLR7)	-	• 65: MAKTTPDRQ	• 29: ISCGTVAGS • 39: APCATYLSK • 60: KTLNSMAKT • 64: SMAKTTPDR • 77: RCIQSTAKS • 78-80: TaK	• 64-67: TtpD	• 9: LRFFTCLVL • 33: TVAGSLAPC • 100: KCGVSIPYP	• 28-33: GTvaGS • 84-89: GLnpSX	• 27: IVASVDAAISCGTVAGSLAPC • 72: AKTTPDRQQACRCIQSTAKSI • 111: IPYPISMSTNCNNIKXXXXXX	• 103: KCGVSIPYPISMSTN
Arabidopsis thaliana (nsLTP-5) (Uniprot: Q9XFS7)	-	-	• 52-54: TtR • 72: RALGSRLNA - 95-97: SaR • 102-104: TvR	• 52-55: TtrD	• 21: AVTGSLGQC • 41: RGCCSGVQR • 48: QRLNSLART • 91: RVRISYPIS	• 16-21: GAvtGS • 23-28: GQcyNY • 38-43: GCcsGV • 65-70: GAarAL • 71-76: GSrlNA	• 15: APMASEAAISCGAVTGSLGQC • 60: ARTTRDRQQACRCIQGAARAL	• 92: ACRVRISYPISARTN

Sequence	N-Glycosilation (start-end): 'modification' (PS00001)	Phosphorilation (start-end): 'modification'				N-Myristoilation (start-end): 'modification' (PS00008)	Posttranslational modification – REDOX metabolism	
		MAPK (NethPhos)	PKC (PS00005)	CK2 (PS00006)	PKA		S-nitrosylation (C)	T-nitration (Y)
Ole e 7 (NCBI: XP_022893508.1)	• ASN 88-91: NYSK	-	• 31: ISCGTVVGQ • 62: KSLYTSAKT • 63-65: SaK • 67: SAKTTADRR - 83-85: SfK • 110: KIDPSTDCS • 111: IDPSTDCSK	• 66-69: TtaD	• 72: ADRRSICYC	• 30-35: GTvvGQ • 55-60: GGikSL • 82-87: GSfkGI • 86-91: GInySK	• 29: VAPLADAAISCGTVVGQLKPC • 99: YSKAAGLPGKCGVNIPYKIDP	• 105: KCGVNIPYKIDPSTD
Lupinus albus (Uniprot: A0A6A5MQ88)	-	-	• 27: QATISCGQV - 32-34: TsK • 65: AAAQSTADK - 109: ISTSTNCAR	• 65–68: StaD	• 106: PYKISTSTN • 117: RDGLSLPVL	• 3-8: GIkvAC • 29-34: GQvtSK • 45-50: GGpvSG • 50-55: GGccGG • 51-56: GCcgGV • 54-59: GGvrAL	• 28: AAPIAQATISCGQVTSKLAPC • 38: CGQVTSKLAPCINFLRFGGPV	-
Lupinus angustifolius (Uniprot: A0A4P1RV83)	• ASN 110-113: NSSS	-	• 3-5: SiK • 27: QATITCGQV • 40: APCLTYLQS • 44: TYLQSGGAV • 78: NCLKSVAAS • 82: SVAASTQFN - 107: YKISTSTNS • 108: KISTSTNSS - 109: ISTSTNSSS	• 65–68: StaD • 112-115: SssE • 113-116: SseE • 114: NSSSSEELM	• 34: QVVSSLAPC • 127: YRHHSKFLV	• 18-23: GApiAQ • 29–34: GQvvSS • 46–51: GAvpGT • 50–55: GTccNG	• 52: LQSGGAVPGTCCNGVKGLVAL	• 123: ELMWWERYRHHSKFL
Glycine max (Uniprot: I1J7M1)	-	-	• 5: MASFTKLAC • 67: NARTTGDRR • 111: ISTSTNCNS • 115-117: SiK	• 66-69: TtgD	• 108: PYKISTSTN	• 26-31: GIrcGQ • 30-35: GQvqGN • 46-51: GGavSR • 52-57: GCcnGV	• 29: VAHNTVQGIRCGQVQGNLAPC	• 105: KCGVNIPYKISTSTN
Arachis hypogaea (NCBI: XP_025656480.1)	-	• 50: GGVPTPTCC	• 3-5: SiR • 31: MSCGTVTVS - 63-65: SaR • 110: KISPSTNCN	• 66-69: TpaD • 124: LKCFTCDGE	• 7: SIRVTCVVL • 28: HGAMSCGTV • 35: TVTVSLTSC • 72: ADRRTVCTC • 80: CLKSSAGQV	• 25-30: GAmsCG • 82-87: GQvpGL • 86-91: GLnlAN • 93-98: GSlpSK	-	-

Sequence	N-Glycosilation (start-end): 'modification' (PS00001)	Phosphorilation (start-end): 'modification'				N-Myristoilation (start-end): 'modification' (PS00008)	Posttranslational modification – REDOX metabolism	
		MAPK (NethPhos)	PKC (PS00005)	CK2 (PS00006)	PKA		S-nitrosylation (C)	T-nitration (Y)
Cajanus cajan (NCBI: XP_020237462)	-	• 21: VAVLSPKAE • 38: VSNLTPCVS	• 21-23: SpK • 50: NGGKTVPVP	• 91-94: SnvD	• 74: PDRQTVCNC • 89: AIPYSKSNV	• 31-36: GQvvSN	• 56: NGGKTVPVPCCNGIKTLYNLA • 103: VDLAAGLPKKCGVNIPYKISP	• 109: KCGVNIPYKISPSTD
Phaseolus vulgaris (Uniprot: D3W146)	-	-	• 3-5: SvK • 39: VPCVTFLQN • 61-63: SaR • 65: SARSTADRR • 108: KISTSTNCA • 109: ISTSTNCAS -	• 64–67: StaD	• 106: PYKISTSTN	• 18-23: GAhtAQ • 24-29: GMtcGQ • 28-33: GQvqSN • 50-55: GCcnGV • 70-75: GIcnCL • 80-85: GAvrGL • 84-89: GLnpNN	• 13: SVKFACVVVLCMVVVGAHTAQ	• 103: KCGVNIPYKISTSTN
Glycine soja (Uniprot: A0A445M2F4)	-	• 48: QNGGTPPSG	• 3-5: SlK • 28: HAAITCGQV • 66: AAAKTTADR • 110: KISTSTNCA • 111: ISTSTNCAT	• 66-69: TtaD	• 35: QVTNSLINC • 108: PYKISTSTN	• 30-35: GQvtNS • 47-52: GTppSG • 52-57: GCcnGV	• 74: AKTTADRQTACNCLKSAASQI	• 105: KCGVSIPYKISTSTN
Lens culinaris (Uniprot: A0AT33)	-	• 29: TSDLSPCLT	• 75: SAAGSITKL • 102: KISTSTNCN • 107-109: TvK	• 58-61: TtpD	• 75: SAAGSITKL • 100: PYKISTSTN	• 17-22: GAisCG • 22-27: GAvtSD	• 21: IAPMAEGAISCGAVTSDLSPC	• 97: KCGVNIPYKISTSTN
Trifolium pratense (Uniprot: A0A2K3M7A7)	-	• 38: QLTLTPCLG • 69: QAKTTPDRQ	• 4: -MASSMLVK • 61: NGIRSLNNQ • 68: NQAKTTPDR • 74: PDRQSVCRC • 96: PAAASILAK	• 68-71: TtpD	• 50: RPGPSVPPP • 85: STALSLPGL • 110: PYKISPSID	• 20-25: GIplAD	• 16: LVKVTCFAMICLVLGIPLADA	• 118: PSIDCNTYISLNQLS • 127: SLNQLSIYFHL****
Spatholobus suberectus (NCBI: TKY63608.1)	-	-	• 5: MKTATLNTR • 15: HELATSLQN • 31-33: SfK - 137: ISTSTNCAR -	• 92-95: TtaD • 148: DLMLSSFLC	• 31: LRMASFKLA • 134: PYKISTSTN • 159: AVRKSELMD	• 46-51: GAhtAQ • 56-61: GQveGN • 78-83: GCcnGV • -108-113: GAvrGL • 112-117: GLnpSN	• 55: VGAHTAQAITCGQVEGNLAPC • 100: ARTTADRRAICNCLKTAAGAV	-

Sequence	N-Glycosilation (start-end): 'modification' (PS00001)	Phosphorilation (start-end): 'modification'				N-Myristoilation (start-end): 'modification' (PS00008)	Posttranslational modification – REDOX metabolism	
		MAPK (NethPhos)	PKC (PS00005)	CK2 (PS00006)	PKA		S-nitrosylation (C)	T-nitration (Y)
Cicer arientinum (Uniprot: O23758)	-	• 65: AAVTTPDRQ	• 3-5: SmK • 64: SAAVTTPDR - 108: KISTSTNCA • 109: ISTSTNCAT • 113-115: TiR	• 64-67: TtpD	• 31: CGRVSAALA • 106: PYKISTSTN	• 53-58: GGvrNL	-	• 103: KCGVNIPYKISTSTN
Vigna unguiculata (Uniprot: UPI0010170F74)	-	-	• 8: LKLASVVAV • 62: RRLNSAART • 66: SAARTTGDR • 67: AARTTGDRR • 79: NCLKSLAAS	• 66-69: TtgD	• 62: RRLNSAART • 72: GDRRTACNC • 108: PYRISPSTN	• 29-34: GQvtSA • 47-52: GVppAQ • 86-91: GLnlNT	• 14: LKLASVVAVMCMVLVTAPLTH	-
Abrus precatorius (Uniprot: UPI000F7C313B)	-	• 39: VNNLTPCIS • 70: MARTTPDRQ	• 69: SMARTTPDR • 90: NSGFTYTSF	• 69-72: TtpD	• 3: –MASLQIR	• 27-32: GAvtCG • 32-37: GQvvNN	-	• 47: PCISYVVYGGNMVPA • 112: KCGVNIPYQISPNTD
Arachis ipaensis (NCBI: XP_020971907.1)	-	-	• 3-5: SiR • 31: ISCGTVTVS • 33: CGTVTVSLA • 75: KTVCTCLKT • 110: KISPSTNCN • 115-117: TiK	• 66-69: TpaD	• 7: SIRVTCVVL • 28: HGAISCGTV • 35: TVTVSLAPC	• 25-30: GAisCG • 82-87: GQvpGI • 86-91: GInlAN • 93-98: GSlpSK	• 74: ARTPADRKTVCTCLKTSAGQV	• 105: KCGVNIPYKISPSTN
Trifolium subterraneum (NCBI: GAU29990.1)	• ASN 90-93: NLSA	• 69: QAKSTPDRR	• 4: -MASSMLVK • 29: NAALSCGQI • 81: RCLKSTIFS • 85: STIFSLPGI • 96: SALASTPTK • 120: NTYFSDHK-	• 68-71: StpD	• 74: PDRRSGCRC	• 88-93: GInlSA	• 101: LSALASTPTKCGINLPYKISP	• 107: KCGINLPYKISPSIN
Prosopis alba (NCBI: XP_028808641.1)	-	-	• 44: SYLQSGGAP • 61: RSLLSAAQT • 65: SAAQTTVDK	• 65-68: TtvD	• 107: PYKISTSTN	• 29-34: GQvtTS • 81-86: GQlpGL	-	• 104: QCKVNIPYKISTSTN

Sequence	N-Glycosilation (start-end): 'modification' (PS00001)	Phosphorilation (start-end): 'modification'				N-Myristoilation (start-end): 'modification' (PS00008)	Posttranslational modification – REDOX metabolism	
		MAPK (NethPhos)	PKC (PS00005)	CK2 (PS00006)	PKA		S-nitrosylation (C)	T-nitration (Y)
			• 66: AAQTTVDKQ • 71: VDKQTVCNC • 110: ISTSTNCAN					
Vigna angularis (NCBI: KOM57753.1)	- ASN. 60-63: NSSR	-	• 3: –MASVKFA • 19: VVVGSHSAV • 61: NILNSSRTT • 108: KISASTNCN	• 64-67: TtpD	• 106: PYKISASTN	• 18-23: GShsAV • 24-29: GMtcGQ • 28-33: GQvqGN • 80-85: GAvrGI • 84-89: GInpNN	• 111: LPYKISASTNCNRYIYYFEVS	• 117: NCNRYIYYFEVSS¨qq
Arachis duranensis (NCBI: XP_015950831.1)	• ASN. 60-63: NGTA • ASN. 112-115: NCSS	-	• 47: KSGGTVSGP - 62-64: TaK • 65: GTAKTTSDR - 67-69: SdR • 78: NCLKSVAGS • 82: SVAGSLGSQ - 110: KISTSTNCS • 111: ISTSTNCSS - 114: STNCSSIK- • 115-117: SiK	• 65-68: TtsD	• 34: FVTKSIAPC • 82: SVAGSLGSQ • 108: PYKISTSTN	• 18-23: GApiAK • 45-50: GGtvSG • 61-66: GTakTT • 81-86: GSlgSQ • 84-89: GSqiNL	• 13: KLAPCVVLMLCMAIVGAPIAK • 28: GAPIAKAAIQCSFVTKSIAPC	• 105: KCGVSIPYKISTSTN
Pisum sativum (NCBI: A0A158V755.1)	• 50: PNNASPPPP	• 4-6: SmK • 68: GAATTTPDR - 117-119: TiK	• 33: CGTVSGDLA • 68-71: TtpD	• 30-35: GTvsGD • 64-69: GAatTT	• 29: IAPMAEAALSCGTVSGDLAPC	• 107: KCGVSIPYKISTSTN		

MAPK: mitogen-activated protein kinase; PKC: Protein quinase C, CK2: Protein quinase 2; PKA: Protein quinase A.

Table A1.
Post-translational modifications: glycosilations, phosphorylations, myristoilations and post-translational modifications related to redox metabolism.

Sequence	T	S	Y
Lupinus angustifolius (Lup an 3)	• 70: ADKRTVCGC (CDI)	• 50: AVPPSCCGG (cdc2) • 77: GCLKSAVGA (cdc2) • 106: PYKISVSTN (cdc2) • 118: YVLFSLF— (CKI)	-
Lupinus angustifolius (Lup an 3.0101)	• 66: LAKTTPDRR (cdk5) • 71: PDRRTACNC (PKG) • 83: AAANTPGLN (GSK3)	• 93: SNAGSLPGK (PKI) • 93: SNAGSLPGK (DNAPK) • 93: SNAGSLPGK (GSK3) • 107: PYKISTSTN (cdc2)	-
Medicago truncatula	• 26: EAAITCGTV (CKI) • 65: AATTTPDRQ (cdk5)	• 3: –MASMKVA (cdc2) • 33: TVTGSLAPC (DNAPK) • 45: LKGGSGPSA (cdc2) • 60: KRLNSAATT (CaM-II) • 106: PYKISTSTN (cdc2)	-
Arabidopsis thaliana (nsLTP-3)	-	• 33: TVAGSLAPC (DNAPK) • 33: TVAGSLAPC (cdc2) • 42: ATYLSKGGL (PKI) • 106: PYPISMSTN (cdc2)	• 40: PCATYLSKG (unsp)
Arabidopsis thaliana (nsLTP-5)	• 53: LARTTRDRQ (cdc2)	• 21: AVTGSLGQC (DNAPK)	-
Olea europea L. (Ole e 7)	-	• 28: DAAISCGTV (CKI) • 59: GGIKSLYTS (DNAPK) • 72: ADRRSICYC (CKI)	• 42: PCLGYVQGG (unsp) • 89: KGINYSKAA (INSR)
Lupinus albus	-	• 33: GQVTSKLAP (cdc2) • 106: PYKISTSTN (cdc2)	-
Lupinus angustifolius	• 71: ADKQTACNC (PKG)	• 34: QVVSSLAPC (cdc2) • 34: QVVSSLAPC (DNAPK) • 106: PYKISTSTN (cdc2) • 114: NSSSSEELM (CKI) • 127: YRHHSKFLV (PKB)	• 41: PCLTYLQSG (unsp) • 103: VNLPYKIST (unsp)
Glycine max	-	• 108: PYKISTSTN (cdc2)	-
Arachis hypogaea	• 50: GGVPTPTCC (cdk5) • 66: ASARTPADR (cdk5) • 66: ASARTPADR (GSK3) • 72: ADRRTVCTC (PKG)	• 38: VSLTSCLGY (CKI) • 94: ANAGSLPSK (CKI) • 94: ANAGSLPSK (cdc2) • 94: ANAGSLPSK (DNAPK)	• 42: SCLGYLQRG (unsp) • 105: VNIPYKISP (unsp)
Cajanus cajan	-	• 4: -MANSGVVK (cdc2) • 91: PYSKSNVDL (cdc2)	• 43: PCVSYVLNG (unsp) • 109: VNIPYKISP (unsp)
Phaseolus vulgaris	-	• 3: –MASVKFA (cdc2) • 32: GQVQSNLVP (cdc2) • 106: PYKISTSTN (cdc2)	-
Glycine soja	-	• 35: QVTNSLINC (cdc2) • 51: GTPPSGCCN (cdc2) • 82: KSAASQISG (DNAPK)	• 42: NCIGYLQNG (unsp)

Sequence	T	S	Y
		• 108: PYKISTSTN (cdc2)	
Lens culinaris	-	• 26: GAVTSDLSP (cdc2) • 29: TSDLSPCLT (cdk5) • 42: GPGPSPQCC (cdk5) • 42: GPGPSPQCC (GSK3)	-
Trifolium pratense	• 38: QLTLTPCLG (cdk5)	• 4: -MASSMLVK (cdc2)) • 85: STALSLPGL (CKI) • 96: PAAASILAK (cdc2) • 112: KISPSIDCN (cdc2)	• 107: VNLPYKISP (unsp) • 118: DCNTYISLN (unsp) • 118: DCNTYISLN (INSR)
Spatholobus suberectus	-	• 149: LMLSSFLCI (cdc2)	-
Cicer arientinum	• 65: AAVTTPDRQ (cdk5)	• 106: PYKISTSTN (cdc2)	• 41: PCLGYLQGG (unsp)
Vigna unguiculata	• 72: GDRRTACNC (PKG) • 72: GDRRTACNC (CKI)	• 36: TSAISPCIG (cdk5) • 108: PYRISPSTN (cdk5) • 108: PYRISPSTN (cdc2)	• 45: PCIGYLRGG (unsp)
Abrus precatorius	• 39: VNNLTPCIS (GSK3)	• 58: AQCCSGVKN (cdc2) • 93: FTYTSFNLN (cdc2) • 115: PYQISPNTD (cdk5)	• 44: PCISYVVYG (unsp) • 91: SGFTYTSFN (unsp) • 91: SGFTYTSFN (INSR) • 112: VNIPYQISP (unsp)
Arachis ipaensis	• 66: AGARTPADR (cdk5) • 66: AGARTPADR (GSK3)	• 80: CLKTSAGQV (PKG) • 94: ANAGSLPSK (CKI) • 94: ANAGSLPSK (DNAPK) • 94: ANAGSLPSK (cdc2)	• 105: VNIPYKISP (unsp)
Trifolium subterraneum	• 69: QAKSTPDRR (cdk5) • 97: ALASTPTKC (cdk5)	• 4: -MASSMLVK (cdc2) • 85: STIFSLPGI (DNAPK) • 85: STIFSLPGI (CKI) • 85: STIFSLPGI (cdc2)	• 43: PCLGYLRNP (unsp) • 107: INLPYKISP (unsp)
Prosopis alba	• 33: GQVTTSLAP (cdc2)	• 34: QVTTSLAPC (DNAPK)	• 41: PCLSYLQSG (unsp)
Vigna angularis	• 65: SSRTTPDRR (cdk5)	-	• 103: VNLPYKISA (unsp)
Arachis duranensis	-	• 82: SVAGSLGSQ (CKI) • 85: GSLGSQINL (DNAPK) • 85: GSLGSQINL (ATM) • 108: PYKISTSTN (cdc2)	• 41: PCFGYLKSG (unsp)
Pisum sativum	• 69: AATTTPDRQ (cdk5) • 91: SRLNTNNAA (RSK)	• 4: -MATSMKLA (cdc2) • 28: EAALSCGTV (CKI) • 50: PNNASPPPP (cdK5)	• 42: PCLTYLQAP (unsp)

Phosphorylations are classified by modified residues. T: threonine; S: serine; Y: tyrosine.

Table A2.
Post-translational modifications. phosphorylations.

Sequence	Carbonylations			
	K	P	R	T
Lupinus angustifolius (Lup an 3)	• 56: SGGAVPPSCCGGVKSLVSSAQTTADKR • 68: VKSLVSSAQTTADKRTVCGCLKSAVGA • 76: QTTADKRTVCGCLKSAVGAIPNYNDAN • 96: PNYNDANAAALPGKCGVSVPYKISVST • 104: AALPGKCGVSVPYKISVSTNCATYVLF	• 20: ACVVLMCMAVVAAPIAQAITCGQVVGN • 36: QAITCGQVVGNLAPCITYLRSGGAVPP • 48: APCITYLRSGGAVPPSCCGGVKSLVSS • 49: PCITYLRSGGAVPPSCCGGVKSLVSSA • 83: TVCGCLKSAVGAIPNYNDANAAALPGK • 94: AIPNYNDANAAALPGKCGVSVPYKISV • 102: NAAALPGKCGVSVPYKISVSTNCATYV	• 42: QVVGNLAPCITYLRSGGAVPPSCCGGV • 69: KSLVSSAQTTADKRTVCGCLKSAVGAI	• 26: CMAVVAAPIAQAITCGQVVGNLAPCIT • 39: TCGQVVGNLAPCITYLRSGGAVPPSCC • 64: CCGGVKSLVSSAQTTADKRTVCGCLKS • 65: CGGVKSLVSSAQTTADKRTVCGCLKSA • 70: SLVSSAQTTADKRTVCGCLKSAVGAIP
Lupinus angustifolius (Lup an 3.0101)	• 24: VLICMVVVSAPLTKAITCGQVTANLAQ • 57: SGGAVPAPCCNGIKNILNLAKTTPDRR • 64: PCCNGIKNILNLAKTTPDRRTACNCLK • 77: KTTPDRRTACNCLKAAAANTPGLNPSN • 97: PGLNPSNAGSLPGKCGVNIPYKISTST	• 21: ACAVLICMVVVSAPLTKAITCGQVTAN • 49: AQCLNYLRSGGAVPAPCCNGIKNILNL • 51: CLNYLRSGGAVPAPCCNGIKNILNLAK • 67: NGIKNILNLAKTTPDRRTACNCLKAAA • 84: TACNCLKAAAANTPGLNPSNAGSLPGK • 88: CLKAAAANTPGLNPSNAGSLPGKCGVN • 95: NTPGLNPSNAGSLPGKCGVNIPYKIST • 103: NAGSLPGKCGVNIPYKISTSTNCASIK	• 43: QVTANLAQCLNYLRSGGAVPAPCCNGI • 69: IKNILNLAKTTPDRRTACNCLKAAAAN • 70: KNILNLAKTTPDRRTACNCLKAAAANT	• 23: AVLICMVVVSAPLTKAITCGQVTANLA • 27: CMVVVSAPLTKAITCGQVTANLAQCLN • 32: SAPLTKAITCGQVTANLAQCLNYLRSG • 65: CCNGIKNILNLAKTTPDRRTACNCLKA • 66: CNGIKNILNLAKTTPDRRTACNCLKAA • 71: NILNLAKTTPDRRTACNCLKAAAANTP • 83: RTACNCLKAAAANTPGLNPSNAGSLPG
Medicago truncatula	• 42: TVTGSLAPCIGYLKGGSGPSAACCGGV • 56: GGSGPSAACCGGVKRLNSAATTTPDRQ • 76: TTTPDRQAACNCLKSAAGAISGLNPNI • 96: SGLNPNIAAGLPGKCGVNIPYKISTST	• 19: VACVLLMMCIIVAPMAEAAITCGTVTG • 36: AAITCGTVTGSLAPCIGYLKGGSGPSA • 47: LAPCIGYLKGGSGPSAACCGGVKRLNS • 66: GGVKRLNSAATTTPDRQAACNCLKSAA • 87: CLKSAAGAISGLNPNIAAGLPGKCGVN • 94: AISGLNPNIAAGLPGKCGVNIPYKIST • 102: IAAGLPGKCGVNIPYKISTSTNCATIR	• 57: GSGPSAACCGGVKRLNSAATTTPDRQA • 68: VKRLNSAATTTPDRQAACNCLKSAAGA	• 26: MCIIVAPMAEAAITCGTVTGSLAPCIG • 29: IVAPMAEAAITCGTVTGSLAPCIGYLK • 31: APMAEAAITCGTVTGSLAPCIGYLKGG • 63: ACCGGVKRLNSAATTTPDRQAACNCLK • 64: CCGGVKRLNSAATTTPDRQAACNCLKS • 65: CGGVKRLNSAATTTPDRQAACNCLKSA
Arabidopsis thaliana (nsLTP-3)	• 43: VAGSLAPCATYLSKGGLVPPSCCAGVK • 56: KGGLVPPSCCAGVKTLNSMAKTTPDRQ • 63: SCCAGVKTLNSMAKTTPDRQQACRCIQ	• 36: AAISCGTVAGSLAPCATYLSKGGLVPP • 48: APCATYLSKGGLVPPSCCAGVKTLNSM • 49: PCATYLSKGGLVPPSCCAGVKTLNSMA • 66: AGVKTLNSMAKTTPDRQQACRCIQSTA	• 68: VKTLNSMAKTTPDRQQACRCIQSTAKS • 73: SMAKTTPDRQQACRCIQSTAKSISGLN	• 14: MAFALRFFTCLVLTVCIVASVDAAISC • 29: CIVASVDAAISCGTVAGSLAPCATYLS • 39: SCGTVAGSLAPCATYLSKGGLVPPSCC • 57: GGLVPPSCCAGVKTLNSMAKTTPDRQQ • 64: CCAGVKTLNSMAKTTPDRQQACRCIQS • 65: CAGVKTLNSMAKTTPDRQQACRCIQST

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Sequence	Carbonylations			
	K	P	R	T
Arabidopsis thaliana (nsLTP-5)	-	• 36: GQCYNYLTRGGFIPRGCCSGVQRLNSL • 83: LGSRLNAGRAARLPGACRVRISYPISA	• 31: VTGSLGQCYNYLTRGGFIPRGCCSGVQ • 37: QCYNYLTRGGFIPRGCCSGVQRLNSLA • 45: GGFIPRGCCSGVQRLNSLARTTRDRQQ • 51: GCCSGVQRLNSLARTTRDRQQACRCIQ • 54: SGVQRLNSLARTTRDRQQACRCIQGAA • 56: VQRLNSLARTTRDRQQACRCIQGAARA • 61: SLARTTRDRQQACRCIQGAARALGSRL • 68: DRQQACRCIQGAARALGSRLNAGRAAR • 73: CRCIQGAARALGSRLNAGRAARLPGAC • 78: GAARALGSRLNAGRAARLPGACRVRIS • 81: RALGSRLNAGRAARLPGACRVRISYPI • 87: LNAGRAARLPGACRVRISYPISARTNC • 89: AGRAARLPGACRVRISYPISARTNCNT	• 19: PMASEAAISCGAVTGSLGQCYNYLTRG • 30: AVTGSLGQCYNYLTRGGFIPRGCCSGV • 52: CCSGVQRLNSLARTTRDRQQACRCIQG • 53: CSGVQRLNSLARTTRDRQQACRCIQGA
Olea europea L. (Ole e 7)	• 37: DAAISCGTVVGQLKPCLGYVQGGNVVP • 58: GGNVVPPPCCGGIKSLYTSAKTTADRR • 65: PCCGGIKSLYTSAKTTADRRSICYCLK • 78: KTTADRRSICYCLKSLAGSFKGINYSK • 85: SICYCLKSLAGSFKGINYSKAAGLPGK • 91: KSLAGSFKGINYSKAAGLPGKCGVNIP • 98: KGINYSKAAGLPGKCGVNIPYKIDPST	• 21: ATCFVLIAVALVAPLADAAISCGTVVG • 38: AAISCGTVVGQLKPCLGYVQGGNVVPP • 50: KPCLGYVQGGNVVPPPCCGGIKSLYTS • 51: PCLGYVQGGNVVPPPCCGGIKSLYTSA • 52: CLGYVQGGNVVPPPCCGGIKSLYTSAK • 96: SFKGINYSKAAGLPGKCGVNIPYKIDP • 104: KAAGLPGKCGVNIPYKIDPSTDCSKVP	• 70: IKSLYTSAKTTADRRSICYCLKSLAGS • 71: KSLYTSAKTTADRRSICYCLKSLAGSF	• 31: LVAPLADAAISCGTVVGQLKPCLGYVQ • 62: VPPPCCGGIKSLYTSAKTTADRRSICY • 66: CCGGIKSLYTSAKTTADRRSICYCLKS • 67: CGGIKSLYTSAKTTADRRSICYCLKSL
Lupinus albus	• 34: IAQATISCGQVTSKLAPCINFLRFGGP • 69: VRALVAAAQSTADKQAACNCLKSAAGA • 77: QSTADKQAACNCLKSAAGAIKFNPTNA • 84: AACNCLKSAAGAIKFNPTNAAALPGKC • 96: IKFNPTNAAALPGKCGVRIPYKISTST • 104: AALPGKCGVRIPYKISTSTNCARDGLS	• 20: ACLVLMCMAVVAAPIAQATISCGQVTS • 37: ATISCGQVTSKLAPCINFLRFGGPVSG • 47: KLAPCINFLRFGGPVSGGCCGGVRALV • 87: NCLKSAAGAIKFNPTNAAALPGKCGVR • 94: GAIKFNPTNAAALPGKCGVRIPYKIST • 102: NAAALPGKCGVRIPYKISTSTNCARDG • 119: STSTNCARDGLSLPVLFALPLQIAGIR	• 43: QVTSKLAPCINFLRFGGPVSGGCCGGV • 57: FGGPVSGGCCGGVRALVAAAQSTADKQ • 100: PTNAAALPGKCGVRIPYKISTSTNCAR • 113: RIPYKISTSTNCARDGLSLPVLFALPL	• 25: MCMAVVAAPIAQATISCGQVTSKLAPC • 32: APIAQATISCGQVTSKLAPCINFLRFG • 66: CGGVRALVAAAQSTADKQAACNCLKSA • 88: CLKSAAGAIKFNPTNAAALPGKCGVRI • 107: PGKCGVRIPYKISTSTNCARDGLSLPV • 109: KCGVRIPYKISTSTNCARDGLSLPVLF
Lupinus angustifolius	• 57: SGGAVPGTCCNGVKGLVALAQSTADKQ • 69: VKGLVALAQSTADKQTACNCLKSVAAS • 77: QSTADKQTACNCLKSVAASTQFNPENA • 96: TQFNPENAASLPGKCGVNLPYKISTST	• 20: ACMVLMCMVVVGAPIAQATITCGQVVS • 37: ATITCGQVVSSLAPCLTYLQSGGAVPG • 49: APCLTYLQSGGAVPGTCCNGVKGLVAL • 87: NCLKSVAASTQFNPENAASLPGKCGVN	-	• 25: MCMVVVGAPIAQATITCGQVVSSLAPC • 27: MVVVGAPIAQATITCGQVVSSLAPCLT • 40: TCGQVVSSLAPCLTYLQSGGAVPGTCC • 51: CLTYLQSGGAVPGTCCNGVKGLVALAQ

Sequence	Carbonylations			
	K	P	R	T
	• 104: ASLPGKCGVNLPYKISTSTNSSSSEEL	• 94: ASTQFNPENAASLPGKCGVNLPYKIST • 102: NAASLPGKCGVNLPYKISTSTNSSSSE		• 66: CNGVKGLVALAQSTADKQTACNCLKSV • 71: GLVALAQSTADKQTACNCLKSVAASTQ • 83: QTACNCLKSVAASTQFNPENAASLPGK • 107: PGKCGVNLPYKISTSTNSSSSEELMWW • 109: KCGVNLPYKISTSTNSSSSEELMWWER
Glycine max	• 78: RTTGDRRAVCNCLKIAAGAVRKLNPYN • 86: VCNCLKIAAGAVRKLNPYNAQALPGKC • 98: RKLNPYNAQALPGKCGVNIPYKISTST	• 38: QGIRCGQVQGNLAPCLGFLQNGGAVSR • 89: CLKIAAGAVRKLNPYNAQALPGKCGVN • 96: AVRKLNPYNAQALPGKCGVNIPYKIST • 104: NAQALPGKCGVNIPYKISTSTNCNSIK	• 28: MVVMVAHNTVQGIRCGQVQGNLAPCLG • 51: PCLGFLQNGGAVSRGCCNGVRSIVNNA • 58: NGGAVSRGCCNGVRSIVNNARTTGDRR • 65: GCCNGVRSIVNNARTTGDRRAVCNCLK • 70: VRSIVNNARTTGDRRAVCNCLKIAAGA • 71: RSIVNNARTTGDRRAVCNCLKIAAGAV • 85: AVCNCLKIAAGAVRKLNPYNAQALPGK	• 23: MVLACMVVMVAHNTVQGIRCGQVQGNL • 66: CCNGVRSIVNNARTTGDRRAVCNCLKI • 67: CNGVRSIVNNARTTGDRRAVCNCLKIA
Arachis hypogaea	• 58: RGGVPTPTCCQGVKNILASARTPADRR • 78: RTPADRRTVCTCLKSSAGQVPGLNLAN • 98: PGLNLANAGSLPSKCGVNIPYKISPST • 106: GSLPSKCGVNIPYKISPSTNCNTINSI	• 21: CVVLMVCMALLSAPLVHGAMSCGTVTV • 49: LTSCLGYLQRGGVPTPTCCQGVKNILA • 51: SCLGYLQRGGVPTPTCCQGVKNILASA • 67: CQGVKNILASARTPADRRTVCTCLKSS • 85: TVCTCLKSSAGQVPGLNLANAGSLPSK • 96: QVPGLNLANAGSLPSKCGVNIPYKISP • 104: NAGSLPSKCGVNIPYKISPSTNCNTIN • 109: PSKCGVNIPYKISPSTNCNTINSILKC	• 45: VTVSLTSCLGYLQRGGVPTPTCCQGVK • 65: TCCQGVKNILASARTPADRRTVCTCLK • 70: VKNILASARTPADRRTVCTCLKSSAGQ • 71: KNILASARTPADRRTVCTCLKSSAGQV	• 31: LSAPLVHGAMSCGTVTVSLTSCLGYLQ • 33: APLVHGAMSCGTVTVSLTSCLGYLQRG • 37: HGAMSCGTVTVSLTSCLGYLQRGGVPT • 50: TSCLGYLQRGGVPTPTCCQGVKNILAS • 52: CLGYLQRGGVPTPTCCQGVKNILASAR • 66: CCQGVKNILASARTPADRRTVCTCLKS • 72: NILASARTPADRRTVCTCLKSSAGQVP • 75: ASARTPADRRTVCTCLKSSAGQVPGLN • 111: KCGVNIPYKISPSTNCNTINSILKCFT • 115: NIPYKISPSTNCNTINSILKCFTCDGE
Cajanus cajan	• 23: VLMATVWVAVLSPKAEAAVTCGQVVSN • 49: NLTPCVSYVLNGGKTVPVPCCNGIKTL • 60: GGKTVPVPCCNGIKTLYNLAHNTPDRQ • 80: HNTPDRQTVCNCIKNAIRAIPYSKSNV • 90: NCIKNAIRAIPYSKSNVDLAAGLPKKC • 101: YSKSNVDLAAGLPKKCGVNIPYKISPS • 102: SKSNVDLAAGLPKKCGVNIPYKISPST	• 22: LVLMATVWVAVLSPKAEAAVTCGQVVS • 39: AAVTCGQVVSNLTPCVSYVLNGGKTVP • 52: PCVSYVLNGGKTVPVPCCNGIKTLYNL • 54: VSYVLNGGKTVPVPCCNGIKTLYNLAH • 70: NGIKTLYNLAHNTPDRQTVCNCIKNAI • 87: TVCNCIKNAIRAIPYSKSNVDLAAGLP • 100: PYSKSNVDLAAGLPKKCGVNIPYKISP • 108: LAAGLPKKCGVNIPYKISPSTDCSRVQ	• 72: IKTLYNLAHNTPDRQTVCNCIKNAIRA • 84: DRQTVCNCIKNAIRAIPYSKSNVDLAA	• 14: MANSGVVKLVLMATVWVAVLSPKAEAA • 29: WVAVLSPKAEAAVTCGQVVSNLTPCVS • 38: EAAVTCGQVVSNLTPCVSYVLNGGKTV • 50: LTPCVSYVLNGGKTVPVPCCNGIKTLY • 61: GKTVPVPCCNGIKTLYNLAHNTPDRQT • 69: CNGIKTLYNLAHNTPDRQTVCNCIKNA • 74: TLYNLAHNTPDRQTVCNCIKNAIRAIP

Sequence	Carbonylations			
	K	P	R	T
Phaseolus vulgaris	• 76: RSTADRRGICNCLKTAAGAVRGLNPNN • 96: RGLNPNNAQALPGKCGVNIPYKISTST	• 36: QGMTCGQVQSNLVPCVTFLQNGGFVPA • 48: VPCVTFLQNGGFVPAGCCNGVRNIMNS • 87: CLKTAAGAVRGLNPNNAQALPGKCGVN • 94: AVRGLNPNNAQALPGKCGVNIPYKIST • 102: NAQALPGKCGVNIPYKISTSTNCASIN	• 56: NGGFVPAGCCNGVRNIMNSARSTADRR • 63: GCCNGVRNIMNSARSTADRRGICNCLK • 68: VRNIMNSARSTADRRGICNCLKTAAGA • 69: RNIMNSARSTADRRGICNCLKTAAGAV • 83: GICNCLKTAAGAVRGLNPNNAQALPGK	• 21: CVVVLCMVVVGAHTAQGMTCGQVQSNL • 26: CMVVVGAHTAQGMTCGQVQSNLVPCVT • 39: TCGQVQSNLVPCVTFLQNGGFVPAGCC • 65: CNGVRNIMNSARSTADRRGICNCLKTA • 77: STADRRGICNCLKTAAGAVRGLNPNNA
Glycine soja	• 58: NGGTPPSGCCNGVKSLNAAAKTTADRQ • 65: GCCNGVKSLNAAAKTTADRQTACNCLK • 78: KTTADRQTACNCLKSAASQISGFKANN • 88: NCLKSAASQISGFKANNAASLPGKCGV • 98: SGFKANNAASLPGKCGVSIPYKISTST	• 21: FLAAVLCMVVVSAPMAHAAITCGQVTN • 49: LINCIGYLQNGGTPPSGCCNGVKSLNA • 50: INCIGYLQNGGTPPSGCCNGVKSLNAA • 96: QISGFKANNAASLPGKCGVSIPYKIST	• 70: VKSLNAAAKTTADRQTACNCLKSAASQ	• 28: MVVVSAPMAHAAITCGQVTNSLINCIG • 33: APMAHAAITCGQVTNSLINCIGYLQNG • 48: SLINCIGYLQNGGTPPSGCCNGVKSLN • 66: CCNGVKSLNAAAKTTADRQTACNCLKS • 67: CNGVKSLNAAAKTTADRQTACNCLKSA • 72: SLNAAAKTTADRQTACNCLKSAASQIS
Lens culinaris	• 50: GGPGPSPQCCGGVKKLLAAANTTPDRQ • 51: GPGPSPQCCGGVKKLLAAANTTPDRQA • 70: NTTPDRQAACNCLKSAAGSITKLNTNN • 78: ACNCLKSAAGSITKLNTNNAAALPGKC • 90: TKLNTNNAAALPGKCGVNIPYKISTST	• 30: GAISCGAVTSDLSPCLTYLTGGPGPSP • 39: SDLSPCLTYLTGGPGPSPQCCGGVKKL • 41: LSPCLTYLTGGPGPSPQCCGGVKKLLA • 43: PCLTYLTGGPGPSPQCCGGVKKLLAAA • 60: GGVKKLLAAANTTPDRQAACNCLKSAA • 88: SITKLNTNNAAALPGKCGVNIPYKIST • 96: NAAALPGKCGVNIPYKISTSTNCNTVK	• 62: VKKLLAAANTTPDRQAACNCLKSAAGS	• 25: APMAEGAISCGAVTSDLSPCLTYLTGG • 33: SCGAVTSDLSPCLTYLTGGPGPSPQCC • 36: AVTSDLSPCLTYLTGGPGPSPQCCGGV • 58: CCGGVKKLLAAANTTPDRQAACNCLKS • 59: CGGVKKLLAAANTTPDRQAACNCLKSA • 77: AACNCLKSAAGSITKLNTNNAAALPGK • 81: CLKSAAGSITKLNTNNAAALPGKCGVN
Trifolium pratense	• 67: PCCNGIRSLNNQAKTTPDRQSVCRCLK • 80: KTTPDRQSVCRCLKSTALSLPGLNLPA • 100: PGLNLPAAASILAKCGVNLPYKISPSI • 108: ASILAKCGVNLPYKISPSIDCNTYISL	• 22: VTCFAMICLVLGIPLADAALPCGQVQL • 29: CLVLGIPLADAALPCGQVQLTLTPCLG • 39: AALPCGQVQLTLTPCLGYLRRPGPSVP • 47: QLTLTPCLGYLRRPGPSVPPPCCNGIR • 49: TLTPCLGYLRRPGPSVPPPCCNGIRSL • 52: PCLGYLRRPGPSVPPPCCNGIRSLNNQ • 53: CLGYLRRPGPSVPPPCCNGIRSLNNQA • 54: LGYLRRPGPSVPPPCCNGIRSLNNQAK • 70: NGIRSLNNQAKTTPDRQSVCRCLKSTA • 87: SVCRCLKSTALSLPGLNLPAAASILAK • 92: LKSTALSLPGLNLPAAASILAKCGVNL • 106: AAASILAKCGVNLPYKISPSIDCNTYI	• 45: QVQLTLTPCLGYLRRPGPSVPPPCCNG • 46: VQLTLTPCLGYLRRPGPSVPPPCCNGI • 60: PGPSVPPPCCNGIRSLNNQAKTTPDRQ • 72: IRSLNNQAKTTPDRQSVCRCLKSTALS • 77: NQAKTTPDRQSVCRCLKSTALSLPGLN	• 36: LADAALPCGQVQLTLTPCLGYLRRPGP • 38: DAALPCGQVQLTLTPCLGYLRRPGPSV • 68: CCNGIRSLNNQAKTTPDRQSVCRCLKS • 69: CNGIRSLNNQAKTTPDRQSVCRCLKST • 82: TPDRQSVCRCLKSTALSLPGLNLPAAA - 117: NLPYKISPSIDCNTYISLNQLSIYFHL

Sequence	Carbonylations			
	K	P	R	T
		• 111: LAKCGVNLPYKISPSIDCNTYISLNQL		
Spatholobus suberectus	• 33: LLHNHFPLRMASFKLACAVLVCMAAVG • 84: HGGPAPAGCCNGVKSILNAARTTADRR • 104: RTTADRRAICNCLKTAAGAVRGLNPSN • 124: RGLNPSNAQALPGKCGVNIPYKISTST • 132: QALPGKCGVNIPYKISTSTNCARDFDL	• 26: LATSLQNLLHNHFPLRMASFKLACAVL • 64: QAITCGQVEGNLAPCIGFLQHGGPAPA • 74: NLAPCIGFLQHGGPAPAGCCNGVKSIL • 76: APCIGFLQHGGPAPAGCCNGVKSILNA • 115: CLKTAAGAVRGLNPSNAQALPGKCGVN • 122: AVRGLNPSNAQALPGKCGVNIPYKIST • 130: NAQALPGKCGVNIPYKISTSTNCARDF	• 28: TSLQNLLHNHFPLRMASFKLACAVLVC • 91: GCCNGVKSILNAARTTADRRAICNCLK • 96: VKSILNAARTTADRRAICNCLKTAAGA • 97: KSILNAARTTADRRAICNCLKTAAGAV • 111: AICNCLKTAAGAVRGLNPSNAQALPGK • 141: NIPYKISTSTNCARDFDLMLSSFLCIH	• 15: KTATLNTRTHELATSLQNLLHNHFPLR • 49: CAVLVCMAAVGAHTAQAITCGQVEGNL • 54: CMAAVGAHTAQAITCGQVEGNLAPCIG • 92: CCNGVKSILNAARTTADRRAICNCLKT • 93: CNGVKSILNAARTTADRRAICNCLKTA • 105: TTADRRAICNCLKTAAGAVRGLNPSNA • 135: PGKCGVNIPYKISTSTNCARDFDLMLS • 137: KCGVNIPYKISTSTNCARDFDLMLSSF
Cicer arientinum	• 76: VTTPDRQAACNCLKSAAGSISRLNANN • 96: SRLNANNAAALPGKCGVNIPYKISTST	• 19: VVCVALIMCIVIAPMAESAITCGRVSA • 36: SAITCGRVSAALAPCLGYLQGGPGPSA • 45: AALAPCLGYLQGGPGPSAQCCGGVRNL • 47: LAPCLGYLQGGPGPSAQCCGGVRNLNS • 66: GGVRNLNSAAVTTPDRQAACNCLKSAA • 94: SISRLNANNAAALPGKCGVNIPYKIST • 102: NAAALPGKCGVNIPYKISTSTNCATIR	• 29: VIAPMAESAITCGRVSAALAPCLGYLQ • 56: GGPGPSAQCCGGVRNLNSAAVTTPDRQ • 68: VRNLNSAAVTTPDRQAACNCLKSAAGS • 84: ACNCLKSAAGSISRLNANNAAALPGKC	• 26: MCIVIAPMAESAITCGRVSAALAPCLG • 64: CCGGVRNLNSAAVTTPDRQAACNCLKS • 65: CGGVRNLNSAAVTTPDRQAACNCLKSA
Vigna unguiculata	• 78: RTTGDRRTACNCLKSLAASFSGLNLNT	• 21: SVVAVMCMVLVTAPLTHAITCGQVTSA • 37: HAITCGQVTSAISPCIGYLRGGGGVPP • 49: SPCIGYLRGGGGVPPAQCCGGVRRLNS • 50: PCIGYLRGGGGVPPAQCCGGVRRLNSA • 96: SFSGLNLNTAASLPGRCRMRIPYRISP • 104: TAASLPGRCRMRIPYRISPSTNCNRIR	• 43: QVTSAISPCIGYLRGGGGVPPAQCCGG • 58: GGGVPPAQCCGGVRRLNSAARTTGDRR • 59: GGVPPAQCCGGVRRLNSAARTTGDRRT • 65: QCCGGVRRLNSAARTTGDRRTACNCLK • 70: VRRLNSAARTTGDRRTACNCLKSLAAS • 71: RRLNSAARTTGDRRTACNCLKSLAASF • 98: SGLNLNTAASLPGRCRMRIPYRISPST • 100: LNLNTAASLPGRCRMRIPYRISPSTNC • 102: LNTAASLPGRCRMRIPYRISPSTNCNR	• 19: LASVVAVMCMVLVTAPLTHAITCGQVT • 23: VAVMCMVLVTAPLTHAITCGQVTSAIS • 27: CMVLVTAPLTHAITCGQVTSAISPCIG • 32: TAPLTHAITCGQVTSAISPCIGYLRGG • 66: CCGGVRRLNSAARTTGDRRTACNCLKS • 67: CGGVRRLNSAARTTGDRRTACNCLKSL • 72: RLNSAARTTGDRRTACNCLKSLAASFS • 91: KSLAASFSGLNLNTAASLPGRCRMRIP
Abrus precatorius	• 24: LAIVCLALGATIPKAQGAVTCGQVVNN • 61: GGNMVPAQCCSGVKNLNSMARTTPDRQ • 81: RTTPDRQTVCNCIKNAVSNSGFTYTSF • 105: TSFNLNLAAGLPRKCGVNIPYQISPNT	• 23: CLAIVCLALGATIPKAQGAVTCGQVVN • 40: GAVTCGQVVNNLTPCISYVVYGGNMVP • 53: PCISYVVYGGNMVPAQCCSGVKNLNSM • 71: SGVKNLNSMARTTPDRQTVCNCIKNAV • 103: TYTSFNLNLAAGLPRKCGVNIPYQISP • 111: LAAGLPRKCGVNIPYQISPNTDCSRVQ	• 68: QCCSGVKNLNSMARTTPDRQTVCNCIK • 73: VKNLNSMARTTPDRQTVCNCIKNAVSN • 104: YTSFNLNLAAGLPRKCGVNIPYQISPN	• 21: LVCLAIVCLALGATIPKAQGAVTCGQV • 30: ALGATIPKAQGAVTCGQVVNNLTPCIS • 39: QGAVTCGQVVNNLTPCISYVVYGGNMV • 69: CCSGVKNLNSMARTTPDRQTVCNCIKN • 70: CSGVKNLNSMARTTPDRQTVCNCIKNA • 75: NLNSMARTTPDRQTVCNCIKNAVSNSG

Sequence	Carbonylations			
	K	P	R	T
				• 90: CNCIKNAVSNSGFTYTSFNLNLAAGLP • 92: CIKNAVSNSGFTYTSFNLNLAAGLPRK
Arachis ipaensis	• 58: RGGAPPLACCQGVKNVLAGARTPADRK • 71: KNVLAGARTPADRKTVCTCLKTSAGQV • 78: RTPADRKTVCTCLKTSAGQVPGINLAN • 98: PGINLANAGSLPSKCGVNIPYKISPST	• 21: CVVLMVCMALLSAPMVHGAISCGTVTV • 38: GAISCGTVTVSLAPCLAYLQRGGAPPL • 49: LAPCLAYLQRGGAPPLACCQGVKNVLA • 50: APCLAYLQRGGAPPLACCQGVKNVLAG • 67: CQGVKNVLAGARTPADRKTVCTCLKTS • 85: TVCTCLKTSAGQVPGINLANAGSLPSK • 96: QVPGINLANAGSLPSKCGVNIPYKISP • 104: NAGSLPSKCGVNIPYKISPSTNCNTIK	• 45: VTVSLAPCLAYLQRGGAPPLACCQGVK • 65: ACCQGVKNVLAGARTPADRKTVCTCLK • 70: VKNVLAGARTPADRKTVCTCLKTSAGQ	• 31: LSAPMVHGAISCGTVTVSLAPCLAYLQ • 33: APMVHGAISCGTVTVSLAPCLAYLQRG • 66: CCQGVKNVLAGARTPADRKTVCTCLKT • 72: NVLAGARTPADRKTVCTCLKTSAGQVP • 75: AGARTPADRKTVCTCLKTSAGQVPGIN • 79: TPADRKTVCTCLKTSAGQVPGINLANA
Trifolium subterraneum	• 30: PGINLSALASTPTKCGINLPYKISPSI • 38: ASTPTKCGINLPYKISPSINCNTYFSD	• 22: VTCLALICLVLNIPLANAALSCGQIQL • 39: AALSCGQIQLTVAPCLGYLRNPGPSVP • 47: QLTVAPCLGYLRNPGPSVPAPCCNGLR • 49: TVAPCLGYLRNPGPSVPAPCCNGLRNL • 52: PCLGYLRNPGPSVPAPCCNGLRNLNNQ • 54: LGYLRNPGPSVPAPCCNGLRNLNNQAK • 17: SGCRCLKSTIFSLPGINLSALASTPTK • 28: SLPGINLSALASTPTKCGINLPYKISP • 36: ALASTPTKCGINLPYKISPSINCNTYF	• 45: QIQLTVAPCLGYLRNPGPSVPAPCCNG	• 36: LANAALSCGQIQLTVAPCLGYLRNPGP • 27: FSLPGINLSALASTPTKCGINLPYKIS • 29: LPGINLSALASTPTKCGINLPYKISPS
Prosopis alba	• 69: VRSLLSAAQTTVDKQTVCNCLKGAAGQ • 77: QTTVDKQTVCNCLKGAAGQLPGLNPQN • 99: LNPQNAQNLPAQCKVNIPYKISTSTNC	• 21: ACMVVLCVALVATPIAEAITCGQVTTS • 37: EAITCGQVTTSLAPCLSYLQSGGAPAP • 48: LAPCLSYLQSGGAPAPACCNGVRSLLS • 50: PCLSYLQSGGAPAPACCNGVRSLLSAA • 84: TVCNCLKGAAGQLPGLNPQNAQNLPAQ • 88: CLKGAAGQLPGLNPQNAQNLPAQCKVN • 95: QLPGLNPQNAQNLPAQCKVNIPYKIST • 103: NAQNLPAQCKVNIPYKISTSTNCANIR	• 57: SGGAPAPACCNGVRSLLSAAQTTVDKQ	• 20: VACMVVLCVALVATPIAEAITCGQVTT • 27: CVALVATPIAEAITCGQVTTSLAPCLS • 32: ATPIAEAITCGQVTTSLAPCLSYLQSG • 33: TPIAEAITCGQVTTSLAPCLSYLQSGG • 65: CCNGVRSLLSAAQTTVDKQTVCNCLKG • 66: CNGVRSLLSAAQTTVDKQTVCNCLKGA • 71: SLLSAAQTTVDKQTVCNCLKGAAGQLP
Vigna angularis	• 43: VQGNLAQCIGFLQKGGFVPPACCSGVK • 56: KGGFVPPACCSGVKNILNSSRTTPDRR • 76: RTTPDRRAVCSCLKAAAGAVRGINPNN • 96: RGINPNNAEALPGKCGVNLPYKISAST	• 48: AQCIGFLQKGGFVPPACCSGVKNILNS • 49: QCIGFLQKGGFVPPACCSGVKNILNSS • 66: SGVKNILNSSRTTPDRRAVCSCLKAAA • 87: CLKAAAGAVRGINPNNAEALPGKCGVN	• 63: ACCSGVKNILNSSRTTPDRRAVCSCLK • 68: VKNILNSSRTTPDRRAVCSCLKAAAGA • 69: KNILNSSRTTPDRRAVCSCLKAAAGAV • 83: AVCSCLKAAAGAVRGINPNNAEALPGK	• 26: FMVVVGSHSAVGMTCGQVQGNLAQCIG • 64: CCSGVKNILNSSRTTPDRRAVCSCLKA • 65: CSGVKNILNSSRTTPDRRAVCSCLKAA • 109: KCGVNLPYKISASTNCNRYIYYFEVSS

Sequence	Carbonylations			
	K	P	R	T
	• 104: EALPGKCGVNLPYKISASTNCNRYIYY	• 94: AVRGINPNNAEALPGKCGVNLPYKISA • 102: NAEALPGKCGVNLPYKISASTNCNRYI		
Arachis duranensis	• 23: LMLCMAIVGAPIAKAAIQCSFVTKSIA • 33: PIAKAAIQCSFVTKSIAPCFGYLKSGG • 43: FVTKSIAPCFGYLKSGGTVSGPCCSGI • 64: PCCSGIQNINGTAKTTSDRQAVCNCLK • 77: KTTSDRQAVCNCLKSVAGSLGSQINLN • 98: SQINLNNAASLPGKCGVSIPYKISTST	• 20: CVVLMLCMAIVGAPIAKAAIQCSFVTK • 37: AAIQCSFVTKSIAPCFGYLKSGGTVSG • 51: CFGYLKSGGTVSGPCCSGIQNINGTAK • 96: LGSQINLNNAASLPGKCGVSIPYKIST • 104: NAASLPGKCGVSIPYKISTSTNCSSIK	• 69: IQNINGTAKTTSDRQAVCNCLKSVAGS	• 32: APIAKAAIQCSFVTKSIAPCFGYLKSG • 47: SIAPCFGYLKSGGTVSGPCCSGIQNIN • 62: SGPCCSGIQNINGTAKTTSDRQAVCNC • 65: CCSGIQNINGTAKTTSDRQAVCNCLKS • 66: CSGIQNINGTAKTTSDRQAVCNCLKSV
Pisum sativum	• 60: NNASPPPPCCAGVKKLLGAATTTPDRQ • 61: NASPPPPCCAGVKKLLGAATTTPDRQA • 80: TTTPDRQAACNCLKSAAGSISRLNTNN • 100: SRLNTNNAAALPGKCGVSIPYKISTST	• 21: ACVALVMCMVVIAPMAEAALSCGTVSG • 38: AALSCGTVSGDLAPCLTYLQAPNNASP • 46: SGDLAPCLTYLQAPNNASPPPPCCAGV • 51: PCLTYLQAPNNASPPPPCCAGVKKLLG • 52: CLTYLQAPNNASPPPPCCAGVKKLLGA • 53: LTYLQAPNNASPPPPCCAGVKKLLGAA • 54: TYLQAPNNASPPPPCCAGVKKLLGAAT • 70: AGVKKLLGAATTTPDRQAACNCLKSAA • 98: SISRLNTNNAAALPGKCGVSIPYKIST • 106: NAAALPGKCGVSIPYKISTSTNCNTIK	• 72: VKKLLGAATTTPDRQAACNCLKSAAGS • 88: ACNCLKSAAGSISRLNTNNAAALPGKC	• 31: VIAPMAEAALSCGTVSGDLAPCLTYLQ • 41: SCGTVSGDLAPCLTYLQAPNNASPPPP • 67: PCCAGVKKLLGAATTTPDRQAACNCLK • 68: CCAGVKKLLGAATTTPDRQAACNCLKS • 69: CAGVKKLLGAATTTPDRQAACNCLKSA • 91: CLKSAAGSISRLNTNNAAALPGKCGVS

The carbons are classified by residues to be modified such as K: lysine; P: proline; R: arginine; T: threonine.

Table A3.
Post-translational modifications. carbonylations.

Protein name and accesión number	ligad	Residues involved in the interactino
Lup an 3 (Uniprot: A0A1J7GK90)	Acid esteárico (STE)	V^{31}, L^{34}, A^{35}, C^{37}, I^{38}, V^{55}, L^{58}, V^{59}, A^{62}, L^{75}, I^{82}, L^{93}, V^{101}, Y^{103}, I^{105}
	Ácid 10-oxo-12-octadecenoic (ASY)	V^{31}, L^{34}, A^{35}, L^{75}, S^{77}, A^{78}, V^{79}, A^{81}, I^{82}, I^{105}
	Lauroil (LAP)	V^{55}, V^{59}, K^{68}, A^{90}, S^{100}, V^{101}, P^{102}, Y^{103}
Lup an 3.0101 (Uniprot: A0A4P1RWD8)	Prostaglandine B2 (E2P)	L^{35}, C^{38}, L^{42}, C^{52}, I^{56}, I^{59}, A^{63}, C^{75}, L^{76}, A^{79}, L^{94}, I^{102}, P^{103}, Y^{104}, K^{105}, I^{106}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	L^{60}, R^{69}, C^{73}, L^{76}, A^{80}, A^{91}, N^{101}, K^{105}, S^{107}, T^{108}, I^{115}
	Ácido 10-oxo-12-octadecenoico (ASY)	T^{32}, L^{35}, A^{36}, L^{76}, A^{78}, A^{79}, A^{80}, N^{82}, T^{83}, I^{106}
Medicago truncatula (Uniprot: A0A072UTH7)	Ácido esteárico (STE)	T^{31}, L^{34}, A^{35}, C^{37}, I^{38}, V^{55}, L^{58}, N^{59}, A^{62}, L^{75}, I^{82}, L^{93}, I^{101}, Y^{103}, I^{105}
	Ácido 10-oxo-12-octadecenoico (ASY)	T^{31}, L^{34}, A^{35}, L^{75}, S^{77}, A^{78}, A^{79}, A^{81}, I^{82}, I^{105}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	N^{59}, R^{68}, C^{72}, L^{75}, A^{79}, A^{90}, A^{100}, P^{104}, K^{106}, C^{107}, K^{114}
***Arabidopsis thaliana* (nsLTP-3)** (Uniprot: Q9LLR7)	Ácido esteárico (STE)	A^{31}, L^{34}, A^{35}, C^{37}, A^{38}, V^{55}, L^{58}, N^{59}, A^{62}, I^{75}, I^{82}, L^{93}, I^{101}, Y^{103}, I^{105}
	Ácido 10-oxo-12-octadecenoico (ASY)	A^{31}, L^{34}, A^{35}, I^{75}, S^{77}, T^{78}, A^{79}, S^{81}, I^{82}, I^{105}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	N^{59}, R^{68}, C^{72}, I^{75}, A^{79}, A^{90}, S^{100}, P^{104}, P^{106}, M^{107}, I^{114}
***Arabidopsis thaliana* (nsLTP-5)** (Uniprot: Q9XFS7)	Ácido esteárico (STE)	T^{19}, L^{22}, G^{23}, C^{25}, Y^{26}, V^{43}, L^{46}, N^{47}, A^{50}, I^{63}, L^{70}, L^{82}, I^{90}, Y^{92}, I^{94}
	Ácido 10-oxo-12-octadecenoico (ASY)	T^{19}, L^{22}, G^{23}, I^{63}, G^{65}, A^{66}, A^{67}, A^{69}, L^{70}, I^{94}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	N^{47}, R^{56}, C^{60}, I^{63}, A^{67}, A^{79}, R^{89}, P^{93}, S^{95}, A^{96}, V^{103}
Ole e 7 (NCBI: XP_022893508.1)	Ácido palmítico (PLM)	V^{32}, L^{36}, V^{43}, I^{57}, L^{60}, Y^{61}, I^{73}, L^{77}, A^{92}, L^{95}, P^{96}, V^{101}, V^{103}, Y^{105}
	Ácido 10-oxo-12-octadecenoico (ASY)	V^{33}, L^{36}, K^{37}, L^{77}, S^{79}, L^{80}, A^{81}, S^{83}, F^{84}, I^{107}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	L^{36}, K^{37}, L^{40}, I^{57}, L^{80}, S^{83}, F^{84}
Lupinus albus (Uniprot: A0A6A5MQ88)	Stearic acid (STE)	T^{32}, L^{35}, A^{36}, C^{38}, I^{39}, V^{56}, L^{59}, V^{60}, A^{63}, L^{76}, I^{83}, L^{93} , I^{101}, Y^{103}, I^{105}
	Ácid 10-oxo-12-octadecenoic (ASY)	T^{32}, L^{35}, A^{36}, L^{76}, S^{78}, A^{79}, A^{80}, A^{82}, I^{83}, I^{105}
	Trifluoroacetil (TFA)	L^{59}, V^{60}, A^{63}, A^{72}, R^{100}, I^{101}, P^{102}, Y^{103}
Lupinus angustifolius (Uniprot: A0A4P1RV83)	Palmitic acid (PLM)	V^{31}, L^{35}, L^{42}, V^{56}, L^{59}, V^{60}, A^{72}, L^{76}, A^{90}, L^{93}, P^{94}, V^{99}, L^{101}, Y^{103}, I^{105}
	Ácid 10-oxo-12-octadecenoico (ASY)	V^{32}, L^{35}, A^{36}, L^{76}, S^{78}, V^{79}, A^{80}, S^{82}, T^{83}, I^{105}
	Stearic acid (STE)	V^{32}, L^{35}, A^{36}, C^{38}, L^{39}, V^{56}, L^{59}, V^{60}, A^{63}, L^{76}, T^{83}, L^{93}, L^{101}, Y^{103}, I^{105}

Protein name and accesión number	ligad	Residues involved in the interactino
Glycine max (Uniprot: I1J7M1)	Palmitic acid (PLM)	V^{32}, L^{36}, L^{43}, V^{57}, I^{60}, V^{61}, V^{73}, L^{77}, A^{92}, L^{95}, P^{96}, V^{101}, I^{103}, Y^{105}, I^{107}
	1-Miristoil-sn-glicerol-3-fosfocoline (LPC)	V^{61}, R^{70}, C^{74}, L^{77}, A^{81}, A^{92}, N^{102}, K^{106}, S^{108}, T^{109}, I^{116}
	Ácid 10-oxo-12-octadecenoic (ASY)	Q^{33}, L^{36}, A^{37}, L^{77}, I^{79}, A^{80}, A^{81}, A^{83}, V^{84}, I^{107}
Arachis hypogaea (NCBI: XP_025656480.1)	Stearic acid (STE)	T^{33}, L^{36}, T^{37}, C^{39}, L^{40}, V^{57}, I^{60}, L^{61}, A^{64}, L^{77}, V^{84}, L^{95}, I^{103}, Y^{105}, I^{107}
	Ácid 10-oxo-12-octadecenoic (ASY)	T^{33}, L^{36}, T^{37}, L^{77}, S^{79}, S^{80}, A^{81}, Q^{83}, V^{84}, I^{107}
	1-Miristoil-SN-glicerol-3-fosfocoline (LPC)	L^{61}, R^{70}, C^{74}, L^{77}, A^{81}, A^{92}, N^{102}, K^{106}, S^{108}, P^{109}, I^{116}
Cajanus cajan (NCBI: XP_020237462)	Palmitic acid (PLM)	V^{33}, L^{37},V^{44}, I^{59}, L^{62}, Y^{63}, V^{75}, I^{79}, A^{96}, L^{99}, P^{100}, V^{105}, I^{107}, Y^{109}
	Ácid 10-oxo-12-octadecenoic (ASY)	V^{34}, L^{37}, T^{38}, I^{79}, N^{81}, A^{82}, I^{83}, A^{85}, I^{86}, I^{111}
	1-Miristoil-sn-glicerol-3-fosfocoline (LPC)	Y^{63}, R^{72}, C^{76}, I^{79}, I^{83}, A^{96}, N^{106}, K^{110}, S^{112}, P^{113}, V^{120}
Phaseolus vulgaris (Uniprot: D3W146)	Prostaglandine B2 (E2P)	L^{34}, C^{37}, L^{41}, C^{51}, V^{55}, I^{58}, A^{62}, C^{74}, L^{75}, A^{78}, L^{93}, I^{101}, P^{102}, Y^{103}, K^{104}, I^{105}
	Ácid 10-oxo-12-octadecenoic (ASY)	Q^{31}, L^{34}, V^{35}, L^{75}, T^{77}, A^{78}, A^{79}, A^{81}, V^{82}, I^{105}
	1-Miristoil-SN-glicerol-3-fosfocoline (LPC)	M^{59}, I^{68}, C^{72}, L^{75}, A^{79}, A^{90}, N^{100}, K^{104}, S^{106}, T^{107}, I^{114}
Glycine soja (Uniprot: A0A445M2F4)	Stearic acid (STE)	T^{33}, L^{36}, I^{37}, C^{39}, I^{40}, V^{57}, L^{60}, N^{61}, A^{64}, L^{77}, I^{84}, L^{95}, I^{103}, Y^{105}, I^{107}
	Ácid 10-oxo-12-octadecenoic (ASY)	T^{33}, L^{36}, I^{37}, L^{77}, S^{79}, A^{80}, A^{81}, Q^{83}, I^{84}, I^{107}
	1-Miristoil-SN-glicerol-3-fosfocoline (LPC)	N^{61}, R^{70}, C^{74}, L^{77}, A^{81}, A^{92}, S^{102}, K^{106}, S^{108}, T^{109}
Lens culinaris (Uniprot: A0AT33)	Stearic acid (STE)	T^{25}, L^{28}, S^{29}, C^{31}, L^{32}, V^{49}, L^{52}, L^{53}, A^{56}, L^{69}, I^{76}, L^{87}, I^{95}, Y^{97}, I^{99}
	Ácid 10-oxo-12-octadecenoic (ASY)	T^{25}, L^{28}, S^{29}, L^{69}, S^{71}, A^{72}, A^{73}, S^{75}, I^{76}, I^{99}
	1-Miristoil-SN-glicerol-3-fosfocoline (LPC)	L^{53}, R^{62}, A^{66}, L^{69}, A^{73}, A^{84}, N^{94}, K^{98}, S^{100}, T^{101}, V^{108}
Trifolium pratense (Uniprot: A0A2K3M7A7)	Palmitic acid (PLM)	V^{33}, L^{37}, L^{44}, I^{59}, L^{62}, N^{63}, V^{75}, L^{79}, A^{94}, I^{97}, L^{98}, V^{103}, L^{105}, Y^{107}, L^{130}
	Ácido 10-oxo-12-octadecenoico (ASY)	Q^{34}, L^{37}, T^{38}, L^{79}, S^{81}, T^{82}, A^{83}, S^{85}, L^{86}, I^{109}
	Group trifluoroacetil (TFA)	L^{62}, N^{63}, A^{66}, V^{75}, N^{104}, L^{105}, P^{106}, Y^{107}
Spatholobus suberectus (NCBI: TKY63608.1)	Stearic acid (STE)	E^{59}, L^{62}, A^{63}, C^{65}, I^{66}, V^{83}, I^{86}, L^{87}, A^{90}, L^{103}, V^{110}, L^{121}, I^{129}, Y^{131}, I^{133}
	Ácid 10-oxo-12-octadecenoic (ASY)	E^{59}, L^{62}, A^{63}, L^{103}, T^{105}, A^{106}, A^{107}, A^{109}, V^{110}, I^{133}
	Myristic acid (MYR)	V^{83}, L^{87}, A^{90}, R^{96}

Protein name and accesión number	ligad	Residues involved in the interactino
Cicer arientinum (Uniprot: O23758)	Stearic acid (STE)	S^{31}, L^{34}, A^{35}, C^{37}, L^{38}, V^{55}, L^{58}, N^{59}, A^{62}, L^{75}, I^{82}, S^{93}, I^{101}, Y^{103}, I^{105}
	Ácid 10-oxo-12-octadecenoic (ASY)	S^{31}, L^{34}, A^{35}, L^{75}, S^{77}, A^{78}, A^{79}, S^{81}, I^{82}, I^{105}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	N^{59}, R^{68}, C^{72}, L^{75}, A^{79}, A^{90}, N^{100}, K^{104}, S^{106}, T^{107}, I^{114}
Vigna unguiculata (Uniprot: UPI0010170F74)	Ácido esteárico (STE)	T^{32}, I^{35}, S^{36}, C^{38}, I^{39}, V^{57}, L^{60}, N^{61}, A^{64}, L^{77}, F^{84}, L^{95}, I^{103}, Y^{105}, I^{107}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	N^{61}, R^{70}, C^{74}, L^{77}, A^{81}, A^{92}, R^{102}, R^{106}, S^{108}, P^{109}, I^{116}
	Ácido 10-oxo-12-octadecenoico (ASY)	T^{32}, I^{35}, S^{36}, L^{77}, S^{79}, L^{80}, A^{81}, S^{83}, F^{84}, I^{107}
Abrus precatorius (Uniprot: UPI000F7C313B)	Ácido palmítico (PLM)	V^{34}, L^{38}, V^{45}, V^{60}, L^{63}, N^{64}, V^{76}, I^{80}, A^{99}, L^{102}, P^{103}, V^{108}, I^{110}, Y^{112}, I^{114}
	Ácido 10-oxo-12-octadecenoico (ASY)	V^{35}, L^{38}, T^{39}, I^{80}, N^{82}, A^{83}, V^{84}, N^{86}, S^{87}, I^{114}
	Grupo trifluoroacetil (TFA)	L^{63}, N^{64}, A^{67}, V^{76}, N^{109}, I^{110}, P^{111}, Y^{112}
Arachis ipaensis (NCBI: XP_020971907.1)	Ácido palmítico (PLM)	V^{32}, L^{36}, L^{43}, V^{57}, V^{60}, L^{61}, V^{73}, L^{77}, A^{92}, L^{95}, P^{96}, V^{101}, I^{103}, Y^{105}, I^{107}
	Ácido 10-oxo-12-octadecenoico (ASY)	T^{33}, L^{36}, A^{37}, L^{77}, T^{79}, S^{80}, A^{81}, Q^{83}, V^{84}, I^{107}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	L^{61}, R^{70}, C^{74}, L^{77}, A^{81}, A^{92}, N^{102}, K^{106}, S^{108}, P^{109}, I^{116}
Trifolium subterraneum (NCBI: GAU29990.1)	Ácido palmítico (PLM)	I^{33}, V^{37}, L^{44}, L^{59}, L^{62}, N^{63}, G^{75}, L^{79}, L^{94}, T^{97}, P^{98}, I^{103}, L^{105}, Y^{107}, I^{109}
	Ácido 10-oxo-12-octadecenoico (ASY)	Q^{34}, V^{37}, A^{38}, L^{79}, S^{81}, T^{82}, I^{83}, S^{85}, L^{86}, I^{109}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	N^{63}, R^{72}, C^{76}, L^{79}, I^{83}, L^{94}, N^{104}, K^{108}, S^{110}, P^{111}, Y^{118}
Prosopis alba (NCBI: XP_028808641.1)	Ácido esteárico (STE)	T^{32}, L^{35}, A^{36}, C^{38}, L^{39}, V^{56}, L^{59}, L^{60}, A^{63}, L^{76}, L^{83}, L^{94}, I^{102}, Y^{104}, I^{106}
	Ácido 10-oxo-12-octadecenoico (ASY)	T^{32}, L^{35}, A^{36}, L^{76}, G^{78}, A^{79}, A^{80}, Q^{82}, L^{83}, I^{106}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	L^{60}, K^{69}, C^{73}, L^{76}, A^{80}, A^{91}, N^{101}, S^{107}, T^{108}, I^{115}
Vigna angularis (NCBI: KOM57753.1)	Ácido esteárico (STE)	Q^{31}, L^{34}, A^{35}, C^{37}, I^{38}, V^{55}, I^{58}, L^{59}, S^{62}, L^{75}, V^{82}, L^{93}, L^{101}, Y^{103}, I^{105}
	Ácido 10-oxo-12-octadecenoico (ASY)	Q^{31}, L^{34}, A^{35}, L^{75}, A^{77}, A^{78}, A^{79}, A^{81}, V^{82}, I^{105}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	L^{59}, R^{68}, C^{72}, L^{75}, A^{79}, A^{90}, N^{100}, K^{104}, S^{106}, A^{107}, Y^{114}
Arachis duranensis (NCBI: XP_015950831.1)	Ácido esteárico (STE)	T^{32}, I^{35}, A^{36}, C^{38}, F^{39}, I^{56}, I^{59}, N^{60}, A^{63}, L^{76}, L^{83}, L^{95}, I^{103}, Y^{105}, I^{107}
	Ácido 10-oxo-12-octadecenoico (ASY)	T^{32}, I^{35}, A^{36}, L^{76}, S^{78}, V^{79}, A^{80}, S^{82}, L^{83}, I^{107}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	N^{60}, R^{69}, C^{73}, L^{76}, A^{80}, A^{92}, S^{102}, K^{106}, S^{108}, T^{109}, I^{116}

Protein name and accesión number	ligad	Residues involved in the interactino
Pisum sativum (NCBI: A0A158V755.1)	Ácido esteárico (STE)	S^{33}, L^{36}, A^{37}, C^{39}, L^{40}, V^{59}, L^{62}, L^{63}, A^{66}, L^{79}, I^{86}, L^{97}, I^{105}, Y^{107}, I^{109}
	1-Miristoil-SN-glicerol-3-fosfocolina (LPC)	L^{36}, A^{37}, L^{40}, V^{59}, A^{82}, S^{85}, I^{86}
	Lauroil (LAP)	V^{59}, L^{63}, R^{72}, A^{94}, S^{104}, I^{105}, P^{106}, Y^{107}

This table presents the main ligands of each nsLTP analyzed and the number of residues involved in the protein-ligand binding site. The three highest scoring ligands are shown for each nsLTP.

Table A4.
Interaction motives in nsLTPs for ligands of lipidic nature.

Sequences	Protein ligands	Type of interaction	Score
Medicago truncatula (Uniprot: A0A072UTH7)	**11420485.** *Calmodulin-binding heat shock protein*	T	0,592
	11419595. *DEAD-box helicase family protein; ATP-dependent RNA helicase DDX1*	T	0,592
	11443121. *Thioredoxin-like protein 1-1; Thioredoxin domain 2; Thioredoxin fold; Thioredoxin-like protein; Uncharacterized protein*	T	0,592
	11413919. *Calmodulin-binding transcription activator*	T	0,52
	11427895. *Calmodulin-binding transcription activator*	T	0,52
	11419142. *Lycopene beta-cyclase; NAD-binding site*	T	0,453
	AET05172. *Profilin (actin-binding-protein)*	T	0,453
Arabidopsis thaliana (nsLTP-3) (Uniprot: Q9LLR7)	**LTP4.**	T, H, C	0,866
	MYB96 *transcription factor-like protein*	T	0,812
	AT2G15325. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein*	T	0,669
	ELP. *Extensin-like protein*	T	0,66
	FAR7. Fatty acid reductase 7	T	0,625
	AT1G62510. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein*	T	0,612
	AT5G05960. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein*	T	0,611
	AT4G33550. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein*	T	0,602
	MYB47. *Putative MYB47 transcription factor*	T	0,597
	EXP3. *Barwin-like endoglucanases superfamily protein*	T	0,573
Arabidopsis thaliana (nsLTP-5) (Uniprot: Q9XFS7)	**AT2G15325**. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein.*	T	0,895
	XYP2. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein.*	T	0,695
	LTPG1. *Glycosylphos-phatidylinositol-anchored lipid protein transfer 1.*	T	0,677
	AT2G16592. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein.*	T	0,643

Sequences	Protein ligands	Type of interaction	Score
	AT4G12825. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein.*	T	
	AT2G13295. *Encodes a Protease inhibitor/seed storage/ LTP family protein.*	T	0,637
	AZI1. *pEARLI1-like lipid transfer protein 1; Probable LTP.*	T	0,623
	DIR1. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein.*	T	0,593
	AT1G10770. *Plant invertase/pectin methylesterase inhibitor superfamily protein.*	T	0,568
	PRK2A. Leucine-rich repeat protein kinase family protein.	T	0,567
Olea europea L. (Ole e 7) (NCBI: XP_022893508.1)	**LTP3.**	T, H, C	0,866
	ELP. *Extensin-like protein*	T	0,67
	AT5G55460. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein*	T	0,664
	AT5G55430. *Unknown protein.*	T	0,649
	AT5G55440. *Protein of unknown function (DUF295).*	T	0,649
	LTI30. *Dehydrin protein family.*	T	0,642
	AT1G62510. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein*	T	0,628
	AT5G05960. *Bifunctional inhibitor/LTP/seed storage 2S albumin superfamily protein*	T	0,624
	AT1G09500. *NAD(P)-binding Rossmann-fold superfamily protein.*	T	0,616
	AT3G58690. *Protein kinase superfamily protein.*	T	0,58
Lupinus albus (Uniprot: A0A6A5MQ88)	**11420485.** *Calmodulin-binding heat shock protein*	T	0,592
	11419595. *DEAD-box helicase family protein; ATP-dependent RNA helicase DDX1*	T	0,592
	11443121. *Thioredoxin-like protein 1-1; Thioredoxin domain 2; Thioredoxin fold; Thioredoxin-like protein; Uncharacterized protein*	T	0,592
	11413919. *Calmodulin-binding transcription activator*	T	0,52
	11427895. *Calmodulin-binding transcription activator*	T	0,52
	11419142. *Lycopene beta-cyclase; NAD-binding site*	T	0,453
	AET05172. *Profilin*	T	0,453
Lupinus angustifolius (Uniprot: A0A4P1RV83)	**11420485.** *Calmodulin-binding heat shock protein*	T	0,592
	11419595. *DEAD-box helicase family protein; ATP-dependent RNA helicase DDX1*	T	0,592
	11443121. *Thioredoxin-like protein 1-1; Thioredoxin domain 2; Thioredoxin fold; Thioredoxin-like protein; Uncharacterized protein*	T	0,592
	11413919. *Calmodulin-binding transcription activator*	T	0,52
	11427895. *Calmodulin-binding transcription activator*	T	0,52
	11419142. *Lycopene beta-cyclase; NAD-binding site*	T	0,453

Sequences	Protein ligands	Type of interaction	Score
	AET05172. *Profilin*	T	
Lup an 3 (Uniprot: A0A1J7GK90)	**11420485.** *Calmodulin-binding heat shock protein*	T	0,592
	11419595. *DEAD-box helicase family protein; ATP-dependent RNA helicase DDX1*	T	0,592
	11443121. *Thioredoxin-like protein 1-1; Thioredoxin domain 2; Thioredoxin fold; Thioredoxin-like protein; Uncharacterized protein*	T	0,592
	11413919. *Calmodulin-binding transcription activator*	T	0,52
	11427895. *Calmodulin-binding transcription activator*	T	0,52
	11419142. *Lycopene beta-cyclase; NAD-binding site*	T	0,453
	AET05172. *Profilin*	T	0,453
Lup an 3.0101 (Uniprot: A0A4P1RWD8)	**11420485.** *Calmodulin-binding heat shock protein*	T	0,592
	11419595. *DEAD-box helicase family protein; ATP-dependent RNA helicase DDX1*	T	0,592
	11443121. *Thioredoxin-like protein 1-1; Thioredoxin domain 2; Thioredoxin fold; Thioredoxin-like protein; Uncharacterized protein*	T	0,592
	11413919. *Calmodulin-binding transcription activator*	T	0,52
	11427895. *Calmodulin-binding transcription activator*	T	0,52
	11419142. *Lycopene beta-cyclase; NAD-binding site*	T	0,453
	AET05172. *Profilin*	T	0,453

Type of interactions with nsLTPs: (E): experimental; (T): theoretical; (H): homology; (C): co-expression.

Table A5.
Analysis of functional interaction between nsLTPs and protein ligands.

Protein name	Mapped IgE and PID epitopes	Motives MEME/ MAST	SVM – aminoacid composition	SVM-dipeptide composition	Blast ARPs
Lup an 3 (Uniprot: A0A1J7GK90)	-	-	Potential allergen	Potential allergen	GSISGVNPNNAAGLPGKCGVNVPY
Lup an 3.0101 (Uniprot: A0A4P1RWD8)	-	-	Potential allergen	Potential allergen	SNLAPCINYVKGGGAVPPACCNGI
Medicago truncatula (Uniprot: A0A072UTH7)	-	-	Potential allergen	Potential allergen	CCNGIRNVNNLARTTPDRRTACNC
***Arabidopsis thaliana* (nsLTP-3)** (Uniprot: Q9LLR7)	-	-	Potential allergen	Alérgeno potencial	VPPACCNGIRNVNNLARTTADRR
***Arabidopsis thaliana* (nsLTP-5)** (Uniprot: Q9XFS7)	-	-	allergen	Potential allergen	SGVKNLNSIAKTTPDRQQACNCIQ
Ole e 7 (NCBI: XP_022893508.1)	-	-	Potential allergen	Potential allergen	CNGVRTINNAAKTTADRRTACQCL
Lupinus albus (Uniprot: A0A6A5MQ88)	-	-	Potential allergen	Potential allergen	AGIPGKCGVNIPYAISQGTDCSK
Lupinus angustifolius (Uniprot: A0A4P1RV83)	-	-	allergen	Potential allergen	CIAYVRGGGAVPPACCNGIRNI
Glycine max (Uniprot: I1J7M1)	-	-	Potential allergen	Potential allergen	NGIRNVNNLARTTPDRQAACNCLK
Arachis hypogaea (NCBI: XP_025656480.1)	-	-	Potential allergen	Allergen	AASIPSKCNVNVPYTISPDIDCS
Cajanus cajan (NCBI: XP_020237462)	-	-	Potential allergen	Potential allergen	NLVAGIPGKCGVNIPYAISQGT
Phaseolus vulgaris (Uniprot: D3W146)	-	-	Potential allergen	Potential allergen	CCNGIRNVNNLARTTPDRRTACNC
Glycine soja (Uniprot: A0A445M2F4)	-	-	Potential allergen	Potential allergen	CCNGIRNVNNLARTTPDRRTACNC
Lens culinaris (Uniprot: A0AT33)	-	-	Potential allergen	Potential allergen	LNLNNAASIPSKCNVNVPYTIS
Trifolium pratense (Uniprot: A0A2K3M7A7)	-	-	allergen	Allergen	KTTADRQTACNCLKQLSASVPGVN
Spatholobus suberectus (NCBI: TKY63608.1)	-	-	Potential allergen	No allergen	VSSSLAPCIGYVRGGGAVPPACCN
Cicer arientinum (Uniprot: O23758)	-	-	Potential allergen	Potential allergen	LKQLSGSISGVNPNNAAALPGKCG

Protein name	Mapped IgE and PID epitopes	Motives MEME/ MAST	SVM – aminoacid composition	SVM-dipeptide composition	Blast ARPs
Vigna unguiculata (Uniprot: UPI0010170F74)	-	-	allergen	No Allergen	GVKNLNSIAKTTPDRQQACNCIQ
Abrus precatorius (Uniprot: UPI000F7C313B)	-	-	Potential allergen	Potential allergen	LNLNNAASIPSKCNVNVPYTISPD
Arachis ipaensis (NCBI: XP_020971907.1)	-	-	Potential allergen	Potential allergen	GSISGVNPNNAAGLPGKCGVNVPY
Trifolium subterraneum (NCBI: GAU29990.1)	-	-	Allergen	Allergen	CCNGVTNLKNMASTTPDRQQACRC
Prosopis alba (NCBI: XP_028808641.1)	-	-	Potential allergen	Potential allergen	SNLAPCINYVKGGGAVPPACCNGI
Vigna angularis (NCBI: KOM57753.1)	-	-	Potential allergen	Allergen	TCGQVSSSLAPCIGYVRGGGAVPP
Arachis duranensis (NCBI: XP_015950831.1)	-	-	Potential allergen	Potential allergen	SGVKNLNSIAKTTPDRQQACNCIQ
Pisum sativum (NCBI: A0A158V755.1)	-	-	Potential allergen	Potential allergen	AGIPGKCGVNIPYAISQGTDCSKV

This table shows IgE epitopes of nsLTPs based on experimentally tested IgE epitopes; MEME / MAST motifs; and they are described as allergens / potential allergen / non-allergen according to SVM analysis based on amino acid and dipeptide composition; they are related to ARPs by blast, and residues contained in the protein appear in red.

Table A6.
IgE binding epitopes.

DOI: http://dx.doi.org/10.5772/intechopen.102889

Proteína	T1	T2	T3	T4	T5	T6	T7	T8	T9
Lup an 3	IKVACVVLMCMAVVAA	YKISVSTNC							
Lup an 3.0101	IVKLACAVLICMVVVSAPLTK	YKISTSTNC	LRSGGAVPA						
Medicago truncatula	MKVACVLLMMCIIVAPM	YKISTSTNC							
Arabidopsis thaliana (nsLTP-3)	LRFFTCLVLTVCIVAS								
Arabidopsis thaliana (nsLTP-5)	MLVTAPMAS	VRISYPISA			VQRLNSLAR				
Ole e 7	VVKATCFVLIAVALVA								
Lupinus albus	LVLMCMAVVAA	VRIPYKISTSTNC	FLRFGGPVS	FNPTNAAAL	VRALVAAAQ	VLFALPLQI			
Lupinus angustifolius	MVLMCMVVVGAPIAQA	YKISTSTNS			VKGLVALAQ	YRHHSKFLV	VVSSLAPCL		
Glycine max	FTKLACMVLACMVVMVAHNTV	YKISTSTNC		VRKLNPYNA	VRSIVNNAR				
Arachis hypogaea	IRVTCVVLMVCMALLSA	YKISPSTNC		LNLANAGSLPS					
Cajanus cajan	VVKLVLMATVWVAVLSPKA	YKISPSTDC	YVLNGGKTV				VVSNLTPCVS		
Phaseolus vulgaris	VKFACVVVLCMVVVGAHTAQG	YKISTSTNC	FVPAGCCNG	VRGLNPNNA	VRNIMNSAR		LVPCVTFLQ		
Glycine soja	VLCMVVVSAPMAH	YKISTSTNC							
Lens culinaris	VVLVMCMVVIAPMAE	YKISTSTNC							
Trifolium pratense	LVKVTCFAMICLVLGIPLAD	YKISPSIDC							YISLNQLSI

Proteína	T1	T2	T3	T4	T5	T6	T7	T8	T9
Spatholobus suberectus	FKLACAVLVCMAAVGAH			VRGLNPSNA				LHNHFPLRM	
Cicer arientinum	MKVVCVALIMCIVIAPMAES	YKISTSTNC			VRNLNSAAV				
Vigna unguiculata	LKLASVVAVMCMVLVTAPLT	MRIPYRISPSTNC			VRRLNSAAR				
Abrus precatorius	IRLVCLAIVCL	YQISPNTDC	YVVYGGNMVPAQ	FNLNLAAGL	VKNLNSMAR		VVNNLTPCIS		
Arachis ipaensis	IRVTCVVLMVCMALLSAPMV	YKISPSTNC	LAYLQRGGAPPL	INLANAGSLPS					
Trifolium subterraneum	LVKVTCLAL—LVLNIPLAN	YKISPSINC		IFSLPGINL					
Prosopis alba	LIKVACMVVLCVALVA	YKISTSTNC			VRSLLSAAQ				
Vigna angularis	VKFACVVVMFMVVVGSHS	YKISASTNC		VRGINPNNA					
Arachis duranensis	VVLMLCMAIVGAPIAKA	YKISTSTNC					IQCSFVTKSIAPC		
Pisum sativum	MKLACVALVMCMVVIAPMAE	YKISTSTNC							

The epitopes are named as Ti, where i is the number assigned to each different epitope.

Table A7.
Identification of T-cell binding epitopes.

DOI: http://dx.doi.org/10.5772/intechopen.102889

Protein	B1	B2	B3	B4	B5	B6
Lup an 3	SSAQTTADKRT	PNYNDANA				
Lup an 3.0101	AKTTPDRRTACN	GLNPSNAG		YKISTSTN		
Medicago truncatula			IGYLKGGSGP	PYKISTSTN		
Arabidopsis thaliana (nsLTP-3)	SMAKTTPDRQQACR					
Arabidopsis thaliana (nsLTP-5)	SLARTTRDRQQACR					
Ole e 7	TSAKTTADRRS			PYKIDPSTDC		
Lupinus albus				PYKISTSTN		
Lupinus angustifolius				YKISTSTNSSSSEE	MWWERYRHHSKF	
Glycine max	NNARTTGDRRA			PYKISTSTNCNS		
Arachis hypogaea	SARTPADRRT			PYKISPSTNCNT		
Cajanus cajan	AHNTPDRQT			YKISPSTDCSR		
Phaseolus vulgaris	NSARSTADRRG	GLNPNNAQA		PYKISTSTN		
Glycine soja	AKTTADRQTACN		LQNGGTPPSG	PYKISTSTN		
Lens culinaris	AANTTPDRQAA	KLNTNNAAA	YLTGGPGPS	YKISTSTNCNT		
Trifolium pratense	NNQAKTTPDRQS		GYLRRPGPS			
Spatholobus suberectus	ARTTADRRA	GLNPSNAQA		PYKISTSTN		NLLHNHFPLR
Cicer arientinum			YLQGGPGS	PYKISTSTN		
Vigna unguiculata	NSAARTTGDRRTACN			YRISPSTNCNR		
Abrus precatorius	MARTTPDRQT			YQISPNTDCSR		
Arachis ipaensis	ARTPADRKT			YKISPSTNCNT		
Trifolium subterraneum	RNLNNQAKSTPDRRSGCR					
Prosopis alba	QTTVDKQTVCN			PYKISTSTN		

Protein	B1	B2	B3	B4	B5	B6
Vigna angularis	LNSSRTTPDRRA	GINPNNAEA		ISASTNCNR		
Arachis duranensis	NGTAKTTSDRQA			YKISTSTNCSS		
Pisum sativum		RLNTNNAAA	QAPNNASPP	YKISTSTNCNT		

The epitopes are named as Bi, where i is the number assigned to each different epitope.

Table A8.
Identification of B-cell binding epitopes.

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Chapter 9

Abiotic and Biotic Stress Factors Affecting Storage of Legumes in Tropics

Habtamu Kide Mengistu

Abstract

Tropical regions such as South Asia (SA) and Sub-Saharan (SSA) do have storage environment that may impose abiotic and/or biotic stress or. This book chapter aims to broaden current knowledge on the 'Abiotic and Biotic Stress Factors Affecting Storage of Legumes in Tropics'. This book chapter is prepared by including all relevant studies and detailed literatures using various scholastic search approaches. Typically, published papers and abstracts are identified by a computerized search of electronic data bases that include PubMed, Science Direct, Scirus, ISI Web of Knowledge, Google Scholar and CENTRAL (Cochrane Central Register of Controlled Trials). Thus, diseases, insects, etc..., are biological factors that cause biotic stress in plants while abiotic stress is caused by either physical or chemical factors. Biotic and abiotic stresses create adverse effects on multiple procedures of morphology, biochemistry and physiology that are directly connected with growth and yield of legume grains. It is, therefore, clear that the most important factors of food grains loss are moisture, temperature, metabolic activity and respiration, insects, mites, micro-organisms, rodents, birds and storage structures. Initial grain condition or quality of the seed for storage can indirectly be affected by abiotic stresses like water scarcity, high salinity, extreme temperatures, and mineral deficiencies or metal toxicities which reduce the crop's productivity. For maintenance of storage of initial grain's quality, grain must be dried and cooled prior to storage, the store must be constructed for blocking rodents and birds, enabling protection from sun and light entrance, allowing aeration to keep the temperature uniform in the store. Also, bringing the temperature of the grain down to below 12°C is necessary, since this temperature is a threshold at which microorganisms' reproductive activity is inhibited. Storage spaces with higher relative humidity (95%) and a temperature of 35°C, are detrimental for storage of legume grains. In general, legume grains should be attaining a temperature of about ≤ 10 °C before placing them in store. For storage safety, it is preferable to place the grain in the storage at moisture content of 13%, or less than 14% on wet basis. Also, combining drying and storage facilities in one and the same structure is economical, and allows further conditioning at later stages if required. In order to reduce postharvest loss from customs of traditional storage by farmers in tropics, governments should mobilize and integrate multidisciplinary management system of storage loss, and monitor precautionary measures of the stored grain throughout the storage period. They should be facilitating the selection and promotion of alternative, cost-effective and appropriate storage structures considering suitability to local conditions and sustainability.

Keywords: abiotic and biotic, legumes, stress factors, storage management, tropics

1. Introduction

There are about 30 species of economically important legumes grown in the tropics [1–3]. Legumes such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris),* lentil *(Lens culinaris),* groundnut (*Arachis hypogaea*), chickpea (*Cicer arietinum*), cowpea (*Vigna unguiculata*), and pigeon pea (*Cajanus cajan*) are the most frequently used species in tropics [1–3].

Legumes production in tropics is common, as these crops majorly aid the countries for securing food, source of income, providing nutrition and maintenance of soil fertility. It is reported that more than 101 million households in Sub-Saharan Africa (SSA) and more than 39 million households in South Asia (SA) grow one or more of these legume crops [4].

Crop losses occur at all stages of the post-harvest. Legume grains should retain both nutritional and all of the essential physiological functions of seeds for growth and therefore, it is preeminent to include storage methods and facilities enabling the quality preservation of these crops for further processing, packaging and marketing.

Storage of legume grains should be provided with certain conditions such as, fumigation to protect the store from undesirable microorganisms and should also be applying ventilation to adjust optimum temperature and humidity in the storage space for keeping quality of legume seeds.

Grain quality is characterized by physical properties, comprising kernel size, and sanitary characteristics, including microorganisms, rodent excrements, toxic seeds, pesticide residue and dust; inherent properties such as nutritional com¬pounds, biological viability and shelf life. However, smallholder farmers of SSA and SA have not yet understood how traditional storage methods affect these quality characteristics.

Therefore, this book chapter seeks to address the factors and major constraints affecting storage of legumes in tropics. It also, discusses conditions of safe storage, grain storage parameters and storage structures; probes management and control of grain loss in storage designs; and finally puts forward recommendations for future work.

2. Nature and properties of legumes

Legumes belong to the botanical family called Fabaceae, which comprises over 750 genera and over 18,000 species, ranking third among other species within this family in the plant kingdom. Legumes are plants which belong to family called Papilionaceae within the order Fabaceae which is also called Leguminosae [5, 6]. Leguminosea can include species of trees, herbs, climbers, and shrubs in which only small number of these are consumed by humans. Legumes grain are the other species commonly served for food consumption by humans.

Common grain legumes include dry beans, lentils, soybeans, peanuts, fava beans, chickpea, mung bean, dry peas and green beans..., etc. [7]. Food legumes are divided into two groups, the first groups are all dry cultivated legume seeds, including pulses which are less in oil content and used for traditional food; the second groups are called oil seeds with high oil content such as soybean and peanuts, and they are used for extraction of edible oil [8, 9]. Legumes are globally consumed as an inexpensive meat alternative and are commonly served next to cereals [6]. Legumes are highly nutritious, providing essential amino acids, complex carbohydrates, fiber, unsaturated fatty acids, vitamins and important minerals [10, 11].

Legumes have been traditionally and widely cultivated crops served as major incorporate of children diet; hence, they are economically cheap and they can be used as alternatives or complements in diets comprising meat [6]. Legumes are highly nutritional containing essential proteins, unsaturated fats, complex carbohydrates, dietary fiber, essential minerals and vitamins [10, 11]. Legumes also possess beneficial bioactive phytochemicals [12] that have major roles in medicines concerning disease such as celiac, diabetes and cholesterol and weight management; as a result, they are recently processed as alternative for replacing animal based food products. Thus, it is obvious that incorporating legumes into various nutrition sensitive intervention programs is highly advisable, especially for developing countries, to reduce malnutrition and as means of income generation. Furthermore, legumes could be a base for the development of many functional foods as well as a range of feed and raw material for industrial products [13].

3. Factors affecting storage of legumes in tropics

It is estimated that about 30% of the world's produced food is lost or wasted [14, 15]. This loss accounts about 1.3 billion milligram (mg) per year in a world where over 870 million people go hungry [16]. World Bank [17] indicated loss of food grains with an estimated cost of 4 billion USD for each year over the last decade. As a consequence, the total amount of grain loss exceeds the total amount of food aid to these countries. On the other hand, such losses are estimated to be equivalent to the annual caloric require-ment of 48 million people.

A significant increase in the food supply in Sub-Saharan Africa could be achieved by investing for reducing post-harvest food losses [17]. Thus, in recent times, experts advocate huge investments on postharvest loss (PHL) reduction to enhance food security [18].

Losses in food grains may fluctuate under different sets of ecological conditions. The quantitative and the qualitative losses occur due to factors of physical: temperature and moisture, biological: insects, rodents, mites, birds and meta-bolic activity of grains, chemical: breakdown of the produce and pesticides and engineering: structural and mechanical aspects. It is, therefore, clear that the most important factors of food grains loss are moisture, temperature, metabolic activity and respiration, insects, mites, micro-organisms, rodents, birds and storage structures.

3.1 Abiotic stress factors affecting storage of legumes in tropics

Moisture content, temperature and initial grain condition are the major abiotic factors affecting storage of legumes in tropics, whereas, the initial grain condition of seed can be negatively impacted for growth, development, yield and seed quality by abiotic stresses such as drought (water stress), excessive watering (water logging), extreme temperatures (cold, frost and heat), salinity and mineral toxicity [19].

3.1.1 Moisture content

All micro-organisms need moisture to maintain life. Keeping the moisture content of legume grains as well as their storage to be low will hinder the growth of microorgan¬isms; therefore, air should be prevented from entering the store. The moisture content below which micro-organisms cannot grow is called the safe moisture content [20]. All legume grains should be below their safe moisture content before they are placed in the storage space. In order to survive and multiply micro-organisms need moisture, and the safe moisture content is somewhat

related to the temperature at the time of storage. Thus, when stored below 27° C, the optimum safe moisture content for both broad bean and cow pea were observed to be 15.0 percent while the optimum safe moisture content for both lentil and pea were posited as 14.0% [21].

Grain stored within the proper moisture content may not remain in that condition, since moisture in the form of water, either from top lid or the side wall of the store may be dropped; or it might be down piped from a bucket elevator. Also, in some cases, moisture through cracks of storage may enter and wet the grain. During cold weather, when a warm grain having temperature of >10° C, or when a grain dried in dryer bin prior to storage, is cooled below -1° C in the store, then condensation happens particularly on the lid and from inside parts of storage space, and therefore, droplets on the amass cause increases in moisture content of the stockpile [22].

Due to excessive humidity, multiplication of fungi particularly *Aspergillus spp*., which produce dangerous toxins (*Aflatoxins*), will make legume grain unfit for human consumption [23]; therefore, The maximum permissible moisture content for safe storage of various crops is the moisture content in equilibrium with 70% relative humidity at about 27°C [21].

It is indicated in **Table 1**, that shelled groundnuts has lowest EMC among the listed legumes, which implies that at any given RH and temperature, legume grains seed which is rich in oil content will maintain lower moisture content than those enriched with other compositions such as lentil which is reach in protein.

3.1.2 Temperature

Besides moisture, temperature is detrimental factor in accelerating or delaying the complex phenomena of the biochemical transformations, especially the "breathing" of the grain that influences the origin of grain degradation. Furthermore, it has a direct influence on the speed of development of insects, molds, yeasts and bacteria and on the premature and unseasonal germination of grain. The temperature within a store can be affected by sun, the cooling effect of radiation from the store, outside air temperatures and the heat generated by the respiration of both the grain and any insects present in the store [20]. It is noted that when the higher the temperature is, the lower must be the moisture of the grain in order to ensure good storage of the legume crops by minimizing the speed of development of these degradation phenomena, so that the temperature and moisture content of the grain conditions the maximal duration of storage.

Crop	EMC
Cowpea	15.0
Pea	14.0
Chickpea	13.5
Pigeon pea	12.5
Groundnuts (shelled)	7.0
Beans	15.0
Soybean	15.0
Common bean	15.0
Lentil	14.0

Table 1.
Equilibrium moisture content (EMC) values during storage of a range of legume crops at 70 percent relative humidity and 27°C.

Moisture content of the stored grain should be monitored as a function of equilibrium moisture content of the air in the storage space. Many grain-degradation phenomena, if not completely blocked, can be slowed down by keeping the relative air humidity below 65–70 percent. In this sense, the "safeguard" moisture content is defined as that corresponding to equilibrium with the air at 65–70 percent relative humidity [21].

3.1.3 Initial grain condition

Initial grain condition can be negatively affected by complex set of biotic and abiotic stresses. Abiotic stresses involve environmental factors that cannot be prevented, and they are the major factors which significantly reduce the crop's productivity and its post-harvest life and the storage life of the legume grain. Abiotic stresses include water scarcity, high salinity, extreme temperatures, and mineral deficiencies, particularly metal toxicities [24].

3.1.3.1 Drought stress

Drought is a term that describes water scarcity in the soil, which can be influenced with seasonal variations. Thus, in general, various factors such as the amount of salt presented in soil causes drought stress which further leads to the flowing out of cellular water, leading to cell death as a consequence of contraction within protoplast of legume cell structure. Water deficit stress is damaging factor, because it inhibits photosynthesis by affecting the thylakoid membranes [25], and reduces nitrogen fixation of legume grains. Drought stress, therefore, is complicated abiotic stress that directly affects the intrinsic growth factors of legume grains imposing physiological deviations which indirectly affect quality of grain during later storage.

3.1.3.2 Extreme temperature stress (hot/cold)

The metabolism of the legume grain cell can be damaged by an increase or decrease in respiration rate due to extreme temperatures. Abnormal anaerobic respiration produces unwanted metabolites that adversely shifts normal protoplasmic streaming with undesired electrolyte efflux imposing alterations occurring within normal cellular physiological metabolism that damages the protoplast. This can be revealed from cellular damage and reduced crop growth, thus, the crop will be rotten, and as a consequence end the life of crop [26]. Also, high temperatures can cause drought stress due to increased water loss by transpiration or evaporation; thus, elevated temperatures in the soil negatively influence the life of crops [27].

3.1.3.3 Salinity stress

Salinity stress of legume grains occurs due to soil salinity or salinization, which is a phenomenon that happens when there is increased amount of salts in soil [28]. It mainly occurs in arid as well as semi-arid environments where the legume grain has higher evaporation and transpiration rates compared to precipitation volume throughout the year. Use of saline water in irrigation purposes, due to modification in soil content, and increased use of fertilizers besides inherent salts in subsoil [29]. Higher salinity in the soil imparts higher osmotic pressure potential and particular ion toxicity [30], that adversely impacts legume seed viability and vitality by inhibiting minerals and water, from being absorbed through leguminous roots, necessary for metabolisms in cytosol of cell membranes of leguminous seed, to enable of germination and normal physiological natural life cycle phases; as a consequence, it reduces the biological nitrogen fixation of legumes.

3.1.3.4 Metal stress

Heavy metals that cause stress, (HMs) in legume plants are toxic inorganic compounds which cannot be biologically broken down into simpler form having negative effects on cells and genes, which impart mutagenic alterations and disruptions in chains of ecosystem surrounding the legume crop [31, 32]. Metals in soils such as iron, manganese, molybdenum, magnesium, zinc, copper, and nickel can be vital micronutrients for serving physiological life cycle of legume grains. Metals such as chromium, lead, cadmium, cobalt, selenium, arsenic, and mercury and silver, are non-essential elements with unknown physiological and biological function [33]. Legume grains require vital metal in smaller amount to carry out for their physiological and metabolic activities in cell, but disproportional coexistence of vital and non-essential metals generally lead to hindrance of normal physiological functioning, disturbance of protein structure as result of non-essential heavy metal bonding with sulpurhydryl building blocks bonding [34], and interfering with functional groups of significant cellular molecules [35].

3.2 Biotic stress factors affecting storage of legumes in tropics

Biotic stresses factors of storage include all living organisms that bring damage to the crop in the form of biological, physical or chemical process. Thus, presence of toxins, productions of unwanted metabolites, deprivation of essential biological components of legume grain will facilitate deterioration of legume crop in storage spaces. It is the climate in which the legume crop lives, determines type of biotic stress that can be imposed on the crop, and influences the ability of the crop species to resist that particular type of stress [19].

3.2.1 Microorganisms

Damages or loss of grains vary generally as a function of crop variety, pest and insects, climate, system of harvesting, system of processing, storage, handling and marketing [36]. The main agents causing deterioration of stored legume grains are microorganisms (fungi, bacteria, yeast and mold), insects and mites, rodents, birds, and metabolic activities. The principal micro-organisms (fungi and yeasts), which attack grains, are very dangerous as they cannot be easily seen with naked eyes and their harmful influence spreads very quickly and renders whole grains waste. Anaerobically respiring species of storage fungi grow more quickly at the optimum growth temperature of about 30°C and below RH of 95 percent [5].

Biotic factors particularly mold (fungi) and insects influence longevity of seed in storage. The two fungi types that attack legumes seed are field fungi and storage fungi. Field fungi affect seed in the field prior to harvesting, and storage fungi attack seed during storage. Field fungi (e.g., *Fusarium spp*.) thrive in high moisture environments, during high moisture level of seed due to rainfall at the time of harvesting [37]. Storage fungi (*Aspergillus spp*.) thrive best when moisture levels of seed are low. Storage fungi do not establish on seed with MC in equilibrium with equilibrium moisture content (EMC) of less than 68% ambient RH [37]. Therefore, when moisture content, temperature, and relative humidity are low, the risk of fungi invasion is minimized. These fungi produce harmful stuff that is injurious to seed cells and cause seed deterioration. Inadequate drying of seed can favor the growth of molds or fungi, hence a decrease in seed quality or quantity. Bacteria prevalence to the stored legume grains may be low. They may, however, invade already damaged portion of the crop products during storage and their

multiplications. Deterioration by bacteria is limited as they require free water to grow. Storage bacteria are active around 90% RH where fungi are already very active [38].

3.2.2 Insects and mites

Insects and mites could seriously attack stored legume grains when there is warm and humid storage environment. They pierce the kernels, consume on the outer covering skin and the inner nutritious endosperm of legume seed, respiring off water which facilitate development of undesirable molds and fungi [36].

Insects are inactive below seed moisture content of 8% while they are active around seed moisture content of 15%. To inhibit growth of insects in the storage, the moisture content of legume grain should be reduced below 8%, while H and temperature within the store should be kept below 40 percent and 10°C, respectively. The most suitable moisture content and temperature of grains for the growth of insects are about 11–15 percent and 28°C - 36°C, respectively [39].

Mites are distinct from insects, hence, at the adult stage they possess eight legs and their bodies are not divided into a head, thorax and abdomen. Thus, insects are generally much smaller than insects. Mites are usually seen, if they are large in number and visible as dust on the surface of bags. Mites are generally not a problem in tropical countries like India because they require low temperature, but when they become active, they spoil 2–3 percent of annual produce [5].

3.2.3 Rodents

Among the various pests detrimental to the wellbeing of man, rodents form an important group and assume great economic importance. During the pre-harvest stages, they cause considerable damage to crops at all stages of growth. In storage they do not only consume large quantities of food stuffs, but also contaminate the food stuff with their excreta, destroy containers by gnawing holes which lead to leakage and wastage of grains and paw into and scatter grains while they eat. Thus, the scattered grains along with that which leak from gnawed holes, are subjected to contamination and admixture with impurities. Damage to grains stored in bulk is less than to grains stored in bags because rodents are unable to burrow into the bulk [21].

3.2.4 Birds

Like rodents, birds also destroy grains by making holes in stacks and feed on grains as well as contaminate the grains through droppings and feathers. Damage directly occurs by birds when grains are being sun dried, and consequent damage occurs when grains are in storage. Losses caused by birds can be avoided by preventing their access to the stored commodities. The birds which cause damage are pigeon (*Columba livia*), crow (*Corvus splendens*) weaver bird (*Ploceus philippinus*), sparrow (*Passer domesticus*) and black bird (*Acrldotheres tristis*) [21].

3.2.5 Metabolic activities

Legume grains are living materials and their normal chemical reactions produce heat and chemical reactions by products [36]. Heat is released as result of exothermic reaction and water is respired off by microorganisms plague, as a by-product of the enzymatic catabolism of nutritious constitutes of seed, used as substrates for synthesis of cell material. Thus, increased temperature and moisture content highly facilitate deterioration of seed in store by microorganisms.

Even though legumes are low in their carbohydrate, microorganisms under aerobic condition will completely convert the small amount of carbohydrates, or endosperm to CO_2, H_2O and produces energy in the form of ATP as shown in the following equation:

$$C_6H1_2O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \text{Energy}(\text{ATP}) \quad (1)$$

Metabolic processes cause two types of losses in the store. The first type is the loss due to the enzymatic catabolism of substrates i.e., synthesis of cell material of grain converted by microorganism to carbon dioxide and water. The other loss occurs when entire of individual grains loose its biological constitutes consumed by microorganism [36].

3.3 Storage structures

The structures and materials from which the store is built, determines safety of grains in store since legume grains should be protected from exposure of sun and rain. Storage structure should facilitate adequate ventilation for monitoring temperature which is appropriate to maintain grain quality in the store. Stores should allow space for inspection and detection for occurrence of disease arising early in the grains [21].

4. Constraints affecting storage of legumes in tropics

Constraints to the development of major tropical grain legumes which are soybean, cowpea, pigeon pea, groundnut and common bean can be technical issues, and are called technical constraints. Thus, to manage and control deterioration of these grains during storage, technical constraints need to be understood for the effect on the crop ecosystem, attributing to abiotic and biotic factors, which are negatively affecting the development and storage of legume grains. Other constraints are institutional which arise from and within the government's agricultural policies and regulations, paying less focus on practicing in solving technical constraints of crop storage management system. Institutional constraints include policies, that do not introduce, motivate and process the release of stress resistant and durable legume varieties; lack of setting regulatory laws on principles that intend for safe storage; lack for investments engaged in research and development of storage equipment and post-harvest storage mechanisms and technologies [4].

Legume grains	Major diseases causing microorganisms
chickpea	*Fusarium oxysporum* causing *Fusarium* wilt in root rots, *Ascochyta* blight, pod borer
common bean	*Xanthomonas oryzae pv. oryzae* causing bacterial blight, *Colletotrichum lagenarium* causing anthracnose, common mosaic virus, bruchids, aphids
cowpea	Viruses, bruchids (storage pest), *Maruca (pod borer)*, aphids, parasitic weeds (*Alectra vogelli and Striga gesnirioides)*
groundnut	Aphids causing rosette, leaf spots, rust
pigeon pea	*Fusarium oxysporum*
soybean	*Maruca* (pod borer) causing rust, frog eye

Table 2.
Major disease causing microorganisms in storage of common legume grains in tropics.

A large number of diseases, insects and parasitic weeds cause varying levels of damage to tropical grain legumes at different stages of growth – from seedling to storage.

It is indicated in **Table 2**, that *Maruca* (pod borer), bruchids, aphids and *Fusarium oxysporum* which causes fusarium wilt are some of the common disease causing microorganisms that are constraints for legume storage in tropics.

5. Conditions of safe storage

The grain, microorganisms and foreign material together form an artificial ecosystem in store. Grain quality can decline in the store as a consequence of chemical, biological and physical processes. These processes are influenced by factors such as are moisture, temperature, carbon dioxide and oxygen, initial biological state of the grain, microorganisms and insects, rodents, birds, whether conditions, cleaning, drying, cooling and ventilation. Among these factors, moisture content and temperature of legume kernels, are major factors to influence for bioprocesses in the grain [21].

Thus, storage spaces with higher relative humidity, which is 95% and a temperature of 35°C, are detrimental for storage of seeds [40]. In order to prevent moisture movement due to temperature gradients within each load, grain should be placed into storage with a temperature ranging 10 to −9°C. In general, the grain should be attaining a temperature of 10°C or below before placing it in store. Moisture values, on wet basis, which are commonly 13, 14 and 15.5 percent are maximum moistures recommended for any storage, thus, should not exceed these, for safe storage of crop load. For storage safety it is preferable to place the grain in the storage at moisture content, on wet basis, of 13 percent, or less than 14 percent [40].

5.1 Drying for safe storage

In general, the life of the seed during storage revolves around its moisture content, storage temperature and humidity. However, the processed seed has better storability. The rate of deterioration of crop seeds increases as respiration goes up with high moisture content (MC). The effect of seed moisture content has been generalized as safe for sealed storage at 6–10% MC at which no pest activity occurs; while fungi, bacteria and insects grow at 12–14% MC and heating occurs at 18–20% MC unless aerated, and in further, germination occurs at 45–50% MC [41, 42]. The safe drying temperatures for seeds with moisture ranging over 22% MC is 55°c and 40°c for seeds with moisture content below 22% [41, 42]. In many cases, facilities for drying and storage are found in one and the same structure. Combining these functions is economical and it allows further conditioning at later stages, if required. However, there are situations where storage is considered quite separately from drying, ranging from the storage of naturally dried crops to the storage of grain dried by a continuous-flow or batch dryer. Utmost, care should be exercised in drying seeds to a safe limit, and thus, good storage should not allow further absorption of moisture.

5.2 Management and control of loss in storage designs and structures for tropical legumes

Since quality of grain can be affected through the entire food chain and this implies storage is concerned only with maintaining the initial quality of legume grains. Hence, clean grain should go into storage, it is necessary to remove weeds

and debris from legume grain seeds. Also, the area presented around the storage site should be free of dirt and the store must be cleaned and kept free from remnants of previously stored grain. Cleaning for harvesting and handling equipments before carrying out the harvest activities will minimize risk factors for grain's quality during storage. During placing legume grain in the store its quality can be facilitated using a rotating grain cleaner, and finally cooling the grain to the existing outside air temperature (that most usually occurs) as soon as it is put into the storage.

5.2.1 Temperature

The temperature at which food is stored is very critical to shelf life. The best range for food storage is a constant temperature between 40 and 60 degrees and void freezing temperatures [21]. Hence, controlling the temperature of small stores is not technically and economically feasible, reducing the moisture content of the stored produce are necessary. In storing dry grain for longer periods or keeping wet grain in stores for a short period of time, it is important to move air through the grain mass, so as to control grain temperature. This become obvious in the spring, when outside air temperatures begin to warm and cause convection air currents inside the store as a consequence of differences in grain temperatures which can move and concentrate moisture in the top center of the storage spaces [21]. Wet grain and molds give off heat through respiration which indirectly contributes for mold growth. Thus, mold growth can be inhibited by keeping the grain and the store cool through application of aeration. Even if grain is dry and cool when placed in storage, aeration is needed to keep temperature uniform within the store to provide the grain mass temperature [21].

5.2.2 Moisture

The moisture content of seed during storage is most detrimental factor affecting the shelf life. Legume grains should have a 10% or less moisture content for long term storage. It has been reported that seed moisture content of about 6–8% is optimum for maximum longevity in storage of most crop species. Keeping oily legume grains below moisture content of 4–6% impose lipid autoxidation. Seeds are hygroscopic in nature, and as a consequence, they can pick up moisture from and releases it to the surrounding air [43]. Moisture levels above safe moisture content can be tolerated when storing seed for short period. The sitting and ventilation of the store are important so as to reduce storage problems due to condensation. Low night temperature can cause the walls of a store cooled below their dew point, as a result, condensation can occur near the edge of the store increasing the moisture in the grain layers.

5.2.3 Microflora, insects and mites

Microorganism's activity can lead to quality deterioration in store by causing loss of grain viability. The microflora activity inside the store is monitored as a function of the correlation between relative humidity in the store, temperature and moisture content of seed and the store. Insect activity in the store increases and reaches maximum with a temperature ranges of 19.5 -°C 33°C and the temperature should be below 17°C. Fumigants and insecticides are chemical methods applied to control insect activity. Applying fumigation, which are highly effective chemical insecticides, environmental friendly and safe for human use, enables control of insects in the store and facilitates longer period of storage [43].

5.2.4 Grain storage parameters and storage structures

For maintenance of initial grain quality storage, grain must be dried and cooled prior to storage; the grain should be protected from insect attack. The store must be constructed in a way to enable blocking of rodents and birds and also enabling protection to sun and light entrance, allowing ventilation so as to keep the temperature uniform in the store. Pulses stored above 12% moisture content (MC) require aeration to maintain quality. Cooling grain in the store cannot be treated with protectants since these chemicals leave harmful residues that may be presented till time of consumption by human.

Application of fumigants and insecticides are the two methods commonly recommended to control pests in store. This requires a gas-tight, sealable storage. Grain Research and Development Corporation (GRDC) noted that efficient handling techniques that minimize physical damage of legume grains should be used in order to minimize the possible attack by insects that may produce additional damage through unwanted chemical and biological processes [44]. Aeration, whereby ambient or artificially cooled air is used, is primarily a grain preservation technique [45–48]. Bringing the temperature of the grain down to below 12°C, is necessary since this temperature is a threshold at which microorganisms reproductive activity is inhibited [45, 48–51].

6. Conclusion

Abiotic and biotic factors are the overall factors contributing to pre-harvest and post-harvest losses of legume crops in tropics. Abiotic stress factors such as drought, salinity, extreme temperature, toxic metals are those determining the crop productivity at the soil stages which, in further, affect initial legume seed's quality for storage. Temperature, moisture and initial grain quality are the most important factors that determine storage of legume crops. Mold and insects are the major biotic factors affecting grain quality in store. Moisture content and temperature of the grain as well as the store has to be monitored throughout storage period. Well -designed storage system should be constructed and provided with adequate ventilation capacity. Regular checking of grain condition and monitoring through proper preventative actions has to be applied before significant deterioration of legume grains happened in the stored. Hence, protectants are not advised to be used as they mostly impart residues which negatively affect the health and safety of consumers, so that it is recommended to selectively use fumigants and insecticides which do not disrupt sustainability of ecosystem, and those which do not leave residues on legume grains so as to avoid negative healthy impacts to human during consumption. For effective control and management of the biotic and abiotic deteriorative factors that affects grain quality in the store, it is important to understand individual and correlated characteristics of the physical, chemical and biological processes related to these deteriorative factors, so that selecting effective way of reducing the initiates of these processes at pre- harvest and post-harvest stage will be possible, helping the design and construction of safe storage.

7. Recommendations

In order to reduce the factors of pre-harvest loss that contributes to the post-harvest loss occurring during storage, the governments in tropics should be establishing soil productivity and preservation polices supported by research studies

and outputs for monitoring and controlling the usage of selected and appropriate fertilizers, establishing grading and storage standards for tropical legume grains, and allocating incentive for private investment in seed production with better storage durability. They should also use an integrated multidisciplinary management, monitoring, and precautionary measures of the stored grain throughout the storage period. The governments should be strategically selecting, promoting of alternative cost-effective/appropriate storage structures, considering suitability to local conditions and sustainability. Moreover, establishing suitable policies and regulations that enable on variety release process in short period of time, increasing investment in agricultural research and development, and many others are pivotal prospects that governments in tropics should focus to reduce loss of legumes, and legumes' quality during storage.

8. Scope of future work

Future work regarding of reducing storage loss in tropics, should focus on assessing and testing grain quality and identify causes of deterioration in the existing traditional storage systems, and filling the gaps along with the overall efforts in improving and promoting of these storage systems. Assessing hygienic quality of farmers and training farmers for principles and procedures in handling and storage of legume grains, in order to avoid risk for deterioration factors during storage. Establishing safe storage moisture limit guidelines for legume crops and monitoring system, which will also ensure functioning of these guidelines during all seasonal variations for storing legumes, indigenous to countries in tropics.

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Chapter 10

Vegetable Soybean and Its Seedling Emergence in the United States

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Abstract

Vegetable soybean or edamame is a specialty soybean (*Glycine max* (L.) Merr.). Unlike grain-type soybean (mainly for oil and source of protein in animal feeds), edamame pods are harvested at a green and immature stage, and beans are consumed by humans as a vegetable. While originally from China, edamame has recently gained much-increased popularity and expanded market needs in the US. However, domestic edamame production is limited in the US because at least 70% of the edamame consumed is imported. Poor seed germination and seedling emergence are one of the major problems in US edamame production. This review focuses on the introduction of edamame, a high-value niche crop, and its low emergence issue in production. Here, we provide a comprehensive exploration of the factors that influence edamame germination and emergence, including the intrinsic factors related to seeds (seed and seedling characteristics), and extrinsic factors related to the biotic (soil/seed-borne diseases) and abiotic (seedbed physical components as well as their interaction with climate) stresses. This information will help farmers and plant breeders to better understand the causes of the poor edamame emergence and may provide a foundation for improved field management of edamame, to increase production of this valuable specialty crop.

Keywords: vegetable soybean, seedling emergence, seed vigor, biotic stresses, abiotic stresses

1. Introduction

Vegetable soybean is a specialty soybean (*Glycine max* (L.) Merr.). Unlike grain soybeans (mainly for oil, source of protein in animal feeds, and processed foodstuffs, including soy sauce, tofu, soy milk, and natto), vegetable soybeans are consumed by humans as a vegetable [1]. Originating from China, vegetable soybean is popular throughout East Asian countries (especially China, Japan, and Korea) due to its long history of consumption. The earliest documentation of vegetable soybean comes from poems by Lu You (1125–1210 AD), a distinguished scholar in China, describing the picking and eating of green soybean pods. Vegetable soybean is commonly called "maodou" among Chinese people [2]. In 1275 AD, the popular name "edamame" appeared in Japan, and a well-known Buddhist Saint Nichiren wrote a note thanking a parishioner for the edamame he left at the temple [2]. Now, edamame, which literally means "stem bean" (eda = "branch" or "stem" and mame = "bean"), is commonly used to refer to vegetable soybean in many countries [3].

Soybean development and maturation can be divided into vegetative and reproductive physiological stages. The vegetative stages are numbered according to how many fully developed trifoliate leaves are present, including emergence (VE), unrolled unifoliate (VC), and a series of stages named by the number of leaves (V1–V(n)) [4]. The reproductive stages are characterized by blooming (R1 and R2), pod development (R3 and R4), seed filling (R5 and R6), and plant maturity (R7 and R8) stages [5]. Unlike grain soybeans that are harvested at full maturity (R8 stage), edamame is harvested in pods between the reproductive stages of R6 and R7, when beans fill 80–90% of the pod width and still retain around 65% moisture content [6]. Harvesting at the R6 stage brings the benefits of having desired edible quality attributes for edamame, such as peak seed weight and sucrose content, lower oligosaccharide and anti-nutrients values, and intense green color [7]. Loss of quality occurs as pods turn yellow; therefore, harvest time is very important in edamame production [8]. Characteristics for high-quality edamame pods are bright green crescent-shaped pods (approximately 5.0 cm in length and 1.4 cm in width) with light pubescence (white to gray) and unblemished pods containing two to three large seeds (seed dry wt >250 mg/seed) with a hilum consisting of a buff or yellow color [9–11]. Edamame varieties can possess different seed coat colors, ranging from yellow, green, brown, or black [12]. For the best quality, seeds should have a smooth and firm texture (but not chewy), higher sugar content (especially sucrose), and distinctive flavors (such as sweet, nutty, buttery, and beany flavors) [13, 14].

In the past few decades, globalization has provided a platform for international edamame trade and allowed more people to enjoy its unique taste well as multiple health and nutritional benefits. Now, edamame is becoming more and more popular all over the world, particularly in the United States.

1.1 Nutritional and functional values

Edamame can be considered a nutraceutical and functional food crop. The nutritional value of edamame is mainly determined by its chemical constituents, such as protein, fiber, starch, and sugars. Compared to grain soybean, edamame has lower oil, lower trypsin-inhibitor levels, fewer indigestible oligosaccharides, and more vitamins [12]. Since edamame is a complete protein source containing all the essential amino acids associated with human health, it is usually considered an alternative to meat and can support vegan, vegetarian, and other plant-based diets by providing viable and more environmentally friendly proteins [15].

Edamame also has superior nutritional content when compared to green peas [16]. Masuda reported that the calorific value (energy) of edamame is about six times that of green peas; edamame bean contains 60% more Ca, and twice the P and K of green peas; the Na and carotene content of edamame is about one-third that of green peas and they have similar quantities of Fe, thiamin (vitamins B1), and riboflavin (B2) [17]. In addition, edamame is a rich source of vitamins A, B1, B2, vitamins C (ascorbic acid), vitamin E (tocopherol), niacin, and health-promoting polyunsaturated fatty acids, such as linoleic acid and linolenic acid [18, 19]. Edamame also contains a significant amount of dietary fiber, which when consumed in sufficient quantities could help to reduce blood cholesterol levels due to its viscosity, solubility, and ability to bind molecules [20].

Moreover, edamame is also regarded as a functional food, mainly because of the presence of phytohormones called isoflavones that are associated with the prevention of several human diseases. The major isoflavones present in edamame are genistein and daidzein [21]. Clinical studies show that they have a positive influence on increasing HDL cholesterol (considered good cholesterol) and lowering LDL

cholesterol (bad cholesterol), reducing the risk of cardiovascular diseases [22]. Isoflavones have also been reported to have a preventive effect on other diseases, such as breast cancer, diabetes, menopausal symptoms, and osteoporosis diseases [21]. However, Roland et al. reported that soybean isoflavones may be associated with astringency and bitterness, two undesired sensory attributes that can impact edamame quality [23]. Some studies also observed the health benefits of edamame seed coat pigments. For example, black and brown seed coats accumulate anthocyanins and procyanidins, two antioxidants that could aid in fighting cardiovascular disorders, preventing inflammation, and scavenging harmful radicals [24–26].

1.2 Versatility as a food ingredient

Vegetable soybean can be either sold fresh as pods on the stem, stripped pods, shelled beans, or sold as frozen or canned products. It is versatile as a food ingredient routinely found in salad bars, stews, soups, stir fry dishes, and sushi restaurants as appetizers, as well as an ingredient in hummus or healthy snacks. Edamame is quite easy to prepare as a snack. Pods are usually lightly cooked in salted/unsalted boiling water for 5–7 min and then the beans can be pushed directly from the pods into the mouth with the fingers [27]. Edamame beans can also be roasted like peanuts. Additionally, some companies use edamame to prepare innovative products, such as processed edamame sweets and desserts, green milk, green tofu, green noodles, and soygurt [27, 28].

1.3 Economic importance in the US

Consumers' widespread appreciation of edamame's benefits has resulted in a dramatic growth in demand for edamame in the US since the early 2000s. Sales of edamame in the US increased from 18 million USD in 2003 to 30 million USD in 2007 and reached 84 million USD in 2013 [29]. Today, edamame is the second-largest soy food in the US with about 30,000 tons consumed annually [7]. Edamame is readily available in the US, found in supercenters (e.g., Walmart), grocery stores (e.g., Kroger and Food Lion), wholesale outlets (Sam's Club and Costco), farmers markets, and local restaurants [30].

It is reported that 70% of edamame consumed in the US is primarily imported frozen from China, which is the largest producer, consumer, and exporter of edamame in the world [31]. Frozen-processing methods used by commercial processing facilities may lower edamame quality drastically [32]. Some studies also reported the introduction of harmful foodborne bacteria, such as *Escherichia coli* and *Listeria monocytogenes*, during processing that cause human illness [33]. With the raising concerns about the safety of imported edamame, consumers are seeking domestically grown edamame. This expanding domestic demand, especially for local fresh edamame, has stimulated interest among plant breeders, growers, and food processors in edamame production in the US.

The US is known as one of the top grain soybean-producing countries in the world with ~30-million-hectares grown each year, valued at more than $40 billion [34]. Compared with grain soybean, edamame is grown on a much smaller scale but has a greater market and economic value. Edamame is a profitable alternative crop, especially for small-scale farmers and urban agriculture growers, seeking to increase income by growing a high-value niche crop [30]. First, farmers can adopt edamame production easily, since edamame shares similar production practices with grain soybeans, such as fertilization and irrigation [5]. Second, farmers can get higher gross returns, because edamame has relatively low startup costs, higher market prices as a specialty vegetable, and large local market potential. It is reported

that the net returns reached $4940–$5434/hectare of land in some parts of the US [35], and a report from Mississippi showed that the net return of edamame could be more than twice the returns from grain soybean production [8]. Third, edamame can serve as a component of crop rotations and diversify crop production for US growers. Edamame can fix atmospheric nitrogen and can be used in the ubiquitous wheat/corn-soy rotations which have benefited US growers for many years. Finally, since organic farming gains increased popularity now with the raising public awareness of the environment and human health, organic farmers may benefit from planting edamame based on its high nutritional and market value.

All of these have resulted in a steady increase in land acreage under edamame in the US. However, edamame production faces some challenges and problems, such as limited genetic resources, poor seedling establishment, lodging, inferior plant structure, susceptibility to seed diseases, low yield potential, and greater perishability compared to grain soybeans [30]. From the standpoint of farmers, poor seedling establishment is considered a critical issue that needs to be solved. Seed germination and seedling growth is the first step in establishing a successful crop. Successful stand establishment eliminates the need for replanting and determines the success or failure of the future harvest.

2. Emergence issues in edamame production

Poor emergence is a common problem in field research for edamame and has been well documented in the literature. Williams reported average emergence below 35% among 136 diverse edamame cultivars [34], which is much lower than a normal plant population (80%) for commercial grain soybeans [18]. Poor crop emergence has also been observed in edamame field trials in many states in the US, including North Dakota, Georgia, Illinois, Pennsylvania, and Virginia, where emergence percentages range from 60 to 85% for different cultivars [18, 36, 37]. Poor emergence influences yield if the plant density is below a critical level. To ensure successful stand establishment under variable field conditions, even when using high-quality seeds, good field management practices are needed for edamame.

Up to now, there is still little known about growing edamame in the US. Most planting decisions are based on grain soybean recommendations. However, edamame differs from grain soybean seeds in several key characteristics, such as larger seed size, which may indicate that not all grain soybean management decisions can be applied to edamame. Edamame emergence has been reported to be highly variable among genotypes, indicating the importance of genetics and seed vigor on seedling establishment. Recent studies also reported that edamame emergence was influenced by several factors, such as seed size, plant depth, and temperature [18, 38–40]. Scientific research publications on edamame emergence are still limited. However, related studies on grain soybean establishment may help us to understand issues surrounding edamame field emergence. In this chapter, we discuss edamame seedling emergence, as well as the factors influencing edamame germination and emergence, including both intrinsic factors related to seeds (seed and seedling characteristics) and extrinsic factors related to the biotic (soil/seed-borne diseases) and abiotic (seedbed physical components and their interaction with climate) stresses in the environment.

2.1 Seed emergence process and critical edaphic factors (soil moisture, temperature, and oxygen) involved

Stand establishment is the most important and vulnerable phase of a crop cycle. High-quality seeds require three appropriate conditions for germination—soil

moisture, temperature, and oxygen [41]. Temperature and water availability are two crucial factors that drive the rate of progress through seed imbibition, germination, and seedling growth to emergence [42]. Soybean seeds need to imbibe at least 50% of their mass water to germinate. Edamame has a larger seed size than grain-type soybean, making them more susceptible to soil water stress since they need more water to fully imbibe. Their larger seed size also requires more time to fully hydrate. Seeds will germinate slowly or fail to germinate if the soil moisture is inadequate. Optimally, seed imbibition can be completed within 24 h of planting and the radicle begins to emerge from germinated seed within 24–48 h [41]. Oxygen is required to meet the rapid increases in seed respiration during this period. Germination cannot occur in flooded or compacted soil due to a lack of oxygen. Once the seeds have germinated, it is essential for the radicle to maintain contact with soil moisture, or the seedling may die [42]. The radicle rapidly grows downward developing into the primary root to extract moisture deep in the soil.

Both the rate of imbibition and radicle growth are dependent on temperature if water and oxygen are adequate. Low temperatures, slow imbibition, and the radicle growth rate because of high water viscosity attached to soil as well as slow seed respiratory and metabolic reactions [43]. Grain soybean germination rates range from 2 weeks or more in cold soil (10°C or less) to about 4 days under optimum soil temperatures (27–30°C) [41]. The base, optimum, and maximum temperatures of grain soybean were reported to be 4, 30, and 40°C, respectively, provided no other factors were limiting emergence [44]. It is still unknown if edamame has the same optimal germination temperatures as grain soybean. Sánchez et al. compared seedling emergence of edamame grown on 4 days/night temperature regimes (60/50, 70/60, 80/70, and 90/80°F) on 12-h cycles, and they found that 70/60°F is optimal for edamame emergence [18]. Edamame sown early may suffer from low night temperatures in the field. Mulching reportedly may help to improve the emergence of early (April) direct-seeded edamame through increasing soil temperature and reducing the variation in soil volumetric water content [45]. Moreover, soil moisture and temperature also greatly influence the activity of soil microbes, which, in turn, largely determine oxygen supply in the soil. Thus, oxygen stress may be greater in hot wet conditions [42].

Soybean seedling emergence is epigeal because the food storage organs or cotyledons are pulled above the soil surface. This is a critical step in seedling emergence, especially for edamame. Edamame has large cotyledons, which can suffer high mechanical resistance moving from below soil to above. Hypocotyls may be unable to completely pull cotyledons out of the crusted soil, resulting in a swollen hypocotyl, or even broken cotyledons, ultimately leading to seedling death before emergence is complete [46]. Other adverse field conditions, such as hypocotyl attack by insects and pathogens, can also contribute to seedling mortality in soil. Optimally, hypocotyl expansive growth can drag cotyledons upward until the arch is exposed to sunlight. Then, the arch straightens and lifts the cotyledons and growing point free of the soil surface [47]. The cotyledons unfold and begin to photosynthesize to make food for seedling growth. Finally, the cotyledons totally emerge from the soil representing the vegetative emergence (VE) stage of growth.

After the growing point and cotyledons are exposed, they become vulnerable to environmental stresses, such as hail, frost, and attacks from pests. The seedlings with necrotic lesions or physical injury to the cotyledons exhibit greatly reduced growth rates. Before the apex can be photosynthetic, cotyledons play an important role in seedling growth. Loss of one cotyledon will have little effect on yield. Loss of both cotyledons without harm to their points of attachment (*i.e.*, apical meristem), will result in 2–7% yield loss. The loss of both cotyledons, as well as their points of attachment, will result in plant death because these points of attachment will be the new growing points for the plant [47].

2.2 Seed vigor is a critical factor in germination and emergence

Successful crop establishment can be considered as a balance between environmental deterioration (such as drought, flood, soil crust, and pathogen activity) and the rate of seedling development. Both are determined by the prevailing environment, but the latter is greatly influenced by vigor [42]. Seed vigor is defined as seed ability to germinate and establish seedlings rapidly, uniformly, and robustly across diverse environmental conditions. Seed vigor measured in a laboratory is often used to predict crop establishment in the field.

Three key seed vigor traits have been identified as necessary for successful stand establishment across a wide range of seedbed conditions. The seed must—(i) germinate rapidly; (ii) have rapid initial downward growth; and (iii) have a high potential for upward shoot growth in the soil of increasing impedance [42]. All these features reduce the time between sowing and seedling emergence before the seedbed deteriorates. Although seeds from various sources germinate well under optimal conditions, they may show vastly contrasting abilities to successfully establish a crop under stressful field conditions due to variations in seed vigor.

Seed vigor is a quantitative trait influenced by the complex interaction between genetic and environmental components. It is a measure of how well seeds germinate particularly under adverse conditions. It is widely known that seed vigor can be highly variable among genotypes. Plant breeders in the US have worked decades in developing new edamame varieties with high vigor and better adaptation to the US soil and climate. On the other hand, the location of seed production, stage of maturity at harvest, seed harvesting techniques, processing, and storage conditions also affect seed vigor even in varieties with high vigor potential. In the next section, we will describe how seed vigor can be influenced by various factors including seed physiological and biochemical parameters, such as seed size, seed exudates, as well as external factors, such as temperature and humidity during storage.

2.2.1 Role of seed size on vigor

One of the biggest differences between edamame and grain soybean that may affect crop emergence is seed size. Edamame seeds are 65–100% larger than grain soybean seeds [38]. Although it is well known that the emergence of most edamame varieties is poorer than the grain type controls [34, 36], little evidence suggests that this response is due solely to large seed size. Crawford and Williams evaluated the emergence of two seed size classes (23.7 g/100-seed and 36.8 g/100-seed) within the same edamame variety. Seed size did not influence total emergence, but small seeds emerged 10% faster than large seeds [40]. This is likely due to the fact that small seeds fully hydrate faster than large ones under the same soil moisture conditions. However, more research is needed to understand the relationship between seed size and the emergence of edamame.

Although few studies on seed size in edamame have been conducted, the effect of the seed size and quality of grain soybean on crop performance has been investigated for several decades. The results are often conflicting and the literature on this topic is voluminous. Several authors have reported that small grain soybean seeds had an advantage over large seeds from the same genetic background in terms of radicle and hypocotyl development. Green et al. showed that small seed size was associated with high laboratory germination and high field emergence [48]. Edwards and Hartwig found that the small seed size (9.5 g/100 seeds) showed faster emergence and greater root development than the large seed (22 g/100 seeds) [49]. A similar finding was also reported by Kering and Zhang [39]. Hoy and Gamble found that small seed size was superior in percent emergence and speed of emergence, especially when seeds

were subjected to greater field stresses, such as low temperature and wet or crusted soils [50]. Adebisi et al. observed that for the seeds ranging from 10 to 15 g/100 seeds within the same variety, the small seed size generally produced higher seed germination and field emergence percentages, whereas large seed size produced the highest number of seeds per plant, pods per plant, and seed yield per plant [51].

There are several possible explanations for inferior germination and the emergence of large seeds in these studies. First, large seeds require more time to imbibe sufficient water to germinate, so they germinate slower compared with small seeds [40]. Second, large seeds are more sensitive to water stress, for example, the soil moisture sufficient for the emergence of small seeds may allow germination of large seeds but could be insufficient to sustain seedling growth and emergence [39]. Since large seeds require more water for normal metabolism, they are more easily damaged by reduced osmotic potential [52]. Moreover, Liu et al. stated that large seeds are more prone to oxygen deficits in the soil to support their germination [53]. Furthermore, large seeds also would likely encounter more physical resistance from soil restricting cotyledons during emergence. Seedlings developing from large seeds could be damaged during emergence in hard-crusted soils, reducing seedling vigor [42]. Finally, large seeds are prone to mechanical damage during threshing and processing prior to planting. Large seeds usually have a higher percentage of cracked seed coats, which has been reported to be negatively correlated with germination percentage [54].

However, other studies found that medium and large seeds tend to produce more vigorous seedlings and better stands than small seeds. Rezapour et al. compared germination of three seed size classes within two cultivars (*i.e.,* 13.20, 12.24, and 8.60 g/100 seeds from one cultivar and 20.16, 16.63, and 14.61 g/100 seeds from the other cultivar). The results showed that medium seeds had a higher germination percentage than those for large and small seed sizes, but no significant variations on germination rate among different seed masses were found [52]. Longer et al. reported that large seeds, in general, had a significantly greater percent emergence and greater shoot, and root fresh weight accumulation than small seeds of the same cultivar, even under stressful conditions [55]. Madanzi et al. found large seeds (19 g/100 seeds) achieved higher stand counts than small seeds (12 g/100 seeds) within the same soybean cultivar [56]. Morrison and Xue also observed that large seeds emerged better in heavier-textured soils, possibly due to the enhanced water-holding capacity which benefited large seeds, while more plants emerged from the small seeds in lighter-textured soils (such as sandy soil) [57]. Burris et al. also observed that larger soybean seeds produced larger embryos, greater cotyledonary and unifoliate leaf areas, exhibited higher respiratory rates, and possessed greater field emergence potential than small seeds. It is interesting to note that seedling emergence declined for the exceptionally large seed-sized lines (>22 g/100 seeds), presumably because of greater soil resistance to the large seed [58, 59].

It is apparent that the large seed size of grain soybean favors seedling growth. Soybean seedlings from large seeds were always larger than seedlings from small seeds [60]. Many studies have shown that the positive effects of seed size on emergence seem to be related to interplant competition [61]. Large seeds have more food storage for embryo growth and development which leads to the vigorous growth of seedlings creating competition for light and soil factors with that of small seeds, leading to higher yield [38, 62]. Finch-Savage and Bassel reported that large seeds have large cells, which have a greater capacity to grow and generate force to perform better than their smaller counterparts under stress conditions for mechanical reasons [42]. Bewley and Black also supported that large seed has abundant reserves to be planted deep in the soil where moisture is available because large seeds have a substantial store of reserves to drive seedling growth [63]. However, this is

contradictory to a recent edamame study by Crawford and Williams, who observed that edamame (large seed size) is more sensitive to planting depth and preferred to be planted in shallower depth than grain soybean (small seed size) [40].

However, it seems that the benefits of large seed on soybean emergence were observed generally for cultivars with seed mass < 20 g/100 seed [36]. In some cases, response-reactions of the small seed are similar to those of deteriorated (low vigor) seed, while in others, they are like "immature" seeds [64]. Soybean seed size is a multigenic trait that ranges in heritability from 44 to 94% [65]. While within a cultivar, maturation environment and position on the plant also affect seed mass accumulation [66]. Soybean seeds produced during drought conditions are usually smaller and less vigorous because the maternal plant's photosynthetic capacity is reduced [67, 68]. Seeds produced in the bottom one-third of a soybean canopy were also smaller and had been reported to exhibit less forces to emerge under compacted soil conditions [69].

Finally, there are other researchers who have been unable to detect any relationship between soybean seed size and germination or field emergence [61, 70, 71]. Seed size effects seem to be less pronounced or non-existent in seeds of extremely high or extremely low vigor, or when seeds are sown under "near-ideal" environmental conditions [72, 73]. This indicates that seed quality and the seedbed environment during crop growth likely play a more dominant effect on edamame emergence than the within-cultivar seed size.

2.2.2 Seed coat

The seed coat plays a significant role in seed longevity since it protects the embryo against harmful microorganisms and unfavorable environmental conditions. The soybean seed coat is extremely hydrophilic and can absorb as much as 3.8 times its fresh weight in water [74, 75]. This water-holding capability assists the seed in avoiding imbibitional injury from the rapid hydration of dry seeds that may cause membrane damage. Abnormal seed coats can influence the rate of water uptake, increase the incidence of imbibitional chilling injury, and decrease field emergence [75]. Green et al. reported that wrinkled seed coats were more numerous in seed from earlier dates of planting and were associated with lower laboratory germination and field emergence [48]. However, Nangju found that there was no clear relation between emergence and wrinkled or discolored soybean seed. He observed that germination percentage was negatively correlated with cracked and purple-stained seed, and positively with smooth clean seed and seedling emergence [54]. Cracked seed coats also leak more electrolytes, which encourages the growth of microorganisms around seeds [76].

Seed coat thickness influences seed coat permeability which, in turn, affects the speed and probability of successful germination [77]. Thick seed coats make seeds absorb water slowly to avoid membrane damage, but an extremely hard or thick seed coat can lead to seed physical dormancy and no germination. Seed coat thickness can also be modified by environmental conditions of the mother plant and hormone treatments of the parent plant around the seeds produced [77]. For example, drought stress leads to thinner soybean seed coats, which are more permeable to water [75]. Seed-coat pigmentation is also closely associated with water uptake speed. Colored seeds usually imbibe more slowly than white-coated seeds and showed lower-level infection by *Pythium* due to less seed leakage during germination [78]. Seed coat color also helps to increase the mechanical resistance of seeds because of polymerized phenols. Soluble phenolic compounds provide a chemical defense against microorganisms [76]. Liu et al. concluded that dark-colored soybean seeds have better storability than light-colored seeds [79]. Moreover,

the expression of the impermeable seed coat trait is also influenced by seed size. The impermeable seed coat trait in soybean is of interest to researchers because impermeable seeds retain viability better than permeable seeds. Larger seeds have a higher incidence of the ruptured seed coat, which is significantly correlated ((r = −0.92**, significant at $p < 0.01$) with the impermeable seed percentage [80]. As larger seeds are more likely to exhibit a permeable response than smaller seeds, they could be more prone to chilling injury.

2.2.3 Chilling injury

Seed coat permeability has been reported to act as a principal factor in regulating imbibition rate and chilling injury. Imbibitional chilling injury is one of the key issues that reduce soybean seed quality and reduce seedling survival. Chilling injury is a physiological disorder typically associated with planting in cold soils [42, 81]. In other words, soybean chilling or imbibition damage is most severe when seeds of low initial moisture content imbibe water too quickly at low temperatures. Imbibition damage is associated with membrane dysfunction, which can reduce seed respiration, enhance the leakage of solutes, and decrease mobilization of food reserves from the cotyledons [82]. Seedlings grown from chilling damaged seeds usually show abnormalities and have less emergence force, requiring a longer period to generate maximum force [81].

As we mentioned above, seed coat color can influence seed hydration rate. Powell et al. also supported this point as they found that white-coated seed lines are more sensitive to imbibition damage than dark-coated seed lines [82]. White coated seeds imbibe quickly because they have loosely adhered testae with free space between the testae and embryo. Once the water has moved into the free space between the testa and cotyledons, embryos of white seeds imbibe rapidly. In contrast, dark-seeded lines have close-fitting testae which only allows slow water infiltration even when water can enter the seed through cracks in the testa. Moreover, cracked seeds also have a relatively high rate of water uptake, which indicates that they are more easily damaged.

Imbibition rate can be regulated by the available water around the seeds. For example, seeds priming at low osmotic potential (e.g., polyethylene glycol solutions) can minimize the effects of imbibitional damage by osmoregulation [42]. Temperature-controlled polymer coatings may also serve the same function by preventing imbibition until seeds reach a specific temperature where imbibitional damage will not occur [81]. Chilling injury easily occurs when seeds are exposed to low temperatures at the initial stages of their imbibition, thus, the critical time of chilling injury seems to be the early phase of water entry in seeds. Injury can be prevented if seeds are first allowed to imbibe only at warm temperatures [63].

2.2.4 Seed exudates

Passive release of exudates occurs as soon as seeds imbibe water and germinate [83]. These exudates are usually "normal products" of seed metabolism and they generally consist of simple sugars, such as sucrose, glucose, fructose and maltose, amino acids, flavonoids, sterols, and salts [84]. Depending on the type and abundance of microorganisms around or in the seeds, seed exudates may increase or decrease seed tolerance to abiotic and biotic stresses and affect seedling emergence. Barbour et al. observed that glutamate, aspartate, and dicarboxylic acids in soybean exudates likely represent the natural chemoattractants for *Bradyrhizobium japonicum*, a species of nitrogen-fixing bacteria that is important for nodule formation in soybean roots [85]. Martins et al. also found that malic acid in seed exudates

of common beans can promote growth and biofilm formation of the biocontrol agent *Bacillus amyloliquefaciens*, which, in turn, confers plant drought tolerance and enhances plant growth [86]. On the other side, seed exudates have also been known to promote the growth of pathogens, such as the soilborne pathogen *Pythium ultimum*, which is the causative agent of soybean seedling damping-off [87, 88].

In most cases, increased seed leakage, during imbibition, is associated with membrane damage of soybean cotyledons [89]. Aging seeds leach more electrolytes during imbibition, contributing to a reduction in seed vigor due to loss of the low molecular weight metabolites from cotyledonary cells. Hoy and Gamble reported that large seeds and low-density seeds had the highest seed leachate conductivity, which was correlated with low seed vigor [72]. Our recent study also observed that edamame seeds released exudates more quickly and showed higher seed leachate conductivity than grain soybean (unpublished results). However, specific reasons for the different rates of seed exudate production are still unknown but may be related to differential membrane leakage.

2.2.5 Seed aging

Seed deterioration during storage is one of the basic reasons for reduced seed vigor. The ability to resist aging during storage is an important physiological factor contributing to both seed viability and vigor. This is particularly problematic for soybeans as its seeds are relatively short-lived, whose longevity is only a few months [90, 91]. The longevity of soybean seeds increases progressively during seed maturation, which occurs from the phenological stage 7.2 onwards. From a developmental standpoint, this is shortly before the end of seed filling and onset of maturation drying during stage R9, corresponding to full physiological maturity [90, 92]. Several studies have reported that large soybean seeds deteriorate faster than small seeds [93]. Our recent study (unpublished data) also supports reduced storage life, since we found that edamame seeds aged faster than grain soybean seed when stored under the same conditions.

The longevity of seeds in storage is influenced by four major factors, (i) genetics, (ii) maturity and quality of the seed at the time of harvest and storage, (iii) moisture content of seed or ambient relative humidity, and (iv) temperature of storage environment [93]. Soybean seed vigor declines rapidly with increasing storage duration, but the severity of reduction varies by genotypes. Heatherly et al. reported that the germination of grain soybean declined from 96 to 12% and 93 to 21% in two cultivars after 20 months of seed storage, while for another cultivar, it only declined from 98 to 75% [94]. Temperature and seed moisture content are the two main environmental factors affecting seed storage longevity. Nkang and Umoh compared soybean germinability after 6 months of storage under storage temperatures 0, 25, 35, 45, and 55°C and relative humidities of 45, 55, 65, 75, and 84%. They reported that optimum storage occurred at temperatures of 25–30°C and relative humidity of 55–65% [95]. Mbofung et al. evaluated germination of soybean seeds stored under 10°C; 25°C; in open storage in a warehouse at ambient humidity. High seed viability was maintained for seeds stored at 10°C (>92%) and moderate in the 25°C (>78%) after 20 months, with almost 0% germination for the seeds stored after 20 months at a warehouse [96].

The hydrophilic nature of the high protein content of soybean seed drives the absorption of water from the environment during imbibition, increasing hydrolytic enzyme activity and increasing seed respiration [93]. Seed deterioration is thought to be due to lipid peroxidation, leading to mitochondrial dysfunction, and less ATP production in seeds [97]. High temperatures and seed moisture accelerate the rate of biochemical processes, causing more rapid seed deterioration resulting in first

reduced vigor and eventually seed death. Moreover, high temperature and seed moisture can also stimulate the growth of storage fungi on seeds that rapidly reducing seed quality.

In addition, the rate of seed deterioration during storage is also affected by packaging materials. Since soybean seeds without hard seed coats are hygroscopic, they will take up moisture from the atmosphere when in open storage. This means that when the relative humidity is high, seed moisture content increases, and when the humidity is low seeds can lose water to the atmosphere. In humid areas to maximize storage life, it is recommended to dry seeds to moisture contents below 14%, the threshold for microbial growth, and store seeds in sealed packaging with a moisture barrier; so, there is no increase in seed moisture during storage. Several studies show that containers with moisture barriers improve the storage life of soybean seeds. Polythene bags are superior to cloth bags because they keep moisture out during seed storage [98]. It is reported that the storability of soybean cultivars could be enhanced by 4 months when storing dried seed in polythene bags compared to cloth bags [99]. Monira et al. reported that cloth bags are not safe for long-term soybean seed storage compared to polyethylene bags or metal containers, since the rate of moisture absorbance was higher in cloth bags with no moisture barrier [100]. They also reported higher fungi growth in cloth bag seed storage and metal containers than in polythene bags. Fungal growth in sealed storage occurs when the seed moisture content is too high at the time of packaging. Others have reported that soybean seeds stored in aluminum foil bags have higher germination followed by polyethylene and wheat bags when stored for the same period of time at the same temperature [101].

Moreover, the storability of soybean seeds is also influenced by many pre- and during-harvest factors, including climate conditions during seed production, pest attacks on seeds and pods, disease infection on developing and maturing seeds, premature or delayed harvest, and how the seeds are harvested and processed [54, 102]. Delayed harvest and intense rainfall during pod maturation can increase seed deterioration during storage. A previous study reported that a delay in the harvest of about 2–4 weeks after optimum maturity reduced seed quality [54].

2.2.6 Seed maturity, harvest, seed shape

Maturity groups are thought to have no influence on seed vigor [96]. However, early maturing soybean plants developing during hot, dry conditions increased the number of seeds with morphological defects. These defective seeds germinated and emerged later than seeds maturing on soybean plants that developed after the hot, dry weather conditions were over [103]. For example, the combined occurrence of heat (air temperature above 30°C) and drought stresses during seed filling can increase the percentage of shriveled soybean seeds; a higher incidence of shriveled seeds was observed on the upper third of the mother plant [104]. Germination and emergence were significantly reduced as the level of shriveling increased [104]. Severe other stresses (such as defoliation) during seed filling can also produce small, flat, shriveled, and underdeveloped seeds with poor germinability and vigor [105]. Moreover, the vigor of normal-looking soybean seeds (not wrinkled or shriveled) formed at high temperatures was reduced in comparison to seeds formed at optimal temperatures [106].

2.2.7 Seed mechanical damage

Mechanical injury is another cause of significantly reduced seed vigor. Soybean seed is very susceptible to mechanical damage since the vital tissues of the embryo (radicle, hypocotyl, and cotyledon) lie under a thin seed coat that offers little

protection [107]. In most cases, the damage may not be sufficient to kill seeds but may cause abnormalities in seedlings or cracks in the seed coats, reducing seedling establishment [107]. Mechanical threshing is one of the processes where seed damage occurs because of the abrasions and impacts when seeds pass through a combine [108]. It is reported that large-sized seed is more prone to mechanical damage during harvesting and processing, as cracked seed coats are more common in large soybean seeds [54]. Harvesting seeds at high moisture content can be used to reduce mechanical damage which is greater when seed moisture contents are extremely low.

2.3 Other environmental factors related to the emergence

The soil seedbed is a complex environment in which seeds and seedlings are exposed to multiple stresses. As discussed previously, soil temperature, oxygen, and water content (Section 2.1) play a critical role in seed germination, seedling vigor, and successful establishment (Section 2.2). In the following section, we discuss the effects of some other environmental factors, including the abiotic factors (soil compaction and the planting depth) and the biotic factors (soil microorganisms and insects) on stand establishment. Biotic and abiotic effects on stand establishment can have a pronounced effect on establishment especially when the seeds are of suboptimal quality.

2.3.1 Soil strength

In addition to soil temperature, moisture, and oxygen availability mentioned above, soil strength, another edaphic factor often the result of crust formation at the surface of soils with high clay content, also plays a key role in pre-emergence seedling growth since the hypocotyl may encounter considerable resistance when pulling the cotyledons through crusted soil. If the cotyledons face more mechanical impedance from the soil than the force exerted by the hypocotyl, the hypocotyl may collapse between the cotyledons, producing an abnormal seedling. Even worse, the hypocotyl may break, resulting in seedling mortality [46].

Soil crusting is likely to occur on high clay content soils when the surface dries rapidly following a heavy rainfall [109]. The hard layers at the soil surface show low permeability and high tensile strength making seedling emergence difficult. Another soil structure problem is compaction, which occurs when soil particles are pressed together, reducing pore space between them and consequently increasing the bulk density [110]. Soil compaction is usually caused by compressive forces applied from wheels of heavy field machinery (such as tractors, trucks, and combines) and pressure from the hooves of livestock or other animals [111]. Increased soil bulk density can reduce root growth as well. Severe compaction can also decrease a soil's permeability to water and air. This decrease in permeability will reduce the activity of soil microorganisms and the rate of organic matter decomposition thus slowing the release of essential mineral nutrients needed for seedling growth. These soil problems can be eliminated by using a rotary hoe mounted on a tractor or other similar equipment.

Soil strength is not likely to affect seed germination [42], instead, the increasing soil strength caused by compaction of heavy soils can impair root elongation, particularly on shoot development of pre-emergent seedlings in severely crusted soils [112, 113]. Seedling response to soil strength is associated with seed vigor. Hyatt et al. reported that soybean emergence declined as compaction increased from low (4.6 kJ m^{-3} CE) to high (22.9 kJ m^{-3} CE); however, the emergence of high-vigor seed lots remained >80% until compaction increased to 13.7 kJ m^{-3} CE, while low-vigor seed lots had low emergence (<50%) even at the

lowest compaction (4.6 kJ m^{-3} CE) level [114]. The authors also observed that seed size had no effect on emergence at any level of compaction. However, other studies claimed that larger soybean seeds should be subjected to greater mechanical resistance due to their large cotyledons [40, 59]. Soil strength is closely related to the capillary pressure of water in the pores holding the soil particles together. Clay soils tend to have a higher degree of saturation (thus greater capillary pressure) resulting in higher soil strength than sandy soils [42]. In an ideal situation, the soil structure will minimize water loss by evaporation while remaining mechanically weak with no barrier to growth [42].

2.3.2 Plant depth

Planting depth is an essential management decision influencing emergence of soybean seeds. Depth is correlated to total, rate, and uniformity of emergence. Deep depth causes delayed emergence which may increase seedling mortality by extending the window of time in which seedlings are vulnerable to soil pathogens, risk of soil-crusting, and anaerobic soil conditions [40]. While shallow planting can also be detrimental to emergence when the upper soil lacks sufficient moisture for seed germination and seedling establishment.

Recommended planting depth of grain-type soybean has been reported to be 2.5–5 cm [40, 46, 115], specifically depending upon the soil type and weather conditions (such as rainfall and temperature). In sandy soils, seeds can be planted deeper, while in heavy clay soil, seeds should be planted shallower [46]. Fehr et al. reported a reduction of an average emergence of 73% from 5 cm to 44% from 10 cm among different grain soybean varieties [116]. Varieties also showed variations in response to deeper planting depth, as the emergence of some cultivars was reduced markedly (as low as 13%) at a depth of 10-cm depth [116], partly due to lower seed vigor.

The optimal planting depth for edamame is unresolved, although a few studies have attempted to address it. Zhang et al. found the hypocotyl and radicle of edamame were significantly longer and wider than that of the grain soybean. As planting depth increased from shallow (1 cm) to deep (5 cm), emergence declined for both grain soybean and edamame in a growth chamber, but the grain soybean seed consistently emerged better than the vegetable soybean seeds. The emergence of both the grain soybean seed and the vegetable soybean was >65% until planting depth increased to 3 cm, while the vegetable soybean seed had the lowest emergence (<30%) at the deepest (5 cm) level. Thus, the vegetable soybean was relatively more susceptible to planting depth than the grain soybean, and 3 cm planting depth was an acceptable depth for both types of soybeans [46]. Crawford and Williams also reported similar findings under field conditions. They compared the emergence of edamame and grain soybeans at depths of 1, 2, 3, and 5 cm in the field, and they found that edamame emerged more completely and quicker at the shallowest depths examined if sufficient soil moisture was available [40]. Other studies recommended a planting depth for edamame seeds not greater than one-half inch deep to avoid reduced emergence [117, 118]. All of these results show that if moisture is adequate in soil, the optimal planting depth of edamame should be shallower than grain-type soybean. However, it is hard to conclude what the optimal depth for edamame should be because of variation among varieties, soil, and weather conditions. Under the drought condition, edamame may need to be planted deeper to access soil water reserves [56]. However, the larger edamame seed size may inhibit emergence, particularly in heavy soils prone to compaction and crusting at deeper depths, reducing emergence, especially with suboptimal seed quality.

2.3.3 Seed and seedling diseases caused by soilborne pathogens

Similar to grain soybean, seed and seedling diseases, caused by soilborne fungal and oomycete pathogens, such as *Fusarium* species, *Phytophthora sojae*, *Pythium* species, and *Rhizoctonia solani*, are also common causes of decreased edamame stands and may cause serious or even complete yield loss [36, 119, 120]. These pathogens can survive in the soil for many years, and they can kill seeds (seed rot) or cause seedling death shortly after emergence (damping-off). The higher sugar content and size of edamame seeds mean that they leach more nutrients into the soil upon imbibition compared to smaller grain soybeans. This leachate feeds and attracts microbial pathogens leading to greater seedling mortality [42, 121]. Fungicides (either as an in-furrow or as a seed treatment) with broad-spectrum fungicides provide the most reliable approach to protect against soilborne pathogens. Recently, it is reported that edamame seedling emergence can be improved using seed treatment with fludioxonil + mefenoxam [36]. However, other studies pointed out that fungicides should be used only when the seeds or soil are contaminated with pathogens [75], otherwise biological N_2 fixation may be severely affected due to the toxicity of most fungicides to bradyrhizobia [122, 123]. In addition, no fungicide seed treatment can consistently improve field emergence of seeds with reduced quality for reasons other than a disease, such as mechanical damage, age, or size [124].

With rising public awareness of the potential environmental and health hazards of agrochemicals, the demand for organic edamame has been increasing and constitutes a large portion of the market. Thus, researchers are charged to search for alternatives to fungicides to improve edamame seedling emergence. Biological seed treatments are playing a pivotal role in sustainable crop production by providing a combination of both effective performance and product safety. In general, biological control agents contain natural active ingredients that can include microbes, such as bacteria and fungi, plant or algae extracts, as well as other organic substances. Previous studies have shown some fungal or bacterial strains, including *Trichoderma harzianum*, *Streptomyces* sp., *Bacillus subtilis*, and *Pseudomonas putida* are used or may be potentially effective as biological seed treatments for grain soybean to control soilborne diseases and improve seedling establishment [125–127]. However, there are still no studies examining the effectiveness of biological seed treatments for improving emergence or crop safety when applied to edamame.

A few studies have also focused on evaluating the disease resistance of different varieties of edamame [128–130]. High susceptibility to *Phytophtora* spp. causing root rot disease was found in cultivars "C784" and "Bunya" in Australia. Different degrees of resistance to *Diaporthe phaseolorum* causing soybean seed decay and stem canker were observed in edamame varieties in Argentina. However, more work still needs to be done to test current commercial edamame cultivars for resistance against more soilborne diseases to assist in developing more disease-resistant cultivars.

2.3.4 Insect pests

There are several insect pests that can attack edamame, but most of them eat the foliage of emerging plants or only affect the pod quality without significantly reducing yield [131]. These pests include various beetles (such as Mexican bean beetle, Japanese beetle, bean leaf beetle, and cucumber beetle), grasshoppers, leafhoppers, thrips, loopers, other worms (such as green cloverworms and defoliating caterpillars), and stink bugs. Only some early-season insect pests, such as

wireworms, seedcorn maggots, and white grubs can damage soybean seeds and seedlings [132]. For example, soybean seeds or cotyledons may be attacked by seedcorn maggots when cool, moist conditions prevail, and germination and early growth are slowed. Generally, insecticide seed treatments and hand picking are enough to achieve control [117].

Other animals, such as slugs, rabbits, birds, and deer can do extensive damage to young seedlings [118]. Deer love edamame leaves, and cotyledons, and can quickly defoliate plants. Repellants, scare devices, and fencing can provide temporary protection [133].

3. Conclusion

This chapter has attempted to define the seed intrinsic and environmental factors associated with edamame germination and seedling establishment. It provides readers with a knowledge of the aspects of environmental influence on seed quality and its subsequent effect on seedling emergence, which can be helpful for a comprehensive understanding of the causes of poor edamame seedling emergence that some farmers now face. It should be emphasized that seed quality still plays a critical role in edamame emergence. There is a high potential for edamame seeds with strong viability and vigor to exhibit excellent emergence (>80%). On the other hand, however, the large seed size of edamame contributes to the emergence problem. First, large seeds are more sensitive to poor seedbed environments, including inadequate soil moisture, improper temperature, and soil obstruction. Second, large seeds are more prone to reduce viability and vigor because of mechanical damage during seed harvest, processing, and are also more likely to age during storage. Third, large seeds leach more nutrition during imbibition, thus attracting soilborne pathogens and increasing disease occurrence. All of these contribute to the lower emergence ability of edamame in the field when compared with that of grain-type soybean seeds.

4. Future perspectives

When the causes of emergence problems are understood, the corresponding strategies could be made to enhance edamame seed performance and establishment. Developing edamame cultivars with high seed vigor and better adaption to the US soil and climate, as well as optimizing the conditions of seed processing and storage will be a goal for plant breeders and seed industries to improve seed quality and edamame emergence. Proper planting (such as optimal planting depth) and field management (such as seedbed preparation, *i.e.*, the soil is warm, moist but well-drained, fertile, and free of weeds) are also critical to ensure robust seeding growth in the field. Since only a few edamame production practices were described in this review, due to the limited research that has been conducted, more research focusing on edamame is needed to develop the appropriate management practices for edamame production. This will ultimately support a more reliable edamame supply into the future.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 11

Soybean in Indonesia: Current Status, Challenges and Opportunities to Achieve Self-Sufficiency

Arief Harsono, Didik Harnowo, Erliana Ginting and Dian Adi Anggraeni Elisabeth

Abstract

Soybean is the third important food crop in Indonesia after rice and maize, particularly as a good source of protein. The demand for soybean consumption tends to increase annually. In 2020, the figure was about 3.28 million tons, while the domestic production was 0.63 million tons, thus around 81% of the soybean needed was imported. Efforts to increase the domestic soybean production have been conducted since the last decade, which is concerned with increasing the current productivity (1.5 t/ha) through introducing the high-yielding improved varieties and extending the harvested area, particularly to outside of Java. The potential planting area is focused on the irrigated lowland after rice (optimal land) and suboptimal lands (dry, acid, tidal, and shaded lands). The series of the study showed that the yield potential of soybean grown in such lands varied from 1.8 t/ha to 3.0 t/ha. A number of soybeans improved varieties adapted to different land types or agro-ecological conditions also have been released and supported with advanced cultivation technology. The results, challenges, and opportunities to achieve soybean self-sufficiency are discussed in this paper.

Keywords: Indonesia, soybean, self-sufficiency

1. Introduction

Soybean (*Glycine max* L. Merr.) is the third most important food crop in Indonesia after rice and maize. Soybean plays an important role as a vegetable protein source for most of the community in the country, which is predominantly consumed as tempe and tofu. In 2020, the average soybean consumption level was around 11–12 kg/capita/year. The need for this commodity tends to increase along with the population increase. During the period 2000 to 2019, domestic production contributed 30–35% to the total need, while the rest (65–70%) was imported. The latest report [1] showed that the domestic production of soybean in 2020 was approximately 0.63 million tons, whereas the total need was approaching 3.29 million tons, thus about 81% of soybean was imported.

This condition was related to the discouraged situation of soybean production during the last 10 years (2010–2020). The average productivity during this period was 1.50–1.54 t/ha and no significant increase was recorded [2]. Also, only a slight increase in the harvested area occurred. A number of problems were noted regarding such conditions, including (a) high competition of land use with other commodities, (b) low stability of the yield as soybeans are highly susceptible to pest and disease attacks, (c) efforts to extend the planting area has not been fully succeeded, (d) relatively low quality of seeds as the soybean seed industry has not been well developed, (e) less conducive of soybean trading system, (f) less intensive cultivation techniques, and (g) low profit of soybean farming relative to other crops.

Soybean was targeted to be self-sufficiency by the Government in 2014 through four main strategies as follows: (1) gradually increasing the productivity (2) improving the roles of public and private sectors as well as local government in soybean development, (3) improving the marketing and trading system to be more conducive to farmers, and (4) improving the source of farming capital and partnerships. As a follow-up of such strategies, action steps were undertaken to achieve soybean self-sufficiency, including (a) supporting the research activities, which concerned on the release of new improved varieties with high yield potential, resistance to biotic and abiotic stress, short maturity; assembling the advanced cultivation technologies; and implementing different methods of dissemination, (b) initiating the growth of seed industry in soybean producing areas, (c) subsidizing the fertilizer prices, and (d) improving the access for agricultural tools and machinery application. However, these efforts have not fully succeeded as the increased rate of soybean productivity at the farmer level was considerably low, the planting and harvested areas were stagnant and even tended to decline, resulting in a decreased domestic production. As a consequence, a large amount of soybean is imported annually, suggesting more efforts and proper strategies are needed to achieve soybean self-sufficiency in Indonesia.

This paper will discuss the soybean production matters in Indonesia, including the current status and predicted soybean production and demand, the national program for increasing production, land availability for soybean development and specific production technologies for the different agroecosystems as well as the essential socio-economic aspects to support the achievement of soybean self-sufficiency in Indonesia.

2. Soybean production and demand

The development of the harvested area, productivity, production, and import of soybean in Indonesia during the period 2016–2020 and the prediction for the year 2024 are presented in **Table 1**. Until 2020, the harvested area and production highly fluctuated, whereas the productivity tended to increase. It is estimated that the soybean harvested area until 2024 will not significantly expand as soybean hardly competes with other commodities, particularly maize. There was a considerable increase in soybean production (49.07%) during 2019–2020 as a result of expanding the harvested area. However, for the next four years, it is predicted that soybean production will tend to decline by 3% per year [3]. This was due to the competition of land use with other profitable commodities, such as corn and chili, resulting in a decrease in the harvested area of about 5% per year. Even though the productivity increased by 2% per year, this value was set below the rate of declined harvested area, thus giving no significant increase in soybean production. As a result, a large amount of soybean needs to be imported with an average of 2.49 million tons per year.

Years	Harvest area (ha)	Productivity (t/ha)	National production (t)	National demand (t)	Net Import (t)	The additional need of harvested area (ha)
2016	576,987	1.49	859,653	3,121,456	2,261,803	1,517,989
2017	355,800	1.51	538,730	3,103,475	2,671,914	1,698,507
2018*	493,546	1.31	650,000	3,215,258	2,565,257	1,958,212
2019*	285,270	1.49	424,190	2,726,091	2,301,902	1,544,900
2020**	381,331	1.65	632,326	3,293,377	2,661,051	1,612,758
2021**	262,612	1.69	613,318	3,279,452	2,666,134	1,577,594
2022**	344,455	1.72	594,629	3,240,236	2,645,607	1,538,144
2023**	326,861	1.76	576,278	3,163,759	2,587,481	1,470,160
2024**	309,849	1.80	558,293	3,030,085	2,471,792	1,373,218

Note:
**Agreement figures of Central Bureau of Statistics (BPS) and the Indonesian Ministry of Agriculture.*
***Forecast of the Indonesian Agricultural Data and Information Center.*

Table 1.
The development and projected of harvested area, production, and import of soybean in Indonesia during the period 2016–2024 [3].

The national demand ranged from 2.73 up to 3.29 million tons during the period 2020–2024, which is mostly for consumption purposes. The consumption level of soybeans during this period is predicted to fluctuate and tends to increase by 1.46% per year. In 2019, the figure was 10.17 kg and it slightly increased to 12.15 kg/capita/year in 2020 [3]. It is assumed to be associated with the global pandemic of Covid-19, which led to a decline in people's purchasing power for animal protein sources and shifting to soybean as an affordable protein source, particularly as tempe and tofu. In addition, the increase in soybean consumption is also influenced by the healthy lifestyle of the middle and upper class who prefer a vegetarian diet. It seems that the consumption level will go back to 10.74 kg/capita/year in 2024. **Table 1** shows that the self-sufficiency in soybean within the next four years (2021–2024) can be achieved with an additional harvested area of 1.3–1.5 million hectares per year and productivity of 1.7–1.8 t/ha. Even though it seems hard to achieve such figures, the Government relentlessly encourages both the Ministry of Agriculture and farmers to increase the national soybean production.

3. National soybean program

Since 2000, the Government has been working hard to increase soybean production in order to achieve self-sufficiency through the program entitled "Gema Palagung", "Bangkit Kedelai", and "Farmer's School for Integrated Crop Management/FSICM for soybean". In 2018, a particular intercropping program between soybean with upland paddy or maize was launched, covering an area of 22 thousand hectares in 22 provinces [4]. Initially, the Government established the target for soybean self-sufficiently in 2014. However, as it unsucceded, the target was postponed to be 2017 and again postponed to be 2018, and then to 2020. In 2017–2018, the Ministry of Agriculture had a target of soybean planting area approaching 2 million hectares. Planting started from October to December 2017 with the first target of 500 thousand ha (approximately 25% of the total target). The remaining 1.5 million hectares expectedly can be fulfilled in the next planting season in 20

provinces, from Aceh in the west to East Nusa Tenggara in the eastern part of Indonesia. Meanwhile, another 500 hectares of land were available from the existing traditional farmers. It is estimated that in 2018, the soybean planting area will be becoming 2.5 million hectares [5] and would meet the domestic demand if the productivity was 1.5 t/ha.

Nevertheless, such a target was hard to be achieved as in fact, the total soybean production was only 650,000 tons in 2018 with a harvesting area of 493,546 hectares. In addition to climate and technical/cultivation factors, this failure was also related to economic aspects. It is obvious that soybean farming requires high input, possesses a high risk of crop failure, particularly due to pest and disease attacks, and inadequate income or less profitability. Planting of soybean starting from land preparation to harvesting and processing costs seven to nine million IRD per hectare and 60% of which is accounted for labor cost. The soybean production process in the field is also inefficient as most of the activities are done manually. In fact, the Government has established the selling price of soybean at the farm level that was about IDR 8,500 per kg in 2017 as Minister of Trade's Regulation no 27/2017. However, the price is normally following the market conditions and frequently is below the selling price determined by the Government, particularly during the harvesting season giving a low profit to soybean farming.

4. Land availability for soybean development

Indonesia has a wide and diverse potential land for the development of soybean. **Table 2** shows that there are 3.8 million hectares of irrigated paddy fields and 3.6 million hectares of non-irrigated paddy fields available (optimal land). In irrigated paddy fields, soybean can be grown using a cropping system of paddy-paddy-soybean, and a paddy-soybean cropping system in non-irrigated paddy fields. The main obstacle of soybean cultivation in optimal land is competition with other commodities that have higher economic value, especially maize. Therefore, soybean development in this optimal land should be selected to those lands that have less water available for growing maize. The need for water to grow soybean is only about half compared to growing maize.

There is also the potential of sub-optimal lands for the development of soybean in Indonesia, including dry acidic land, dryland with dry climate, and tidal land area, accounting for 4.5 million ha, 1.2 million ha, and 0.8 million ha, respectively (**Table 3**). The acidic land showed the least favorable for soybean production due to

Islands as central of soybean production	Irrigated lowland (ha)	Non-irrigated lowland (ha)	Drylands (ha)
Sumatera	676,816	852,985	3,655,378
Jawa	2,258,066	1,549,255	2,613,514
Bali+Nusa Tenggara	197,316	245,619	921,281
Kalimantan	214,298	432,462	1,605,806
Sulawesi	430,621	508,033	1,981,629
Maluku	10,094	9,448	252,032
Papua	17,180	8,558	468,358
Indonesia	3,804,391	3,606,360	11,497,998

Table 2.
Irrigated and non-irrigated lowlands available for soybean development in Indonesia [6].

Island	Dry acidic soil (× 1,000 ha)			Dryland with dry climate (× 1,000 ha)			Tidal swampland (× 1,000 ha)			Total (× 1,000 ha)
	AOU	AFC	AFP	AOU	AFC	AFP	AOU	AFC	AFP	
Sumatera	536.6	104.3	659.5	24.9	34.0	58.3	137.4	13.5	271.2	1,839.7
Jawa	46.3	0.0	202.2	8.7	0.0	31.6	0.3	0.0	0.0	289.1
Bali+NT	1.6	0.0	0.0	257.8	10.7	30.4	0.0	0.0	0.0	300.5
Kalimantan	329.9	227.9	1,297.8	0.0	0.0	0.0	82.1	1.6	46.5	1,985.8
Sulawesi	25.8	14.2	0.0	61.0	42.8	0.0	0.8	0.0	0.0	144.6
Maluku	0.0	39.6	0.0	0.0	0.0	0.0	2.7	3.3	0.3	45.9
Papua	11.0	304.3	671.4	9.7	163.5	437.2	0.4	84.8	128.0	1,810.3
Indonesia	951.2	690.3	2,830.9	362.1	251.0	557.5	223.7	103.2	446.0	6,415.9

Note: AOU = Area of Other Uses, AFC = Area of Forest Conversion, AFP = Area of Forest Production, NT = Nusa Tenggara.

Table 3.
The suboptimal lands available for soybean development in Indonesia [7].

lower fertility, potential toxicity from soluble forms of microelements such as Al, Mn, and Fe, and unfavorable physical properties [8–10]. Therefore, to obtain high soybean productivity in this type of land (soil), use of ameliorants and high doses of inorganic fertilizers are needed. On the dry land with a dry climate, the main constraint faced is the short wet month that is only around 3–4 months/year with a rainfall >200 mm/month. In this region, soybean needs to compete with other staple food crops, such as upland rice and maize. In tidal swampland, constraints like water-saturated root, high pyrite, the toxicity of Al, Fe, and Mn, as well as deficiencies of N, P, K, Ca, and Mg may limit soybean production [10, 11]. Therefore, specific cultivation technology is essential for such different types of land.

5. Cultivation technology for various agroecosystem

5.1 Lowland

Soybean cultivation in the irrigated paddy lowland generally follows the cropping pattern of paddy-secondary food crop, while the pattern is paddy-secondary food crop in the non-irrigated paddy land (rainfed land). It seems that soybeans yet have to compete with other commodities, especially maize or other food crops. Currently, the productivity of soybean using existing farmer's technology is about 1.5–1.8 t/ha. Using high-yielding improved varieties and good environmental management through the application of advanced cultivation technology makes it possible to achieve soybean productivity as high as 3.0 t/ha in the lowland.

A number of new improved soybean varieties have the yield potential of more than 3.0 t/ha, namely Dega1, Detap 1, Mutiara 1, Dering 2, Biosoy 1, and Demas 2 [12] as presented in **Table 5**. In additon to new improved varieties, plant spacing is also an important factor in achieving high yield through optimal plant populations. Planting Burangrang, Grobogan, and Anjasmoro varieties at a spacing of 20–30 cm × 40 cm, two plants per hole with optimal fertilization in Malang, East Java gave a grain yield of 3.96 t/ha, 3.93 t/ha, and 3.36 t/ha, respectively [13]. Thus, to achieve the soybean yield >3.0 t/ha, the population of >340 thousand plants/ha which is obtained using a plant spacing of 30 cm × 15 cm needs to be applied as well

Soybean variety	Plant spacing (cm), two plants/hill		
	50×15	40×15	30×15
	Number of crops can be harvested (×1,000)		
Dega 1	240.68bc	255.20 b	345.29 a
Detap 1	204.41 c	252.01 b	344.62 a
Derap 1	202.60 c	249.16 b	350.24 a
Devon 1	204.72 c	260.55 b	358.90 a
	Seed yield (t/ha)		
Dega 1	1.98 d	2.21 d	3.12 b
Detap 1	2.14 d	2.61 c	3.53 a
Derap 1	1.90 d	1.97 d	3.15 b
Devon 1	2.11 d	2.69 c	3.75 a

Note: The values within the same observation followed by the same letter are not significantly different at 5% DMRT level.

Table 4.
The yield of soybean varieties in several plant spacing in irrigated paddy fields in Banyuwangi-East Java [14].

as planting 2 plants/hole and optimal fertilizer application *i.e.*: 11.5 kg/ha N + 36 kg/ha P_2O_5+ 30 kg/ha K_2O at 10 days after planting, and 21.1 kg/ha N + 11.1 kg/ha S at 25 days after planting (**Table 4**).

A study in the rainfed Alfisol soil of Maros, South Sulawesi, which had a pH level of 6.2–6.7 and moderate soil fertility showed that soybean yield increased from 1.6 t/ha (existing technology) to 2.7 t/ha through the application of advanced cultivation technology [15]. This technology consisted of using good quality seed, sufficient fertilizer (30 kg/ha N + 48 kg/ha P_2O_4 + 30 kg/ha K_2O), rhizobium inoculant 250 g/50 kg of seeds, and organic fertilizer (1.5 t/ha). The performance of soybean crops grown after paddy in the irrigated lowland is presented in **Figure 1**. Using such technology, the labor cost accounts for the largest portion of the total production costs, reaching about 65% and 72% for advanced and existing technology, respectively. Nevertheless, both the R/C and B/C ratio of applying the advanced technology is higher relative to those of the existing technology (**Table 5**).

Figure 1.
The performance of soybean crop grown after paddy in the irrigated low land.

Components	Soybean cultivation technology	
	New technology	Existing (Farmers') technology
Production costs (IDR/ha)		
a. Production facilities	2,593,000 (34.7%)	1,470,000 (27.5%)
b. Labor	4,876,667 (65.3%)	3,880,000 (72.5%)
Total costs (IDR/ha)	7,469,667 (100.0%)	5,350,000 (100.0%)
Productivity (kg/ha)	2,725	1,590
Total revenue (IDR/ha)*	16,350,000	9,540,000
Total profit (IDR/ha)	8,880,333	4,190,000
R/C ratio	2.2	1.8
B/C ratio	1.2	0.8

Note:
**With a selling price of soybean IDR 6,000/kg.*

Table 5.
Financial analysis of soybean farming for advanced and farmer's technologies in the rainfed land of South Sulawesi in the dry season (May to August) of 2017 [15].

5.2 Dryland

The cropping patterns in the dryland are generally maize-maize, upland paddy-maize, maize-peanuts, or maize-soybeans. Meanwhile, in a dryland with a dry climate, farmers normally only grow maize or upland paddy during the rainy season. The rainfall in the dryland with a dry climate is approximately <2000 mm per year with a dry period >7 months per year (<100 mm rainfall per month). This type of agroecology is mostly found in Bali and Nusa Tenggara, Sulawesi, and Java [11]. However, the insufficient and non-uniform distribution of rainfall in the dryland considerably results in drought stress during the growing period of soybean and may cause yield reduction and even harvesting failure [16]. In this particular land, soybean development can only be performed through intercropping with maize as it is one of the major staple foods as well as a source of cash income for farmers [17]. Maize productivity in the dryland is relatively low, which ranges from 2.5 to 5.0 t/ha [2]. This is caused by the erratic distribution of rainfall and less optimal maize cultivation by farmers. The introduction of soybean in the dryland through intercropping with maize is expectedly would increase the land productivity and farmer's income. Intercropping system has been adopted all over the world as it can increase land-use efficiency [18, 19].

The use of adapted cultivars and optimal plant spacing in soybean intercropping systems can increase land productivity, reduce the risk of crop failure, increase crop yields and farmers' income [19–21]. The cropping pattern of soybean monoculture in the dryland with a dry climate could produce dry seed about 1.4–2.4 t/ha depending on the variety used and distribution of rainfall. However, this cropping pattern is difficult to be developed in the dryland as such a pattern was less profitable relative to growing maize [9]. Therefore, the development of soybean in the dryland, particularly in the maize producing area should be done by intercropping. Soybean intercropping with a plant spacing of 30 cm × 15 cm, planting two seeds per-hill and planting maize in a double row with a plant spacing of (40 × 20) cm × 200 cm and one seed per hill (**Figure 2**) is able to produce high maize yield and increase the farming profit. Intercropping soybean variety of Dena 1 with maize in the dry land with dry climate (Tuban, East Java) showed higher benefit than using Argomulyo and Dega 1 varieties (**Table 6**). Dena 1 variety is particularly

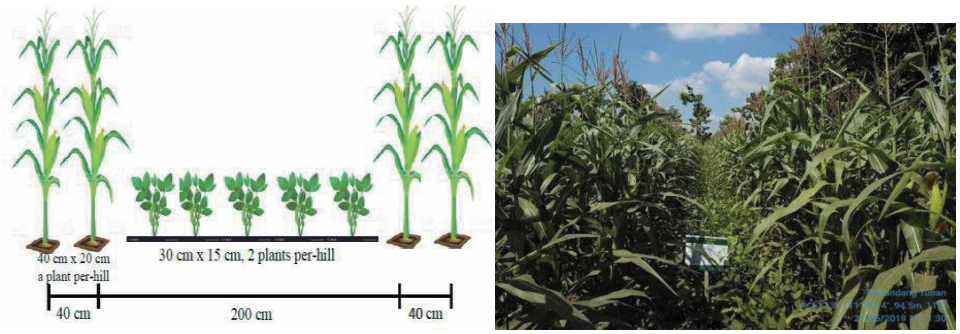

Figure 2.
The optimal crop layout for soybean intercropping with maize in the dryland (a) and the crop performances in the field (b) [9].

Planting patterns	Yield (t/ha)		Total revenue (IDR 000/ha)	Cost production (IDR 000/ha)		Total cost (IDR 000/ha)	Total benefit (IDR 000/ha)
	Maize	Soybean		Maize	Soybean		
Semanding							
'Maize NK212' monoculture	5.488	0	21,952	8,032	0	8,032	13,920
'Argomulyo' monoculture	0	2.430	15,795	0	7,022	7,022	8,773
'Dena 1' monoculture	0	1.873	12,174.5	0	6,802	6,802	5,372.5
'Dega 1' monoculture	0	1.417	9,210.5	0	6,622	6,622	2,588.5
'Maize NK 212' + 'Argomulyo'	4.876	1.447	28,909.5	7,972	4,540	12,512	16,397.5
'Maize NK212' + 'Dena 1'	6.297	1.017	31,798.5	8,252	4,400	12,652	19,146.5
'Maize NK212' + 'Dega 1'	5.635	0.820	27,870	8,047	4,180	12,227	15,643
Merakurak							
'Maize NK212' monoculture	5.648	0	22,592	9,737	0	9,737	12,855
'Argomulyo' monoculture	0	2.880	18,720	0	7,342	7,342	11,378
'Dena 1' monoculture	0	2.280	14,820	0	6,962	6,962	7,858
'Dega 1' monoculture	0	3.060	19,890	0	7,542	7,542	12,348
'Maize NK212' + 'Argomulyo'	3.657	1.927	27,153.5	9,817	4,520	14,337	12,816
'Maize NK212' + 'Dena 1'	4.157	1.687	27,595.5	9,927	4,360	14,287	13,306.5
'Maize NK212' + 'Dega 1'	3.367	1.613	23,952.5	9,787	4,380	14,167	9,785.5

Notes: The population of maize crops 100% (plant spacing of 80 cm × 20 cm, 2 seeds per-hill) was 62,500 crops/ha and soybean 333,333 crops/ha. The selling price of maize and soybean (dry seeds) were IDR 4,000/kg and IDR 6,500/kg, respectively.

Table 6.
Farming income of soybean intercropping with maize, Tuban District, East Java, Indonesia, planting season 2019 [9].

released for intercropping purposes as it is tolerant to shading up to 50%. Other soybean varieties that are suitable for intercropping with other crops, including young plantation crops are Dena 2, Denasa 1, and Denasa 2 (**Table 5**). Also, there are soybean varieties tolerant to drought stress, namely Dering 1, Dering 2, and Dering 3 (**Table 7**).

Variety	Seed coat color	100-seed weight (g)	Protein (% dw)	Fat (% dw)	Potential yield (t/ha)	Specific characters	Year of release
Gepak Kuning	Yellow	8.3–10.3	35.4–41.1	13.4–15.1	2.9	Adaptive in irrigated lowland and upland, both in rainy and dry seasons	2008
Dering 1	Yellow	10.7	34.2	17.1	2.8	Drought tolerant; adaptive in irrigated lowland and dry land (upland)	2012
Dering 2	Light yellow	14.8	35.9	19.7	3.3	Drought tolerant during the reproductive phase	2019
Dering 3	Light yellow	13.9	40.5	17.5	3.0	Drought tolerant during the reproductive phase	2019
Gema	Light yellow	11.3–11.9	37.8–39.1	15.6–19.1	3.1	Adaptive in irrigated lowland and dryland (upland)	2011
Dena 1	Yellow	14.3	36.7	18.8	2.9	Tolerant up to 50% crop-shading	2014
Dena 2	Yellow	13.0	36.5	18.2	2.8	Highly tolerant up to 50% crop-shading	2014
Demas 1	Yellow	13.0	36.1	19.9	2.5	Adaptive in a dryland with acidic soil; good planted at the altitude of 0–600 m asl	2014
Demas 2	Light yellow	14.9	37.5	19.7	3.3	Adaptive in dryland with acidic soil; early maturity; large-seed size	2019
Demas 3	Light yellow	14.4	37.2	17.7	2.9	Adaptive in dryland with acidic soil; early maturity; large-seed size; break-pods tolerant	2019
Devon 1	Yellow	14.3	34.8	17.3	3.1	High isoflavone content (2219.7 μg/g)	2015
Devon 2	Yellow	17.0	37.9	18.8	2.9	High isoflavone content (303.7 μg/g)	2017
Anjasmoro	Yellow	14.8–15.3	41.8–42.1	17.2–18.6	2.3	Broadly adaptive in all land conditions	2001
Panderman	Light yellow	18.0–19.0	36.9	17.7	2.4	—	2003
Grobogan	Yellow	18.0	43.9	18.4	3.4	Broadly adaptive in all land conditions, particularly irrigated lowland	2008
Burangrang	Yellow	20.0	39.0–41.6	14.9–17.0	2.5	—	1999
Argomulyo	Yellow	19.3–20.8	37.0–40.2	18.0–19.0	2.0	—	1998
Dega 1	Yellow	22.9	37.8	17.3	3.8	Adaptive in irrigated lowland	2016

Variety	Seed coat color	100-seed weight (g)	Protein (% dw)	Fat (% dw)	Potential yield (t/ha)	Specific characters	Year of release
Detap 1	Yellow	15.4	40.1	16.2	3.6	Resistant to leaf rust	2017
Deja 1	Yellow	12.9	39.6	17.3	2.9	Highly tolerant to water saturation stress	2017
Deja 2	Yellow	14.8	37.9	17.2	2.8	Tolerant to water saturation stress	2017
Depas 1	Yellow	11.9	39.8	19.5	2.8	Adaptive in tidal land type C; good planted at the altitude of 0–600 m asl	2020
Depas 2	Yellow	11.4	39.7	19.2	2.9	Adaptive in tidal land type C; good planted at the altitude of 0–600 m asl	2020
Denasa 1	Yellow	18.1	36.4	19.6	3.4	Highly tolerant up to 50% crop-shading	2021
Denasa 2	Light yellow	18.6	34.1	20.6	3.4	Tolerant up to 50% crop-shading	2021
Biosoy 1	Yellow	21.7	39.7	18.4	3.3	Gamma irradiated soybean	2018
Biosoy 2	Yellow	22.4	40.5	20.1	3.6	Gamma irradiated soybean	2018
Mutiara 1	Yellow	23.2	37.7	13.8	4.1	High production in irrigated lowland; adaptive in irrigated lowland and dryland (upland)	2010
Mallika	Black	9.0–10.0	37.0	20.0	2.9	Well adaptive in low land and high land; in rainy and dry season	2007
Detam 1	Black	14.8	45.4	13.1	3.5	High protein, suitable for soy sauce	2008
Detam 2	Black	13.5	45.6	14.8	3.0	High protein, moderate drought tolerant, suitable for soy sauce	2008
Detam 3 Prida	Black	11.8	36.4	18.7	3.2	Moderate drought tolerant; early maturity	2013
Detam 4 Prida	Black	11.0	40.3	19.7	2.9	Drought tolerant; early maturity	2013

Note: db = dry basis.

Table 7.
Physicochemical composition and specific characteristic of Indonesia soybean varieties [12, 22, 23].

5.3 Acidic soil

As discussed previously, acidic soils are the least favorable condition for soybean cultivation, therefore the use of ameliorants and high doses of inorganic fertilizers is essential in terms of increasing productivity. The application of 23 kg/ha N + 27 kg/ha P_2O_5+ 30 kg/ha K_2O + 1,500 kg/ha organic fertilizers and

rhizobium biofertilizer 0.25 kg/50 g seeds in acidic soil with a pH of 5.30 and Al saturation of 30% exhibits a good growing performance of four soybean varieties, namely Anjasmoro, Panderman, Dega 1, and Demas 1 [24]. These varieties give a yield of 2.52 t, 2.29 t, 2.72 t, and 1.78 t per hectare, respectively. Demas 1, Demas 2, and Demas 3 varieties are tolerant to acid soil with a potential yield ranging from 2.5 t up to 3.3 t/ha (**Table 7**). Biofertilizers also have a significant role in increasing soybean yield through the natural processes of nitrogen fixation, solubilizing phosphorus, stimulating plant growth, improving soil texture, pH, and other soil properties [25, 26].

In the acidic soil of Banten with a pH of 5.5, the use of 200 g/ha of biofertilizer could substitute 50% of the recommended inorganic fertilizer [27]. Another study in acidic soil in Lampung reported that the use of Rhizobium biofertilizer tolerant to acidic soil about 1.5 t/ha and organic fertilizer enriched with P and Ca, could replace the use of 100% N and P, and 50% of K. The yield also increased more than 50% relative to control and gave higher yield compared to recommended NPK dosage [28]. The performance of soybean crops grown in acidic soil is presented in **Figure 3**.

5.4 Tidal swampland

In tidal swampland, water-saturated roots, high pyrite, the toxicity of Al, Fe, and Mn, deficiencies of N, P, K, Ca, and Mg are the major constraints in soybean development [8, 10]. Among such limitations, low soil pH and high Al saturation are more concerned regarding soybean growth as they may cause a decrease in nitrogen fixation and nutrient uptake, particularly phosphorus which is important for cell growth and photosynthesis. It was reported that liming can improve the growth and yield of soybean in the tidal swampland of South Kalimantan [10]. The highest yield was obtained at a rate of liming equivalent to 10% of Al saturation, which was applied by mixing the lime with soil up to 20 cm depth. Another study in tidal swampland of South Kalimantan investigated that using dolomite to decrease the Al-dd saturation by 20% by using organic fertilizers (1.25 t/ha), application of bio-fertilizer (0.25 kg/50 kg seeds), and inorganic fertilizer (23 kg/ha N, 27 kg/ha P_2O_5 and 30 kg/ha K_2O) gave the yield about 2.0 t/ha [24].

In addition, soil water management can be applied to reduce the pyrite content as the soil is in a reductive condition [29]. The response to water-saturated conditions varied among soybean varieties. Tanggamus and Anjasmoro, the yellow-

Figure 3.
The performance of soybean crop at 40 days after planting in the acidic soil in Lampung, Indonesia.

seeded soybean are classified as adaptive varieties, while the black-seeded soybean varieties, such as Cikuray, Ceneng, and Lokal Malang are less adaptive when grown under the saturated condition in tidal swampland. However, using the technology called water-saturated soybean farming [30], which consisted of appropriate application of Ca (dolomit) and NPK fertilizers with optimal plant population, the yield of soybean cultivation in tidal swampland in South Sumatera could reach 3.2–3.5 t/ha. There are some soybean varieties adapted to tidal swampland, namely Depas 1 and Depas 2 (**Table 7**).

A study on soybean cultivation in tidal swampland of South Kalimantan [22] also reported that the use of technological package (listed as an alternative technology in **Table 8**) consisting of the application of dolomite until soil Al saturation is reduced to 30%, NPK fertilizer with a dosage of 23 kg/ha N + 27 kg/ha P_2O_5 + 30 kg/ha K_2O + 1,500 kg/ha organic fertilizers, and rhizobium inoculant of 0.25 kg/50 kg seed as well as the saturated soil culture (SSC) technology was able to increase the number of filled pods per plant and yield per hectare relative to farmer's existing technology. Using the SSC and alternative technology packages, the seed yield increased by 27% and 17%, respectively compared to that of farmers' existing technology (**Table 8**). The performance of soybean crops treated with an alternative technology is presented in **Figure 4**.

5.5 Shaded land

In addition to several types of agroecosystem as described previously, growing soybean under shading is also potential for soybean development. Shaded land is available under young high state crop plantations, such as teak, palm oil, and

Technological package	Number of filled pods/plant	100 seeds weight (g)	Seed yield (t/ha)	Increased yield (%)
Existing	30.70 b	15.52 a	2.067 a	100
SSC	34.55 ab	15.40 a	2.422 b	117
Alternative	40.80 a	15.45 a	2.625 c	127

Note: The values followed by the same letter do not differ at the 5% DMRT level. SSC = Saturated Soil Culture.

Table 8.
Number of filled pods, 100-seed weight, and soybean seed yield obtained from the application of different technological packages in tidal swampland. Wanaraya District, Barito Kuala Regency, South Kalimantan [24].

Figure 4.
An example of the performance of 40 days after planting of soybean crops in tidal swamps with soil Al saturation of 30% in South Kalimantan Province, Indonesia.

eucalyptus trees. The land associated with teak and eucalyptus trees is generally under the management of State Company, namely Perhutani where the lands/areas are managed by the local community (FACI/Forest Area Community Institution), while the land planted with palm oil crops belongs to the Government. However, there is no accurate data regarding the potential shaded land that can be used for soybean development. This includes the dry land agroecology with flat or hilly topography. Therefore, soybean planting in this agroecology can be only done in the beginning of the rainy season.

The yield of soybean grown under the shading of four to six-year-old of palm oil tree (50% shading) was relatively lower (0.54 t/ha) than that of without shading (2.6 t/ha). Burangrang, Anjasmoro, and Grobogan varieties show similar tolerance to such shading. The recommended N fertilizer application is 100–150 kg/ha [31]. In another study, the application of 34.5 kg/ha N + 36 kg/ha P_2O_5 + 60 kg/ha K_2O + 20 t/ha manure and planting space of 20 cm × 20 cm using three soybean varieties (Dena 1, Anjasmoro, and Grobogan) were able to produce seeds of about 1.8 t/ha at 25% shading level and about 1.4 t/ha at 50% shading level [32]. In particular, Dena 1, Dena 2, Denasa 1, and Denasa 2 varieties are released for shading cultivation of soybean (**Table 7**).

Components of performance	Soybean variety				
	Dega 1[1]	Dena 1[1]	Anjasmoro[1]	Argomulyo[1]	Local[2]
Average of productivity (t/ha)	1.35	1.10	1.05	0.99	0.63
a. Production input (IDR/ha)	3,844,000	3,844,000	3,844,000	3,844,000	3,844,000
b. Labor (IDR/ha)	1,350,000	1,350,000	1,350,000	1,350,000	1,350,000
Total production cost (IDR/ha)	5,194,000	5,194,000	5,194,000	5,194,000	5,194,000
Total revenue* (IDR/ha)	9,450,000	7,700,000	7,350,000	6,930,000	4,410,000
Total income (IDR/ha)	4,256,000	2,506,000	2,156,000	1,736,000	(784,000)
R/C ratio	1.8	1.5	1.4	1.3	0.7
B/C ratio	0.8	0.5	0.4	0.3	

Note:
[1]*Planting spacing was 40 cm × 15 cm (technology of Iletri).*
[2]*Planting spacing was 20 cm × 20 cm (existing technology).*
**Revenue = the average of yield multiplied by the selling price of soybean seeds i.e. IDR 7,000/kg. Figure in the bracket showed total income was minus or soybean farming lost.*

Table 9.
Farming income of soybean farming under teak shade, Blora Regency, Central Java, 2018 [33].

Figure 5.
Soybean grown under the teak stands (left) and eucalyptus trees (right) in Blora, Central Java.

In terms of soybean grown under the two-year-old teak tree in Blora, Central Java, using the technological package of NPK fertilization (30 kg/ha N+ 66 kg/ha P_2O_5 + 30 kg K_2O), biofertilizer (20 g/10 kg of seed), "legowo" planting space (30 cm–50 cm × 15 cm) or regular planting space (40 cm × 15 cm), gave a yield about 1.5 t/ha. Meanwhile, using the existing technology (farmer's method), only 0.75 t/ha of seeds was obtained (**Table 9**) [33]. Soybean grown under the young teak stands and eucalyptus trees is presented in **Figure 5**.

6. Challenges and opportunities to achieve soybean self-sufficiency

6.1 Challenges

There are three primary challenges in terms of increasing the soybean production in Indonesia in order to achieve self-sufficiency, i.e. low fertility of the available land, less competition of existing soybean varieties in terms of the quality traits, and relatively low selling price of locally produced soybean.

Java Island is the most fertile and largest planted area of soybean in Indonesia. Shifting the soybean planting area to outside of Java has been started since the 1980s. The available land for crop cultivation in such areas, including soybean, is more than 40 million hectares, however, the major soil type is ultisol. This mostly exists in Sumatra, Bali, Kalimantan, Sulawesi, and Papua. Constraints, like acidity, low content of organic matter, and phosphorus (P) availability naturally occurred in ultisol soil, thus more inputs are needed to provide optimal conditions for producing soybean [34].

Quality traits of local or domestic soybean are also important to drive or push the production of soybean in Indonesia. However, there is a limited quality trait of local soybean to compete with imported soybean. Previously, the improved soybean varieties belonged to small and medium-seeded, which is not desired for tempeh ingredients. Large-seeded (> 14 g/100 seeds) is favored for tempeh preparation as it would give a good appearance and high volume development, while small to large seed sizes are suitable for tofu making [22]. Therefore, for the last two decades, a number of improved varieties with large seed sizes have been released (**Table 7**) to meet such preferences. However, the released varieties concerning health benefits, such as Devon 1 and Devon 2 with high isoflavone content (**Table 7**) that has antioxidant activity, have not been attractive for consumers and farmers based on this superiority or character as the market is not yet available. Therefore, lack of market quality traits is also an essential challenge for producing local soybean.

In the case of price, the imported soybean always has a lower price than the local soybean. It is calculated [35] that the profitable price for farmers is minimally IDR 9,000 per kg or US$ 0.6/kg (US$ 1 = IDR 14,000). With this selling price, farmers would be able to cover the expenses for soybean production activity and gain some profit. However, the price of local soybean at the farm level is frequently around IDR 6,500 per kg, causing less interest of farmers to grow soybean. Therefore, the current average soybean productivity at the farm level (1.5 t/ha) needs to be increased to at least 3.0 t/ha, thus soybean farming income can compete with those of other commodities, such as maize as presented in **Table 10**.

6.2 Opportunities

Indonesia has a good chance to increase soybean production and fulfills domestic needs. This opportunity can be seen from the market demand, land and improved varieties availability, and the Government's strong will. Soybean demand as food

Parameter	Commodity farming		
	Maize	**Soybean (Farmer technology)**	**Soybean (Improved technology)**
Productivity (t/ha)	5,648	1,873	3,060
Selling price (IDR/kg)	4,000	6,500	6,500
Revenue (IDR/ha)	22,592,000	12,174,500	19,890,000
Production cost (IDR/ha)	9,737,000	6,800,200	7,542,000
Profit (IDR/ha)	12,855,000	5,372,500	12,348,000
B/C	1.32	0.79	1.64

Table 10.
Income of maize farming compared to soybean farming using existing farmer technology and improved technology [9].

and feed increases continuously and be expected to increase in the next years. The highest portion of demand comes from processed food mainly tempeh and tofu. Another high demand is coming from the cattle feed industry which is expected to increase continuously as part of increasing cattle production. Therefore, by increasing the national soybean production, the Government wants to fulfill these demands by using national production and reducing imports [36].

Other potential opportunities are the availability of source seeds, especially in the form of "Breeder Seeds" for the production of certified seed of "Foundation Seeds", "Stock Seeds", and "Extension Seeds" to fulfill the need for quality soybean seed for the area of production. The "Breeder Seeds" available are various soybean varieties with a various specific traits, including the variety tolerance to pod borer and pod sucking insect, shading, flooding, and drought. The readiness of soybean production technology for various agroecosystems can also be stated as an opportunity because those significantly contribute to the high productivity and also for the production of soybean in the country.

7. Conclusion

Soybean in Indonesia is the third important staple food after rice and maize. The need for this commodity continuously increases every year due to the increase in population. The trend of domestic soybean production tended to decline and do not meet the demand leading to the increase of soybean import every year. There are three challenges that require drastic changes so that local soybean production is able to meet domestic needs. First, the current productivity at the farm level, which is around 1.5 t/ha must be increased to at least 2.0–3.0 t/ha. It will also help soybean farming income compete with those of other commodities. Second, the soybean harvested area which only reaches 0.3 million hectares in 2019 must be increased at least become 1.7 million hectares. The potential soybean planting areas in Indonesia are the optimal land including irrigated lowland and rainfed after paddy (rice), as well as suboptimal lands such as dryland, acidic land, tidal land, and shaded land under young plantation crops. Soybean productivity in those kinds of agroecosystems can reach 1.8–3.0 t/ha, depending on the type of land, the improved varieties used, and the applied of cultivation technological package. Third, it is necessary to develop agricultural machinery that can reduce the farming cost, so that soybean farming is more efficient and able to provide higher profit.

Some efforts should be made to increase national soybean production to achieve self-sufficiency, including improving the attractiveness point of soybean farming, launching the program(s) to increase soybean production starting from the central government to the regions, accelerating technology transfer dan adoption of the high yielding improved varieties, reducing soybean import gradually, improving the cooperation among stakeholders, and providing a good market guarantee for soybean farming.

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Conflicts of interest

We declare that we have no conflicts of interest on the entire manuscript.

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Chapter 12

Enzymatic Process for Pigeon Pea

Mukesh Nathalal Dabhi

Abstract

Pigeon pea is generally used as a dhal i.e., in split form therefore, it is important to check its splitting i.e., hulling efficiency and maximum dhal recovery. Pre-treatments are commonly given for loosening and removing of the seed coat with retaining its edible portion. Enzymes viz. xylanase, pectinase and cellulose were applied to evaluate the dehusking properties of pigeon pea grains. The effect of four enzymatic parameters, i.e., enzyme concentration (20–50 mg 100 g^{-1} dry matter), incubation time (4–12 h), incubation temperature (35–55°C) and tempering water pH (4.0–6.0) on dehusking efficiency were optimized with statistical package response surface methodology (RSM). In which the hulling efficiency with a high value for the coefficient of determination R^2 (0.92) described satisfactorily quadratic model. It predicted 76.54–82.80% hulling efficiency, 20.70–25.30% protein content and 12.42–15.10 min cooking time at optimized enzyme concentration of 27.64–31.34%, incubation time 7–9 h, incubation temperature 43–45°C and 5–6 pH value for different varieties of pigeon pea as compared to 66.00–78.30% hulling efficiency, 18.74–21.81% protein content and 13.23–18.00 min cooking time for traditional oil treatment. It shown that increased hulling efficiency and protein content and decreased cooking time for enzyme pretreated pigeon pea compared to the oil pretreated method.

Keywords: pigeon pea, enzyme, grains

1. Introduction

Pulses are mostly consumed as a dhal, it is important to dehusk and then split into two parts. Pigeon pea is very hard to dehusk hence pre-treatment is essential before milling practice. Pre milling treatments are commonly carried out to loosen the seed coat to eliminate husk without dropping any fit for human consumption element and higher dhal recovery. Pigeon pea is commonly processed to mend their cooking and nutritional traits. Dehusking of pigeon pea also aids to get rid of antinutritional compounds which include polyphenols observed in the seed coat. Pretreatment for loosening of the seed coat from the grain is one of the essential stages in dehulling of pigeon pea. This process is usually completed by way of the use of mechanical means. Grain pretreatment is commonly intended to harden the hull and slacken the gummy bond between the hull and the cotyledon and to strengthen the cotyledon to lessen damage. Removal of the seed coat at some stage in dehusking is conventionally completed either through moist or dry methods [1]. Pretreatments may additionally include thermal treatment only or soaking in water, chemical solutions, etc. [2–5]. These treatments results shape deformation of split or poor cooking quality of splits. These treatments needs more labour and consume more time.

Several preceding research pronounced that the husk of grain adhered to the cotyledons due to the presence of calactomonus disaccharide, glucoronai acid and glycol protein [6]. Swamy et al. [7] reported that for adherence of husk to the cotyledons, arabinogalactan type polysaccharide is responsible, which possess the gummy and hygroscopic nature. Those complicated biological compound makes the removal of seed coat of pigeon pea a tough technique. Hence, making of dhal without pretreatment consequences in low dhal availability. Saxena [8] suggested that pre-treatments has an essential function in increasing dhal recuperation by means of slackening seed coat from cotyledons. Consequently Phirke and Bhole [3] advised specific pretreatments viz., water soaking, water spray with oil treatment, sodium bicarbonate treatment and enzyme treatment except sodium bicarbonate treatment induced widespread loss in protein content of cotyledons over untreated samples. Saxena [8] said that the outcomes of seed coat elimination by chemical treatment of pigeon pea grain through the usage of calcium hydroxide, sodium hydroxide and sodium bicarbonate aqueous solutions was observed and among them sodium bicarbonate solution turned into the very much result of dhal availability. Sharanagouda et al. [9] suggested the use of mustard oil treatment for Gulyal variety to get higher unhulled grains during milling (79.4%) and dhal (68.8%) in comparison of Maruti and Asha variety. Whereas Maruti (76.5%) and Asha (56.9%) variety resulted higher unhulled grains by acetic acid treatment. 'Sirka' may be utilized instead of oil for pigeon pea milling [10]. Dhal availability in this procedure became extra or less identical as in case of oil treatment.

It was reported that pigeon pea is tough-to remove seed coat because of the existence of mucilage and gum bring into being a sturdy bond among the seed coat and cotyledons. The mucilage and gums exist in between the husk and cotyledons show an essential function within the removal process of seed coat of pigeon pea due to its chemical nature [4]. Cosgrove [11] observed that mucilages and gums of pigeon pea grains are complex of cellulosic micro fibrils fixed in a medium of non-starch polysaccharides (NSP) and proteins. Through the enzymatic reactions, fractional hydrolysis of those NSP and/or proteins also enable the easy removal of seed coat of pulses [12, 13]. Sreerama et al. [14] mentioned enzyme treatment better than thermal treatment as xylanase intervened degradation of cell wall polysaccharides of horse gram bring about in enlargement of the grain with stepped forward nutritional and functional properties. Sreerama et al. [15] reported protease or sodium bicarbonate pre-treatments develop the physical and enlargement properties of pigeon pea and horse gram.

Reddy et al. [16] studied the protein deposition pattern in pigeon pea seed and reported that the outer layers of the cotyledons are richer in protein in evaluation to inner layers of seed. From vitamins point of view, that is a considerable that dehulling no longer reduces protein-rich germ but additionally the outer layers of the cotyledons wherein distinctly extra protein components are covered. Fortuitously, the protein high-quality in phrases of amino acids is not adversely laid low with removal of seed coat. Singh and Jambunathan [17] similarly pronounced that removal of seed coat process also reduces about 20% calcium and 30% iron. To maintain the beneficial value of pigeon pea seed and minimizing the nutrient losses for the process of dehulling it is essential that extra effective dehulling process is developed and transferred to rural areas wherein through and large milling continues to be executed with the aid of inefficient old-age strategies. In line with Kurien [1] in control situations the dhal recovery obtains the most efficiently up to 80–84% however at industrial the recovery stays round 70%. It was mentioned the reason of different variety (72–82%) for dhal yield. Consequently, it could be expected that for a mixture of a different variety and a competent pigeon pea process, there is possibility to reduce the nutrient losses.

Enzyme pre-treatment resulted 13.81% higher recovery of dhal compared to oil treatment for pigeon pea [18]. Murumkar et al. [19] reported the dhal recovery

(76.60%) and milling efficiency (96.19%) with optimized enzymatic hydrolysis parameters. Enzymes makes the possibility of the fractional disruption/degradation of non-starch polysaccharides and/or proteins of mucilage that is found at interface between hulls and cotyledon. Green gram and black gram pretreated with protease resulted better yield of dehulled grain. In case of horse gram xylanase pre-treatment was very powerful in improving the dehulling process as compared to protease. Whereas for red grain, protease pre-treatment produced greater dehulled than xylanase. It is also evident that the enzyme dehulling pre-treatments no longer only expanded the dehulling performance, however additionally decreased the quantity of powder and fines [20]. Enzyme dispensed with object grains have been observed to make reduction of dehusking time as compared to water treated grains utilized in traditional milling. The enzyme treated grains were resulted to be brighter in contrast to untreated grains. Additionally, there have been adjustments found in the quantity of damaged grains and powder formation i.e., after processing of the grains, the powder formation and wide variety of broken grains decreased extensively which bolsters the overall purpose for application of enzymes for dehusking [21].

Pre milling treatments are commonly employed to loosen the seed coat to dispose of husk without losing any suitable for eating portion. There are many milling strategies like wet milling, dry milling, Central Food Technological Research Institute (CFTRI) technique, Pantnagar method, Central Institute of Agricultural Engineering (CIAE) method and Indian Institute of Pulse Research (IIPR) method advanced for pigeon pea milling. The above stated techniques are time ingesting, require almost four to seven days for the entire milling of pigeon pea. Also, the survey work of few pulse mills in Gujarat revealed that the dry milling treatments achieved at some stage in the milling for pigeon pea take longer processing time, approximately seven to eight days depending upon climate as sun drying is needed to get agreeable milling after pre-treatment [22]. But, these kinds of techniques do not allow easy elimination of seed coat in the course of the following processing operation of pigeon pea milling. Furthermore, those pre-treatments cause enhanced processing charge, longer processing time and labour consuming for pigeon pea milling. It was revealed that the exquisite potentiality of technology up gradation exists to get higher recovery of dhal in addition to lessening in processing time and energy required [22].

This necessitated the proper pre-treatment for pigeon pea milling which could shorten the processing time and improve the product value. The charge for the milled product is fixed on the idea of number of grains with intact husk (in part or entirely) in the pattern, chipping of edges of the cotyledons, volume of floor scouring of the grain, and the variety of the pigeon pea. Dhal with a lesser or no husk, natural luster, yellow in coloration and sharp edges of break up cotyledons, can be sold in the market at a better price.

It is far important to have unique pre-treatment to dissolve the glue that binds the cotyledons of pigeon pea grains to the seed coat. It is almost obvious that de-hulling quality is particularly depending on physical quality of grains and pre-treatments. Selection of pre milling treatment also relies upon on the characteristics of the grain. In addition, pre-treatments given to pigeon pea grains earlier than de-hulling considerably influence the cooking time. The cooking quality of pigeon pea is essentially assessed with the aid of its cooking time [23].

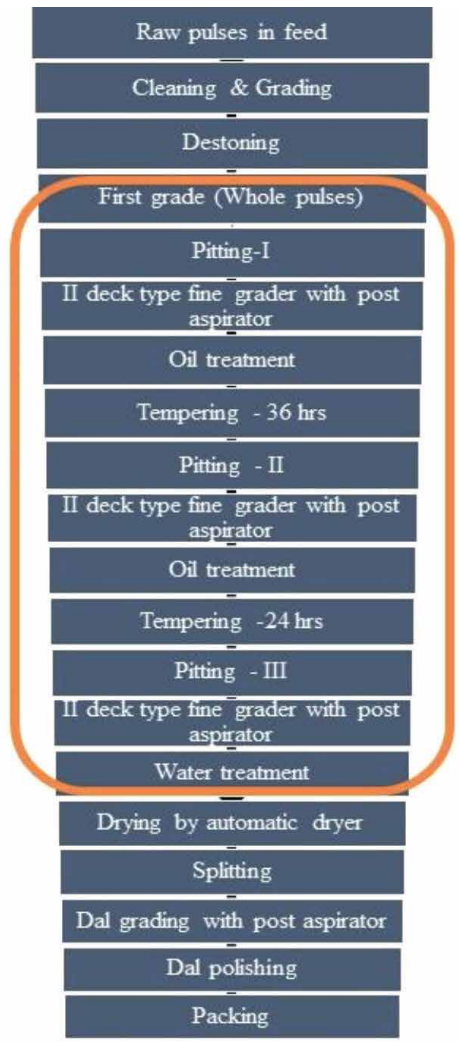
The mechanism of enzymatic activity is governed by using four interacting parameters, i.e., grain moisture content material, enzyme concentration, reaction time and incubation temperature [24]. Foremost ranges of those parameters are necessary to get most recovery and higher quality of dhal. Facts on the effect of above parameters on de-hulled fractions and cooking high-quality seems to be missing. Several reviews are to be had for food grade activities of enzymes i.e., xylanase and cellulase as husk loosening agent in many grains. By way of this reaction of

enzymatic treatments lesser force will be required to result in the de-husking and thereby lower in processing time and cost.

Chemical composition and binding material at the interface of seed coat and cotyledon decides the choice of enzymes. Saxena and Srivastava [25] suggested that bio-bleaching agent for lignin isolation is the xylanase. Cellulose to beta-glucose and pectin to pectic acid converted by cellulase and pectinase, respectively. Consequently, xylanase, cellulase, and pectinase are the important enzymes that ruin down the binding factors that lead to multiplied efficacy.

2. Material and methods

Preliminary trials are essential to achieve standard proportions of enzymes, i.e., xylanase: pectinase: cellulase to get the most out of the husk removal. The outcome of selected enzyme combination on husk removal of pigeon pea grain is to be assessed keeping the enzyme concentration, incubation time, incubation



Process flow chart for traditional method for milling of pigeon pea

Process flow chart for milling of pigeon pea with enzyme pre-treatment

temperature and tempering water pH constant based on the technical specifications of the products delivered by manufacturer.

Following equations are to be used to calculate husk removal and hulling efficiency [26].

$$Husk\ removed\ (HR)\% = \frac{Husk\ Removed\ during\ dehusking}{Total\ husk\ content} \times 100 \tag{1}$$

$$Coefficient\ of\ hulling\ (Ch) = 1 - \frac{Weight\ of\ unhulled\ grain\ after\ milling}{Weight\ of\ unhulled\ grain\ used\ for\ milling} \tag{2}$$

$$Coefficient\ of\ wholeness\ of\ kernel\ (Cwk) = \frac{W_f}{W_f + W_b + W_p} \tag{3}$$

where W_f = weight of finished product; W_b = weight of brokens; W_p = weight of powder.

$$Hulling\ efficiency = Ch \times Cwk \times 100 \tag{4}$$

3. Enzymatic pre-treatment

The enzyme solutions are to be made with the standardized percentage of all three decided enzymes. On this enzymatic pre-treatment method, the degumming is probably because of the reaction of different enzymes used for pre-treatment, i.e., xylanase, pectinase and cellulase. Because the enzyme activities relies upon on temperature, pH and incubation duration, crucial parameters at the side of the enzyme proportions, temperature, pH and incubation duration is to be taken into consideration.

4. Dehulled sample separation

The dissimilar fractions of the milled product which include whole dehulled grains, divided dehulled grains, in part dehulled and unhulled grains, broken, husk and powder are to be separated using suitable sieves (BS sieves no. 4, 6, 18). A grain is to be taken into consideration completely dehulled whilst there has been no husk adhering to it.

5. Cooking time

Pigeon pea dhal are to be cooked in a stainless steel pan having a ratio of dhal: distilled water as 1:10. For observation of cooking time, throughout boiling, the level of water is to be maintained by means of regular addition of boiled water. Boiling is to be persisted and grains to be drawn at 1 min interval to test the cooking time by way of pressing between the thumb and the forefinger till no hard core is left as described by way of [23]. Full cooking time is to be documented as the time while ninety percent of the dhal became gentle sufficient to masticate [27].

In an experiment the observation of different enzyme pretreatment were recorded. The best combination of enzyme concentration, incubation temperature, incubation time and pH were selected with respect to hulling efficiency, cooking time and protein content.

The statistical analysis was carried out of experimental data and the significant effect of enzyme concentration, incubation temperature, incubation period and tempering water pH along with their interactions on hulling efficiency, cooking time and protein content were calculated.

6. Results and discussion

6.1 Effect of enzyme pretreatment parameters on hulling efficiency

The enzymatic pre-treatment for pigeon pea process resulted hulling efficiency in the range of 76.90–82.80% which was higher than dry milling method which was in the range of 66–78.30%. This is due the effects of incubation temperature on hulling efficiency ($p < 0.001$). This finding was confirmed by [18, 19]. Hulling efficiency was also significantly affected by tempering water pH. Sangani et al. [18] additionally mentioned effect of pH on hulling efficiency. Hulling efficiency was significantly affected by enzyme concentration [19–21] but [18] observed the non-significant effect of enzyme concentration on hulling efficiency. Outcomes of incubation time have been determined large effect on hulling efficiency ($p < 0.01$) [18, 19]. Opoku et al. [28] marked tempering is vital for reaching better dehulling results after soaking and drying or steaming and drying.

6.2 Effect of enzyme pretreatment parameters on protein content

The enzymatic treatment for pigeon pea process resulted protein content in the range of 20.70–25.30% which was higher than dry milling method which was in the range of 18.74–21.81%. Singh and Jambunathan [17] reported that dehulling process resulted scarification of outer layers of cotyledons and hence 12% yield loss as powder fraction. The outer surface of cotyledons is an affluent supply of protein, sugar, fiber, and ash but scanty in starch. Protein content of dhal by enzymatic pre-treatment was affected by enzyme concentration, incubation period and pH. However, outcomes of incubation temperature had significant effect on protein content ($p < 0.01$). Chandini et al. [21] also reported that crude protein in pigeon pea was affected by higher soaking time. This may because crude protein possess hydrophilic property which could have leached out while soaking in water. Murumkar et al. [19] reported enzyme pre-treatment to pigeon pea increased 2.96% protein content. Tiwari et al. [29] also reported increases of the protein content due to pre-treatment. The pectinase having high polygalacturonase activity was the most effective preparation in terms of protein release. Rommi et al. [30] reported enzymatic carbohydrate hydrolysis correlated with increased protein extractability at tempering water pH 6. Das et al. [31] reported increase in proteins by cellulase pre-treatment in milled rice.

6.3 Effect of enzyme pretreatment parameters on cooking time

The enzymatic treatment for pigeon pea process resulted cooking time in the range of 12.42–15.10% which was lower than dry milling method which was in the range of 13.23–18.00%. It was reported that effects of enzyme concentration, incubation time and tempering water pH had significant effect on cooking time ($p < 0.001$). However,

results of incubation temperature changed into non-significant impact on cooking time. Sangani et al. [32] showed the significant effect ($p < 0.05$) of enzyme concentration and tempering water pH, and they observed highly significant effect ($p < 0.01$) of incubation time. He also determined non-significant effect of all the interplay on cooking time. Bhokre and Joshi [33] also pronounced that the cooking time reduces by soaking of cowpea. Tiwari et al. [29] also mentioned the effect of conditioning on cooking time. The effect of enzyme pre-treatment on cooking time was reported for pigeon pea, chick pea and other legumes. [19, 34]. Inversely Sreerama et al. [20] was observed no noteworthy change inside the cooking times of dehulled splits for control and enzyme (xylanase and protease) pre-treated with legumes.

Thus it could be concluded that the enzymatic pre-treatment for pigeon pea process resulted higher hulling efficiency, higher protein content and lower cooking time as compared to dry milling method of pigeon pea processing. This method not only giving better recovery and quality, but it reduces the time for processing from 5 days to 1 day.

7. Conclusions

Important parameters for pigeon pea processing are hulling efficiency, protein content and cooking time requirement. It was found that traditional method of oil treatment for pigeon pea processing resulted in the range of 66.00–78.30% hulling efficiency, 18.74–21.81% protein content and 13.23–18.00 min cooking time; whereas enzymatic pretreated pigeon pea processing resulted 76.54–82.80% hulling efficiency, 20.70–25.30% protein content and 12.42–15.10 min cooking time at optimized enzyme concentration of 27.64–31.34%, incubation time 7–9 h, incubation temperature 43–45°C and 5–6 pH value. This process not only increased the hulling efficiency but it reduces the time requirement of process.

Conflict of interest

I declare that it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright holder.

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Chapter 13

Grass-Legume Seeding: A Sustainable Approach Towards Reclamation of Coalmine Degraded Lands in India

Sneha Kumari and Subodh Kumar Maiti

Abstract

Most of the ecosystem services undergo significant degradation during coal mining activities with negative impacts on ecology, biodiversity and local people's livelihoods. The cumulative effect of such large scale environmental changes is reflected in rising pollution load, earth's temperatures and deforestation. There is no eloquence to it that coal is and will continue to be the primary fossil fuel in global energy production, there is a need to embrace sustainability as a key aspect throughout all phases of mining. The cheapest, easiest and eco-friendly approach to accelerate the trajectory of ecological restoration towards a reference state is the introduction of versatile and pioneering plant life forms like grasses and legumes. These species works on basic scientific principles based on ecological theories and incorporating them in post-mined landscapes provides multitudinous environmental benefits coupled with economic and social development. Keeping this in mind the chapter aims to emphasize the importance of grass-legume seeding during ecological restoration of mine degraded lands concerned with the concepts of sustainability.

Keywords: Coal mining, Ecological restoration, Grass-legume seeding, Sustainable development

1. Introduction

In coal powered India, a paradigm shift towards mining sector for energy needs has tremendous negative repercussions in environmental and socio-economic arenas. The idea of '*more hole more coal*' without any conservative measures leaves atrocious footprints on the landscapes like abandoned quarries and discarded dumps devoid of vegetation, including plant stocks and seeds capable to re-germinate. Mining is linked to all the Sustainable Development Goals (SDGs) in many ways. A multi-objective approach towards ecological restoration of mining areas keeping with the principles of sustainable development is the need of the hour [1]. "Pioneer" plant species like grasses and legumes are cost-effective and use basic scientific principles based on ecological theories therefore, incorporating them in post-mined landscapes (**Figure 1**) has shown multitudinous environmental benefits coupled with economic and social development [2]. There is no eloquence to it that coal is and will continue to be the primary fossil fuel in global energy production,

Figure 1.
Ecologically restored coal mine dumps under Bharat Coking Coal Limited (BCCL), India showing (A) growth of grasses on the overburden dump slope near Bhowra area (B) closer view of grass (Pennisetum pedicellatum) and tree growth in the Gokul Park dump of Lodna area, (C & D) distant view of dense and diverse vegetation cover and closer view of legume (Stylosanthes hamata) growth on the Chandan opencast project dump.

there is a need to embrace sustainability as a key aspect throughout all phases of mining (**Figure 2**). Keeping this in mind the chapter aims to emphasize the importance of grass-legume seeding during ecological restoration of mine degraded lands concerned with the concepts of sustainability.

Figure 2.
Criterias for sustainable mining practices.

1.1 Current scenario of coal mining in India

The 'Coal Vision 2025' brought out by the Ministry of Coal, Government of India (GOI), has flagged coal as an essential commodity. It reports an increase in coal production from 777.7 million tonnes (MT) in 2020 to 1.2 billion tonnes (BT) in 2025. In addition data suggests that 67% of India's energy demands depend on fossil fuel, out of which coal makes up to approximately 59%. The major outcomes of the vision are:

1. The annual growth in demand for coal is expected to increase 1147 MT (7% GDP growth) and 1267 MT (8% GDP growth) till 2025.

2. The total production of domestic coal is predicted to increase to 1086 MT in 2025, out of which 83% (902 MT) will consist of open-cast production.

3. The coal vision 2025 would double the land requirement from 1,47, 000 to 2,92, 500 hectares adversely affecting 1,70, 000 families and increasing the need for rehabilitation.

4. The requirement of forest land would increase three-folds from current 15–25% of the projected total land requirement.

As per the vision outcomes and past records, the demand of coal will increase (**Table 1**) and also predicted land degradation escalating environmental complications. There is no data available on how much post-mined lands has been reclaimed in India, however the MONGABAY 2020 article on land reclamation for the year (2018–2019) states that the 52 open-cast coal mines projects of Coal India Limited (CIL) constitutes a total excavated area of 255 square kilometers (sq km) out of which 61 sq. km has been biologically reclaimed, 100 sq. km is under technical reclamation and 95 sq. km is under active mining. The National Mineral Policy (2019) which regulates mining activities in India has therefore stressed about the importance of land reclamation to bring back mined out landscapes to the pre-mining state.

Production Year	Total Coal Demand	
	Domestic production (MT)	Import (MT)
2010–2011	532	76
2011–2012	540	105
2012–2013	556	141
2013–2014	566	169
2014–2015	609	212
2015–2016	639	200
2016–2017	651	191
2017–2018	689	208
2018–2019	734	235
2019–2020	729	248
2020–2021	716	196

Table 1.
Total coal demand in India for the last 10 years (in million tons).

1.2 Multi dimensional impact of coal mining

Coal can be mined through open-cast and underground extraction methods based on the site specific geological condition [3]. An open-cast mining operation affects the ecosystem services as a whole (**Figure 3**). It involves generation of huge mass waste (overburden materials) due to mining activities like blasting, drilling etc. [4]. Coal mining is usually associated with land degradation and the excavated toxic waste materials create serious environmental and socio-economic problems in the adjoining areas. The most severe post-mining impact on the ecosystem are environmental damage such as deforestation, air and water pollution, detoriation of topsoil quality, loss of biodiversity and landscape destruction by invasive species [5–8]. Coal mining activities in Nokrek Biosphere Reserve, India adversely affected the native vegetation and greatly reduced the density of trees and shrubs [9]. The phenomenon of spontaneous heating through interconnected oxidative and thermal process affects various coal mines in the country leading to mine fires. Data estimates report that 10% of total national coal resources are in the fire affected regions. Mine fires give rise to several ecological problems besides safety hazards and economic losses [10]. Coal mining activities puts tremendous pressure on economic–socio-cultural aspects of the people residing around mine areas. Mining induced displacement and rehabilitation is accompanied by loss of social assets including income earning resources, networking, cultural identity, homes and productive land etc. [11, 12]. Coal combustion releases dangerous levels of toxic gaseous pollutants including coal bed methane and dust particles adversely affecting human health, local and global environment as well [13]. The negative effects of mining over large stretch of lands persist for years and can get the better of by relevant planning and policy making ensuring sustainable development. An ongoing challenge for the coal mining industries is sustainable development owing to rising demand for coal in the energy sector. Overcoming these challenges will require ecological resolution pertaining to technical, economic, environmental and social performances.

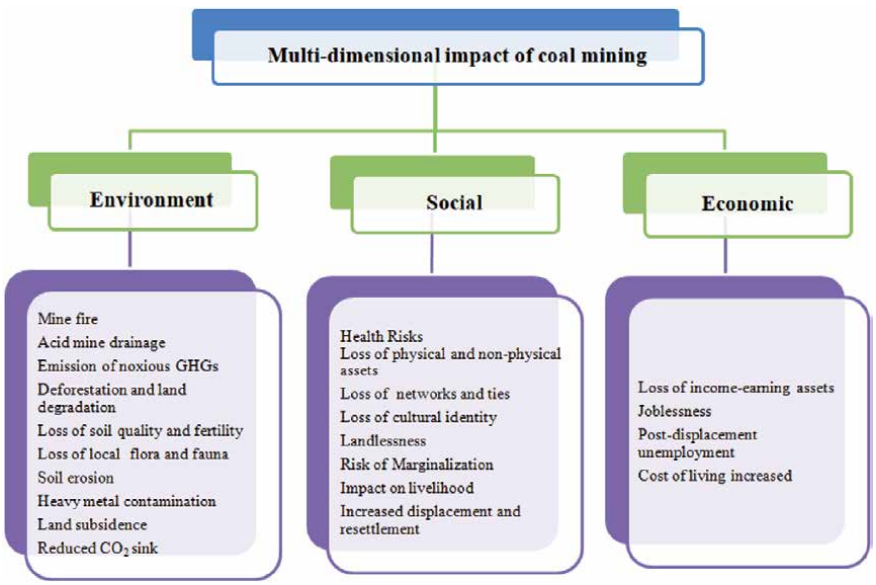

Figure 3.
Multi-dimensional impact of coal mining activities.

2. Ecological restoration

Abrupt changes in natural environment have become an indispensable part of mining activities, still mining cannot be ignored nor can environmental protection be sidelined. Therefore, a balance has to be worked out between mining and environment for sustainable development. Ecological restoration ultimately aims to attain a self-sustainable ecosystem by reconstructing ecosystem functions and structures and may be regarded as identical to secondary succession after the site recovers sustainably on its own [3]. Furthermore, following coal excavation, besides the environmental detoriation, result in a series of social and economic issues. Thus the ecological restoration in mined out lands not only means ecosystem reconstruction but should also include enhancement of environment as well as social and economic development [14]. Ecological restoration provides a solution for sustainable resource management and environmental protection in mining industry through ecological interventions [15–17]. Primary steps involved in ecological restoration are shown in **Figure 4**.

2.1 Reclamation approaches during ecological restoration

Reforestation/revegetation of barren mined out lands over time can bring it to a more or less pre-mining state. The main challenges faced during re-establishment of vegetation on hostile mine lands that has lost their upper soil horizon is finding plant species that will grow under harsh conditions. The success of reclamation depends on the adaptive potential of plant species to the highly variable and newly formed reclaimed mine soils. Surface Mining Control and Reclamation Act (SMCRA) of 1977 have recommended the use of native grass and legume species in mine degraded areas. Forage mixtures containing legumes plays an expanded role

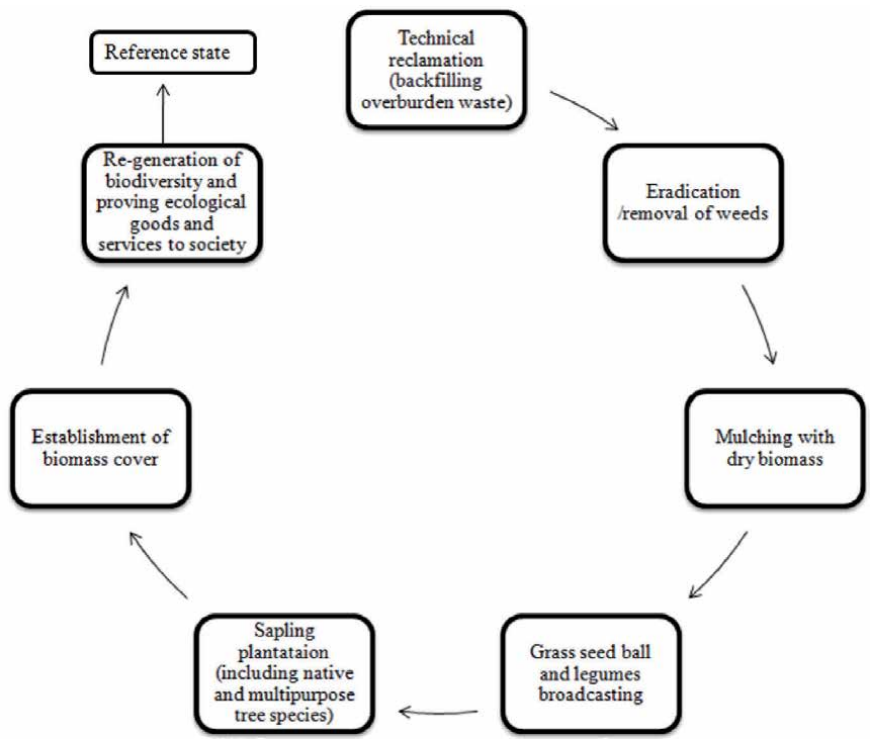

Figure 4.
Primary steps involved in ecological restoration process towards a reference state.

in the nitrogen (N) economy, lowers carbon (C) footprints and out-yield monocultures [18]. Native trees and a more species/genetic diversity accelerate the recovery to a self-sustaining ecosystem (forest) [19, 20]. The development of forest and the trajectory with which it develops on mine degraded sites depends upon geo-climatic conditions and reclamation practices. Successful and sustainable reclamation practices must focus on bringing the disturbed ecosystem back to normalcy leading to restored ecological, aesthetical, and socioeconomic functioning of the post-mining area [21]. Different reclamation approaches have been proposed for various disciplines like forestry, archeology, mining, landscape architecture etc. [22]. The reclamation approaches for mining sector has been discussed below

2.1.1 Forestry reclamation approach

The forestry approach (FRA) has been promoted as a desirable method to reclaim productive forest in coal mined land under the SMCRA act of 1977 [23]. The main features of the approach are:

1. Creating a suitable rooting medium with appropriate available material up to a depth of 4 feet for growth of deep rooted tree species. Preferred rooting medium can be topsoil, weathered rock materials etc. with pH range of 5 to 7, soluble salts less than 1 mmhos cm^{-1}, low pyritic sulfur (S) concentration, and good texture for proper drainage.

2. Excessive soil compaction due to heavy operating equipments on mine soils reduces the growth of planted trees. To re-establish productive forest post-mining, growth medium (topsoil or its substitutes) should be loosely graded to minimize compaction and favor growth.

3. Support groundcover vegetation compatible with growing trees. Groundcover species should include slow growing legumes and grasses that are tolerant to a wide range of soil conditions. This groundcover will ensure balance between erosion control and competition for resources (light, water, and space) required by trees over long-term to form a mature forest

4. Planting diverse tree species for early succession, supporting wildlife and soil improvement, over commercially valuable crop tree species.

5. Proper tree plantation techniques should be adopted. Improper planting of tree seedlings leads to poor survival rate. Tree seedlings should be dormant and stored in a cool environment away from direct sunlight until planted.

6. The revegetation method under FRA commonly used for coal mining areas involves planting bare root tree seedlings and secondly hydro seeding grass and legume seeds. The method suggests use of less competitive and tree compatible grass and legume species. This will minimize competition with the growing tree seedlings and help to establish a tree compatible ground cover. Further it also suggests using fewer amounts of seeds and N fertilizers.

2.1.2 Holistic reclamation approach

Holistic approach has been promoted by Dan Dagget in mining areas. Local environmental microclimatic conditions sometimes prevent forest succession, therefore in such areas establishment of rangelands may be a better option. A holistic approach

requires necessary knowledge of ecological (biotic and abiotic) components along with good drainage patterns. The main features of the approach are:

1. Grade the best available material according to the required topography for establishment of vegetation cover. If topsoil is not available as growth medium than topsoil substitutes can be prepared on site using early succession species like native grass biomass combined with livestock residues.

2. Propagating early succession native species like grasses and other plants

3. Mulching the area to provide the initial forage required for livestock.

4. Establishing paddocks or livestock with managed grazing techniques to heal the land by balancing production and forage use.

5. If the aim is to establish a wildlife area/natural park, as the keystone species returns via ecological succession or are introduced into the system, livestock can be reduced gradually or eliminated.

2.1.3 Integrated reclamation approach (three-tier plantation)

Several countries have opted for plantation of fast-growing exotic tree species during reclamation of post-mined areas. Such single-tier plantation is successful to provide green canopy cover but remains unsuccessful in controlling erosion, groundwater recharge and re-establishing biodiversity. Moreover, the selections of exotic species are not considered to meet socio-economic requirement of the local community. In view of all such drawbacks an integrated approach was proposed which favored plantation by three-tier method [24]. The objective is to replicate natural forest with native species and biodiversity revival as existed prior to mining. The main features of the approach are:

1. Vegetation/plantation should comprise of native species (native to nearby forest) and must consider meeting socio-economic requirement locally.

2. Mine dumps are amply invaded by invasive/exotic weeds like *Parthenium hysterophorus*, *Xanthium strumarium, Lantana camara* etc. Removal of weeds from mine degraded land creates better opportunity for the native species to germinate and re-establish biodiversity.

3. The three-tier plantation involves native species consisting of herbs and grasses (lower level), understory vegetation including shrubs/bushes (middle level) and trees (top level).

4. The lower level vegetation will provide nutrients to the soil and habitat for micro-organisms and arthropods. Overall the three-tier plantation system will improve local climate and attract flora, fauna and other organisms to re-establish biodiversity. At last completely developed forest with food chain and food web shall establish along with improved socioeconomic condition.

3. Sustainability aspects of grass and legume species

Both grasses in woody bamboo forms while legumes as shrubs and trees have their origin from the tropical forests. The grasses belong to the Gramineae family of

monocotyledons with around 780 genera and 12,000 species [25]. The fifth largest flowering plant family currently appears to be most widespread throughout the world and adapted to conditions from rain forest to dry deserts and seashores to cold mountain tops. Grasses are the most versatile and pioneering plant life forms. Grasses have greater digestible fiber compared to legumes. Their adaptability to a diverse ecosystem is due to the fact that they grow very close to soil surface therefore safe from environmental damage including grazing and fire. Grass species recommended for reclamation of coal mine degraded lands are listed in **Table 2**.

Legumes belong to the Fabaceae family that comprises almost 770 genera and more than 19,500 species. It is the third largest family of flowering plants that comprises economically important trees and shrubs adapted to a wide variety of ecological and climatic regime [27]. Research on legume nodulation started in the mid 1960 [28]. Legumes are rich in nutrient composition including crude protein, energy and micronutrients compared to grasses. Legumes contain symbiotic N-fixing bacteria (*Rhizobia*) within root nodules structures hence, a key component in crop rotation. Legumes are often referred to as "green manure" and alternating between legumes and grasses during rotational cropping produces good results by providing ample amount of N compounds [29, 30]. Legume species recommended for reclamation of coal mine degraded lands are listed in **Table 3**.

3.1 Forage production

A grass and legume mixture represents prime example of diversification and adaptation in plant community. Incorporating grasses and legumes as a forage in mine degraded lands started from the early 70's [31]. The main aim of grass-legume mixed seeding in any system is to produce higher yields and improve natural resource use efficiency than monoculture. Legumes (*Stylosanthes hamata*) and grass (*Cenchrus ciliaris*) seeding offer great potential to cope with the prominent challenge of mine reclamation to produce adequate biomass cover where no commercial N-fertilizer is applied [2]. It is generally accepted in studies that the grass species have a competitive advantage over legumes and therefore can dominate pastures. A balance between grasses and legumes is advisable to maintain high biomass productivity [33]. Grass (*Miscanthus sinensis)* and legumes (as a functional group) enhance diverse plant communities, greater biomass and less toxic forage for rapid reclamation of mine degraded lands [34]. This is because legumes improve the functioning of soil systems through bacterial symbiosis [29]. Irreversible changes due to coal mining activities threaten the economy and sustainability of local livelihood such as agriculture and livestock production [35]. Improved animal productivity is associated with the lower fiber contents and higher ruminal rates of passage which are characteristic feature of legume forages compared to grass forages [36]. Forage legumes can overcome the insufficient dietary problem that limits animal production. Grass-legume mixtures produce more forage biomass and feed with less resources therefore improving resource use efficiency in animal production. The high proportion of protein and soluble carbohydrates in legume foliage enables digestion by ruminants (herbivorous mammals). These nutritional benefits of legumes will be most evident with young and lactating ruminants, because their requirements for crude protein are higher than mature ruminants [37]. The quantity of milk produced was significantly higher in livestock's feeding on forage legume (*Stylosanthes)* supplements compared to natural pasture. Experimental results suggested that 3 kg of *Stylosanthes* dry matter (DM) was the optimal level of supplement for the milk production of 1.8 L day^{-1} [38]. Multipurpose forage legumes like *Stylo* spp. is a potential environment-friendly feed strategy to supply crude protein to grazing livestock's during drought conditions when availability of protein rich

Grass species	Distribution	Climate/Annual rainfall	Yield (t ha^{-1})	Type	Characteristic features
Brachiaria brizantha (Palisade grass)	Native to Africa	Warm and humid	GF:120	Warm season	Remains green throughout the year Compatible with legume species if adequate phosphorus concentration is maintained
Brachiaria mutica (Buffalo grass)	Native to Brazil	Warm and humid/ 900 mm.	GF: 1950–2755	Warm season	Shows rapid growth. Compatible with legume species Tolerant to saline salinity
Cenchrus ciliaris (Buffel grass) *Cenchrus setigerus* (Dhaman grass)	Native to South Africa, south Asia (east to India)	Arid and semi-arid /125 to 1250 mm	DM:6–11 GF:35–40	Warm season	Drought tolerant Suitable for soil conservation
Chloris gayana (Rhodes grass)	Native to South Africa	Warm and moist	DM:17	Warm season	Early establishment in soil Compatible with legume species Adapted to a range of soil and climatic conditions
Chrysopogon fulvus (Dhwalu grass)	Native to India and East Africa	Arid and semi-arid /250 to 850 mm	DM:4–10		Acts as good soil binder Can grow on gravel and stony soils Shows luxurious growth during summers when other grasses dry out
Cynodon dactylon (Bermuda grass)	Native to India	Semi arid/ 300 to 2000 mm	DM:4–5 GF:16	Warm season	Drought resistant Tolerant to salinity and alkalinity Controls erosion Ensures stabilization of slopes Compatible with legume species

Grass species	Distribution	Climate/Annual rainfall	Yield (t ha^{-1})	Type	Characteristic features
Digitaria decumbens (Pangloa grass)	Native to Transvaal	Humid /1015 mm	GF:7–13	Cool season	Controls erosion Compatible with legume species Insect resistant
Eragrostis curvula (Weeping love grass)	Native to India and Tanzania	Mild temperate/500 to 1000 mm	GF:20–30	Warm season	Good soil binding capacity Controls erosion Highly tolerant of soil acidity
Panicum antidotale (Sudan grass)	Native to Australia	Arid and semi-arid/100to 1000 mm	GF:20	Warm season	Suitable for pasturage Shows fast re-growth
Panicum maximum (Guinea grass)	Native to Africa	Warm and moist/ variable rainfall	GF:50–60	Warm season	Suitable for soil conservation
Paspalum notatum (Bahia grass)	Native to Brazil	warm and moist/ 1500 mm.	GF:20–40	Warm season	Good soil binder Suitable for soil conservation
Pennisetum pedicellatum (Dinanath grass)	Distributed in West Africa and India	warm climate/ 800 to 1250 mm.	GF:55–60 DM:14	Warm sseason	Suitable to grow on nutrient poor soil Very tall, robust grass Rapid growth under moist, warm conditions Useful windbreak species
Setaria sphacelata (Golden timothy grass)	Native to Africa	warm climate/1500 mm	GF:24	Warm season	Good soil binder Compatible with legume species
Vetiveria zizanoides (Vetiver grass)	Native to Asia	semi-arid /500–5000 mm.		Warm season	Tolerant to extreme drought conditions Suitable for soil conservation

Adapted from Trivedi [26]; DM = Dry matter, GF = Green forage.

Table 2.
Grass species recommended for reclamation of coal mine degraded lands.

forages is scarce. Several forage legumes also possess tannins and polyphenoloxidase (plant secondary metabolites) [39]. Tannins protect proteins degradation in the rumen, and subsequently ruminants excrete less urinary N and greater fecal N.

Legume species	Distribution	Climate/ Annual rainfall	Yield (t ha^{-1})	Type	Characteristic features
Calopogonium mucunoides (Calopo)	Native to South America	Hot humid tropical /1525 mm	GF:56	Warm season	Good Nitrogen (N) fixer Well adapted to grow in acidic soil
Centrosema pubescens (Centro)	Native to South America	Hot humid/ 1525 mm.	GF:15–20	Cool season	Good N fixer and increases soil N content Compatible with grasses like *Panicum, Pennisetum, Digitaria, Brachiaria* etc.
Stylosanthes guianensis (Stylo) *Stylosanthes hamata* (Carribean stylo) *Stylosanthes humilis* (Townsville stylo) *Stylosanthes scabra* (Shrubby stylo)	Native to Brazil	Warm humid tropical/ 500–1270 mm	GF:15–41 DM:5–10	Warm season	High quality forage Drought resistant Provide permanent vegetation cover N-fixation capability Improves soil quality by adding organic matter and N Compatible with grasses like *Cenchrus, Pennisetum* and *Chloris gyana* etc
Medicago sativa (Alfalfa)	Native to South West Asia	Temperate and tropical	GF:150 DM: 9	Cool season	Pest and insect resistance Drought and salt resistant. High N-fixation capability
Desmodium intortum (Green leaf desmodium)	Native to South America	Sub-tropical / 900 to 1275 mm	GF:19 DM:6–13	Warm season	Builds the soil organic matter Conserves soil moisture It contributes large quantity of N to soil Compatible with grasses
Desmanthus virgatus (Dashrath grass)	Native to Argentina	Hot climate/ 250 to 2000 mm	GF:15–25	Warm season	Tolerant to soil salinity Drought resistant Good N fixer
Trifolium repens (White clover) *Trifolium pretense* (Red clover)	Native to Europe	Temperate climate /750–1200 mm	DM:7–18	Warm season Cool season	Used as green manure Excellent N-fixation capability Increases soil fertility Compatible with grasses like *Lolium prenne, Cynodon dactylon, Pennisetum* etc.

Adapted from: Trivedi [26]; DM = Dry matter, GF = Green forage.

Table 3.
Leguminous species recommended for reclamation of coal mine degraded lands.

This is environmentally beneficial because it reduces the conversion of urinary N to ammonia and nitrous oxide, a potential greenhouse gas (GHGs). In addition, several studies have reported that high quality forage can also reduce enteric methane emissions, other powerful GHGs [39, 40]. Livestock grazing legume (*Medicago sativa)*-grass mixture reported 25% reduced enteric methane emissions compared to only grass pastures [41]. Adopting strategic use of grass-legume mixtures in ruminant's diet can be beneficial for health of livestock, sustainable use of resources and environment by mitigating GHGs in addition to benefits like enhanced productivity and reducing shift towards N fertilizer. The linkage between mining and engagement of local communities in mining activities is not only complex but also contentious. However, legume inclusive mining systems can turn in line with sustainability principles at food, animal, human and environmental level.

3.2 Soil fertility

Grass-legume mixture is widely accepted for restoration of coal mine dumps (**Table 4**). Grass-legume mulch residues act as soil conditioner to enhance soil physical properties via moisture conservation, reducing soil erosion and moderating soil temperature. The branching fibrous roots of grasses lowers the bulk density of compacted mine soil which accelerates the recovery of soils physical conditions at surface 10 cm depth [48]. Under drought stress conditions, root length and root area of grasses are more than legumes at the 30–60 cm depth of soil, therefore grass-legume mixture having different water use strategies can be opted for restoration of fragile areas [49]. The aggressive taproot system of legume species penetrates to a depth of 6–8 feet into soil. The N rich high protein legume residues stimulates earthworm burrows which in turn increases soil porosity, movement of air and water to deeper soil depths. Furthermore, legumes have extended value because they are naturally high quality forage that could enhance the quality and productivity of associating species specially grasses by biologically fixing atmospheric N [50]. Legumes can furnish up to 90% of their own N therefore when associated with grasses legume can regulate soil nutrient balance. When legumes are grown with grasses, the amount of atmospheric N fixed depends on three factors (1) available soil N, (2) legume proportion in mixture, and (3) the rate of biological N fixation (BNF). Soils that are N-deficient, legumes will out-compete grasses to grow and produce greater biomass/forage due to their N-fixing ability. Moreover in such situations BNF may be very similar to monoculture. In contrary if the soil contains adequate amount of available N to support grasses they will usually out-compete legumes for available soil N (**Figure 5**). In such situation the leguminous species will be stimulated and BNF will be greater compared to monoculture however, the total atmospheric N fixed will be lower in mixture because of lower legume biomass accumulation and competition with grass species. Adding grasses as an intercrop can increase the competitive aspects between grass and legume plant species but will continue to retain and recycle more total N than their pure strands (**Figure 6**). Non-competitive interferences may be the direct stimulation between species, for example the N fixed by a legume species becoming available to non-legumes. Grass-legume mixtures can yield more N than legumes monocultures due to mutual stimulation of N uptake via symbiotic and non-symbiotic rhizospheric micro-organisms and endophytic association as illustrated in (**Figure 7**) to sustainably improve the soil processes [51, 52]. Soil N management is necessary to reduce negative environmental impacts. The unused or excess N can lead to eutrophication in surrounding water bodies and nitrate poisoning in livestock. The concept of using mixture of N scavenging grasses with N addition legume will maintain the N balance under proper management strategies.

Sl. no	Study type	Vegetation type	Country	Type of soil	Positively affected parameters	Reference
1	Field experiment	Grass-legume mixture with leguminous and non leguminous tree species	India	Coal mine soil	Soil fertility Biomass yield Carbon sequestration	[19]
2	Field experiment	Grass-legume mixture	India	Coal mine soil	Soil fertility Forage/ biomass yield CO_2 flux	[2]
3	Field experiment	Multipurpose tree species and leguminous trees	India	Coal mine soil	Soil fertility	[42]
4	Field experiment	Grasses with leguminous and non leguminous tree species	India	Coal mine soil	Soil fertility N mineralization Biomass yield	[43]
5	Field experiment	Grasses with leguminous and non leguminous tree species	India	Coal mine soil	Soil fertility Reduction in air pollutants Water conservation potential Improved esthetic view	[44]
5	Field experiment	Grasses with leguminous and non leguminous tree species	India	Coal mine soil	Soil fertility and soil quality Biomass yield Soil CO_2 flux Soil enzymatic activity	[45]
6	Field experiment	Grass-legume mixture	India	Coal mine soil	Soil fertility Forage/biomass yield	[46]
7	Field experiment	Grasses, shrubs with leguminous and non leguminous tree species	India	Coal mine soil	Soil fertility Heavy metal reduction	[47]

Table 4.
Various field experiments in India using grass-legume mixture and the positively affected mine soil parameters post- reclamation.

A grass-legume association potentially accumulates high quality organic substrates in soil with soil organic carbon (SOC) and N pool accretion and promoting beneficial soil micro-organisms [53–55]. The difference in the chemical composition of grass-legume mixture incorporated in soil shifts the nutrient cycling via mineralization which stimulated the soil microbial activities [56]. Soil microorganisms are a necessary link between plant–soil interaction for productivity, nutrient availability and cycling thus, legumes are one of the necessary components to increase soil microbial activity accelerating the process of ecological restoration in mined areas [29]. Legumes add high quality of soil organic matter (SOM) because of their low biomass C:N ratio that can be readily decomposed by soil microbes improving soil biodiversity, deep taproot system and high water infiltration [57]. Also, legumes provide additional benefits to strengthen ecosystem services like (1) protection from pests

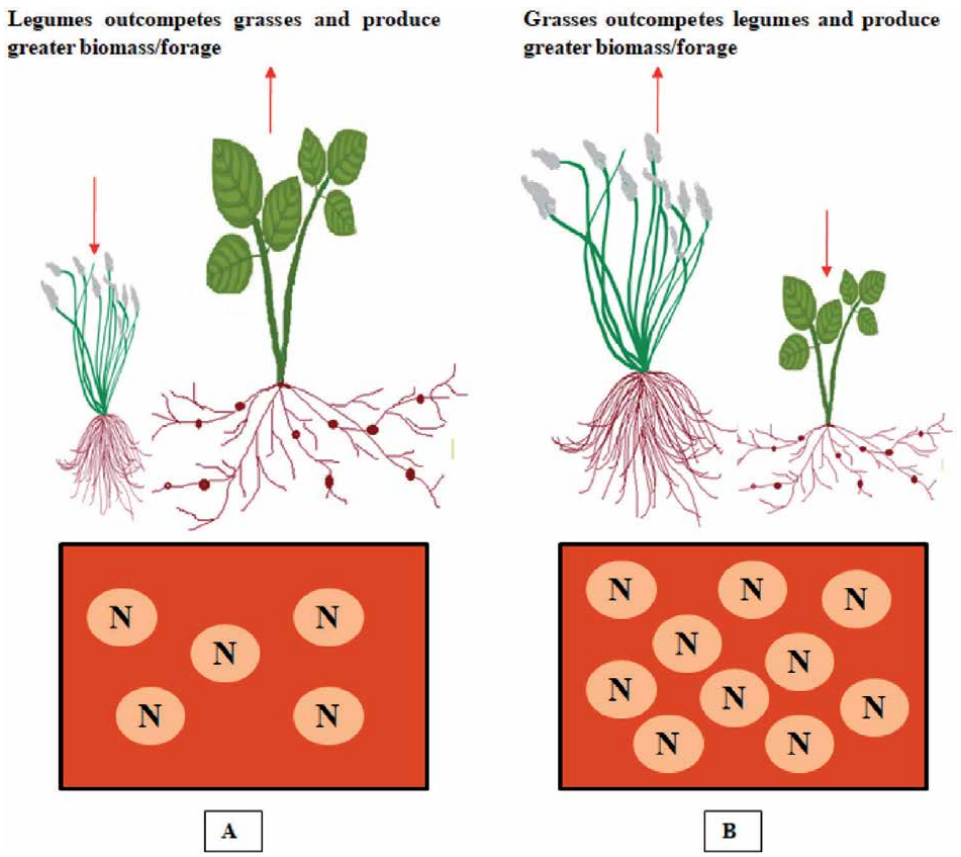

Figure 5.
Competitive aspects of grass-legume mixture under varying soil nitrogen (N) concentration.

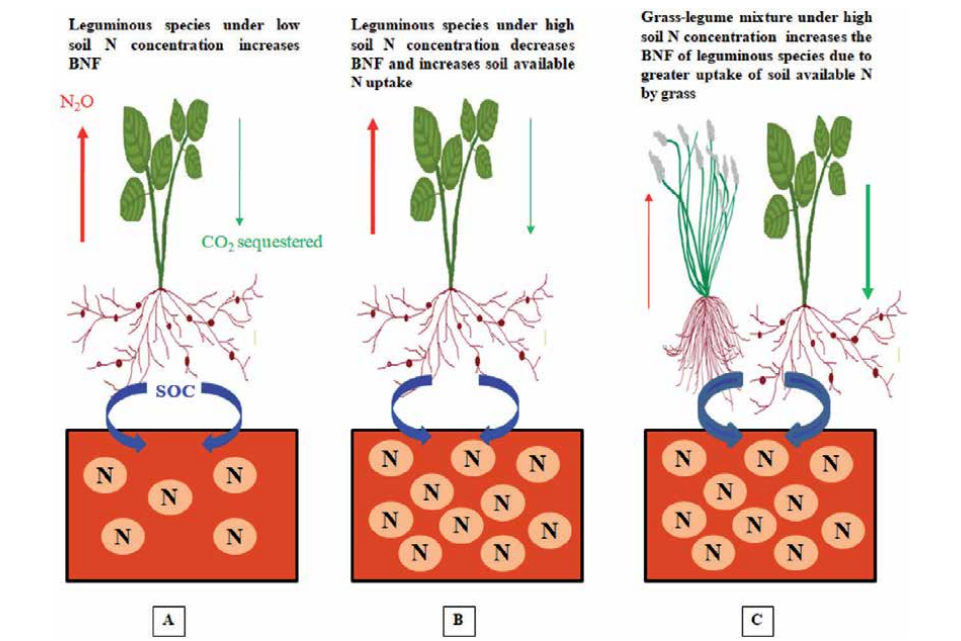

Figure 6.
Potential benefits of diverse species mixture in comparison to monoculture under varying soil nitrogen (N) concentration in binary nitrogen fixation (BNF), nitrous oxide emission (N2O), carbon sequestration and soil fertility.

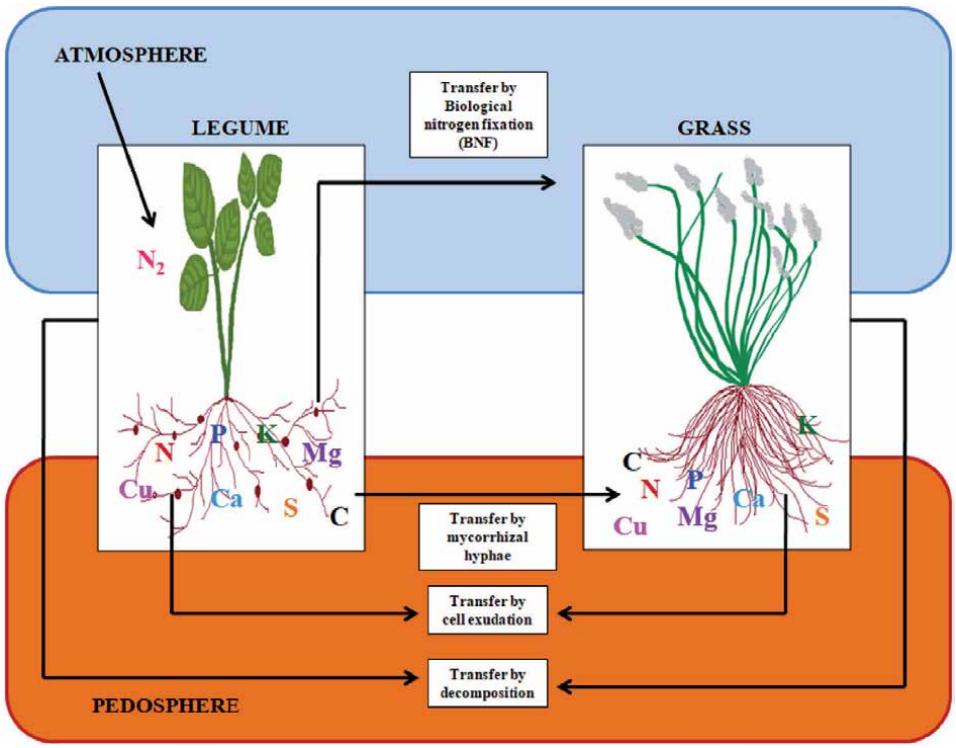

Figure 7.
Pathways of soil nitrogen (N) and other nutrients transfer between associating grass and legume species.

and diseases (2) *Rhizobium*-legume symbiosis accelerates the removal of soil pollutants. *Rhizobium* is a burgeoning component of the degrading microcosm in polluted soil and controlling tool for hazardous metal bioremediation reclaiming soil fertility [58, 59]. Some of the promising leguminous species used to remediate soil pollution are *Dalbergia sisso, Acacia auriculiformis, Albizia lebbeck,* and *Pongamia pinnata* while grasses are *Vetiveria zizanoides* and *Cymbopogon flexuosus* [44].

3.3 Carbon sequestration

Carbon sequestration is the natural process of capturing atmospheric CO_2 into the soil C pool through conversion of biomass residues into stable humus forms. It is one of the most important determinative biological factors of soil quality, productivity, and fertility [60]. Nearly 80% of total terrestrial C accounting to 2500 gigatons (GT) is found in soil out of which 1550 GT is organic C and 950 GT is inorganic C. The amount of C found in living plants and animals is relatively very small (560 GT) compared to soil C [61]. Plant biomass residues increase C sequestration through decomposition of their residues which links soil C sequestration to elevated biomass production and hence to soil fertility. Increasing soil fertility is the most effective way of rapidly accelerating SOC storage and can be accomplished through addition of soil N fertilizers. In contrast the role of legumes in supplying eco-friendly N through fixation is being favored more because of co-benefits like GHGs stability by reducing emissions. Grass-legume based vegetation system contributes to accelerate biomass production which improves the SOC stock and maintains a high amount of sequestered soil C [19, 29, 62]. The potential of C sequestration varies between different species depending on rate of decomposition and rate of conversion of soil liable C to recalcitrant C [57]. Perennial legumes like *Medicago sativa, Lespedeza davurica* and *Astragalus adsurgens* growing on arable lands increased the

soil C sequestration by 79, 68 and 74% respectively [63]. Several practices have been reported to increase forage biomass yields, including better pasture management, fertilization, organic amendments, improved irrigation, grass-legume mixture, reduced tillage and crop rotations. All these techniques are associated with reduced C loss and increased C input however, the rates of C sequestration vary with different management practices and inclusion of legumes or N sources. Land degradation due to coal mining disturbs the ecological processes of photosynthesis, decomposition and soil respiration and consequently to depletion of SOC pool. These anthropogenic activities negatively affect the global climate by rapid inputs of CO_2 and other GHGs to the atmosphere [64]. The French "4 per mile" initiative signed by more than 100 countries at Conference of parties (COP21) states that increase in soil C by 4% (0.4%) a year we can halt the annual CO_2 increase in the atmosphere. A Grass-legume mixture management strategy provides an opportunity for sequestering C back into soil reducing exacerbation of GHGs and climate change.

3.4 N fertilizer and N_2O emission

Legumes owing to their N fixation capabilities have little exogenous fertilizer requirement except the starter dose of application depending on site-specific conditions. The effect of previous legume in rotational cropping also reduces the need for fertilization in succeeding plant cover. Without fertilization legumes like *Trifolium* spp. have reported N fertilizer savings of (160–310 kg ha^{-1}) through BNF [65]. At current times when the chemical inputs like fertilizer application is not a viable option for environment along with increased cost of natural gas-based N fertilizers we need to consider legume as an eco-friendly option to sustain fertility and yields over longer time periods compared to fertilizer [29]. Nitrous oxide (N_2O), powerful GHG is 300 times more potent compared to CO_2 in relation to global warming potential. Nutrient poor or degraded soil requires greater amount of N fertilization to sustain biomass cover and increase yields. The emission of soil N_2O increases linearly with the quantity of N fertilizer applied to soil thus, BNF via legumes will become an essential aspect in all systems. Diverse mixture with legume addition improves biomass yield, in some cases equivalent to mineral N fertilization at the rate of 33–150 kg ha^{-1} and reduce soil N_2O emissions by 30–40% [66]. The study of [67] also showed consistent lower N_2O emissions in binary grass-legume mixtures compared to only grass with N fertilization. The reduced emission rate is associated with species complementarities between grasses and legumes which creates a synchrony in the timing of N mineralization and N demand. Soil systems including grass-legume mixture significantly lower the annual N_2O emissions saving N fertilizers and thus GHGs and a considerable potential for climate change mitigation [50].

3.5 Weed control

Weed invasion on post-mined lands negatively affects plant survival and biomass yield and therefore needs to be fully eradicated. Use of herbicides for weed removal can be effective at times but not environmental friendly and induces GHGs emission. Plant diversity (grass-legume mixture) can effectively suppress weed invasion. Sanderson et al., [68] found consistently lower weed abundance in legume-dominated mixtures compared to monocultures. Weed management system should be consistent with the principle of control, prevention and eradication. Organic mulches including grass and legume mulch residues can suppress the invasion of weeds [69] in several ways like (1) blocking germination by intercepting light (2) lowering soil temperature (3) greatly humidified day and night temperature fluctuations (4) thick mulch layer lowers weed seeds to germinate than non-mulched soil (5) organic

mulches enhances competition of resources, favors plant growth eradicating weeds. Study on weed suppression reported 52% less weed biomass across mixtures varying in species proportions. Weed invasion can be lowered via forage species combination and plant diversity and persistence traits in systems designed to reduce reliance on N fertilizer [70]. Nitrogen is not required for legumes or grass-legume mixture establishment. Application of N in such conditions can deter N fixation by legumes and in turn will accelerate competitive growth of grasses and weeds.

4. Case study: a successful case study promoting sustainable mining in India

Objective of the study: To conserve and enhance the biodiversity along with generating natural resources to cater the needs of local community and better esthetic view of the mined area.

4.1 Study area description

Ecological restoration (using 3-tier plantation model) of Tetulmari coal mine dump under Bharat Coking Coal Limited (BCCL), India was carried out to reverse the environmental degradation post-mining. The total area cover was 8–10 hectares located at 23°48′210“ N and 86°20’527” E and at an elevation of 704.9 m above mean sea level. Prior to restoration the mined out area was 14 years old and fully invaded by exotic weeds (*Lantana camara, Eupatorium odoratum, Heptis suaveolens*). The area was completely devoid of grass cover and native tree species.

4.2 Restoration approaches

- Based on the geological condition of post-mined sites, various restoration approaches were applied. Biological reclamation approach by fast growing single tier species plantation was the first effort of BCCL to restore the coal mine dump. This approach was not suitable for ecological restoration. The monoculture plantation method failed to develop on nutrient deficient rocky structure of mine dumps and also did not allure animals, birds and micro-organisms etc.

- Following the above scenario an ecological restoration approach based on three tier plantation model using grasses, herbs shrubs and trees was developed during three years (2011–2014) time period. A total of 13,000 plants of different species including grasses, legumes and horticulture species were planted in the coal mine dump (**Table 5**). The species were propagated through direct seeding, culms, seed balls, stem cutting, bulbils and seedling planting. Further, for attaining a sustainable and more stable ecosystem at the mine degraded area, a biodiversity enhancement initiative was carried out from 2016 to 2018. The initiative includes steps such as weed eradication, mulching, topsoiling, pitcher irrigation technique.

4.3 Results

Re-vegetation status: The ecological restoration approach was successful in establishing dense and diverse vegetation (trees, shrubs, herbs and grasses) cover on the mined dump within three years of restoration. Vegetation analysis during the course of restoration showed that among planted species *Dalbergia sissoo* was the most successful at the site with a maximum density of 514.3 tree ha^{-1}. The total

	Seed mix (sown)		Seed mix (soil balls)		Species planted	
Sl. No	**Species**	**Family**	**Species**	**Family**	**Species**	**Family**
1	*Acacia nilotica*	Mimosaceae	*Bamboosa bambos*	Poaceae	*Albizia lebbeck*	Fabaceae
2	*Aegle marmelos*	Rutaceae	*Cenchrus ciliaris*	Poaceae	*Albizia procera*	Fabaceae
3	*Albizia lebbeck*	Mimosaceae	*Cenchrus setigerus*	Poaceae	*Azadirachta indica*	Meliaceae
4	*Albizia procera*	Mimosaceae	*Cynodon dactylon*	Poaceae	*Bamboosa bambos*	Poaceae
5	*Bauhinia purpurea*	Caesalpiniaceae	*Panicum nitidum*	Poaceae	*Bombax ceiba*	Bombacaceae
6	*Bombax ceiba*	Bombacaceae	*Saccharum benghalense*	Poaceae	*Cassia fistula*	Fabaceae
7	*Cassia fistula*	Caesalpiniaceae	*Stylosanthes hamata*	Fabaceae	*Dalbergia sissoo*	Fabaceae
8	*Dalbergia sissoo*	Fabaceae	*Trifolium repens*	Fabaceae	*Madhuca indica*	Sapotaceae
9	*Melia azaderach*	Meliaceae			*Mangifera indica*	Anacardiaceae
10	*Moringa oleifera*	Moringaceae			*Emblica officinalis*	Euphorbiaceae
11	*Crotalaria juncea*	Fabaceae			*Pongamia pinnata*	leguminoceae
12	*Pongamia pinnata*	Fabaceae			*Psidium guajava*	Myrtaceae
13	*Dodonaea viscosa*	Sapindaceae			*Syzygium cumini*	Myrtaceae
14	*Indigofera trita*	Fabaceae			*Terminalia arjuna*	Combretaceae
15	*Mimosa pudica*	Mimosaceae			*Zizyphusnummularia*	Rahmnaceae
16	*Mucuna pruriens*	Fabaceae				
17	*Withania somnifera*	Solanaceae				

Table 5.
Species composition under the three-tier plantation method during ecological restoration of Tetulmari coal mine dumps, India.

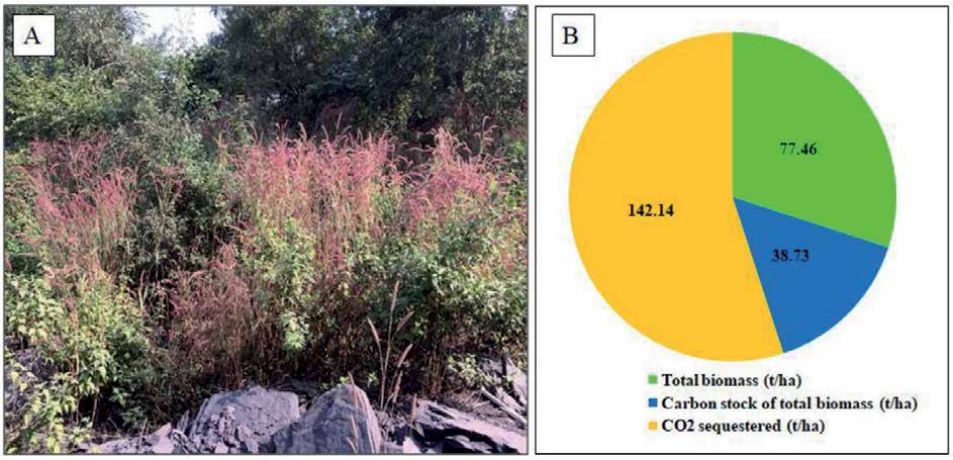

Figure 8.
(A) Closer view of dense and diverse vegetation cover of understory biomass and tree growth (B) biomass carbon stock and CO2 sequestered after ecological restoration of Tetulmari coal mine dumps, India.

shrub and herbs density was 1114 Ind ha^{-1} and 6.79 Ind m^{-2}. Similarly *Cenchrus ciliaris, Cenchrus setigerus* were found to be the promising grass species whereas *Pennisetum pedicellatum* was the first grass species to colonize the site. Successful horticultural species includes *Emblica officinalis, Mangifera indica Syzygium cumini* and *Psidium guajava.* Horticulture and grasses-legume species besides providing ecological stability were able to cater the needs of local communities and adjoining societies by providing food, fodder, timber resources and livelihood opportunities.

Nutrients status: Besides successful vegetation establishment, a notable change is soil physicochemical and biological properties were also observed in the span of three years. The soil pH increased from 6.0 to 7.1. SOC and total N concentration increased by 46% and 180% respectively after ecological restoration. The total biomass (77 t ha^{-1}) accumulated on the dump surface accumulated 39 t ha^{-1} C stock in soil equal to 141 t ha^{-1} CO_2 sequestered (**Figure 8**). The ecological restoration of mine degraded land considerably increased the ability of biomass and soils to sequester C. The development of terrestrial C sinks reduces ill-effects of polluting gases (GHGs) caused to the climate change.

Biodiversity status: The diverse vegetation started attracting different types of faunal species including birds, butterflies, insect, reptiles and naturally re-colonizing animals like foxes, rabbits, jackals etc. The enhanced biodiversity also facilitates to support food chains and better esthetics at the eco-restored area.

5. Conclusions

The mining process is not only ecological and socially devastating but also extremely demanding on natural resources like water land and energy. The post-mined areas are highly susceptible to weed invasion and prone to erosion that can cause mine waste to pollute adjoining soil and water resources. The rising demand of coal is likely to escalate ecosystem damage in several ways. The agronomic benefits' of grass and legume species has lead us to recognition of its environmental and socioeconomic advantages in mined-out landscapes (**Figure 9**). Sustainable mining is essential for the survival of humankind. The review of literature presented here in ascertains that grass-legume based management practices hold a vast potential to advance mine sustainability owing to benefits of BNF, soil

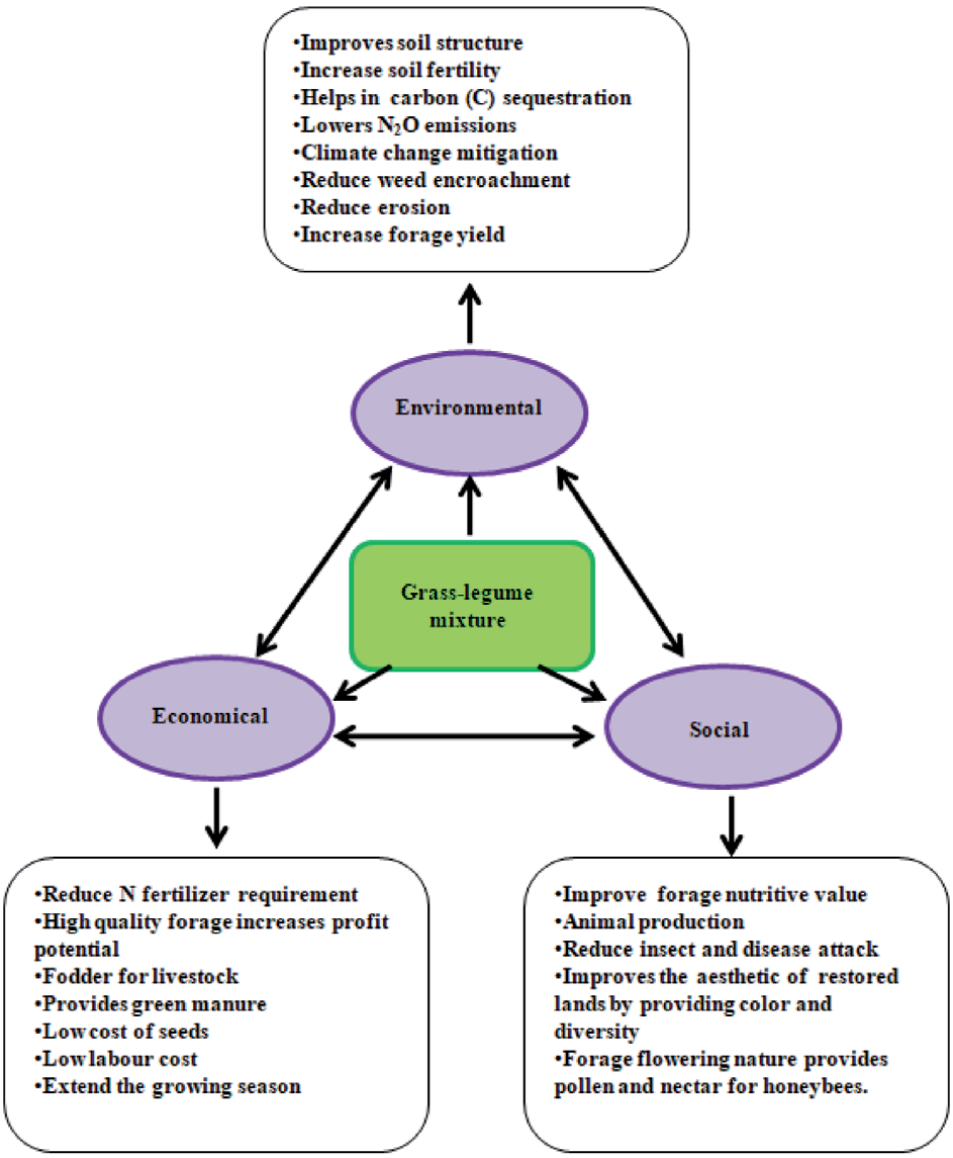

Figure 9.
Sustainability aspects of grass-legume mixture in environmental, social and economic arenas.

regeneration, creating terrestrial C sinks, weed control, reducing GHGs emissions and socioeconomically viable by increasing profit potential. Future perspective ascertains the need of ecological restoration using grass-legume seeding aimed towards sustainable intensification of mine degraded lands besides supporting livelihoods of millions.

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Declaration of competing interest

The authors do not have any conflict of interest.

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Chapter 14

Faba Bean Agronomic and Crop Physiology Research in Ethiopia

Dereje Dobocha and Debela Bekele

Abstract

Faba bean is an important pulse crop in terms of protein source, area coverage, and volume of annual production in Ethiopia. The aim of this paper is to assess the agronomic and crop physiology investigations in the past two decades in Ethiopia. The production limiting factors of this crop are low input usage, natural disasters, depletion of macronutrients, and unavailability of essential nutrients. Phosphorus is among the main limiting nutrients in soil systems in Ethiopia. Seed yield and biomass yield of faba bean were increased from 1338 to 1974 kg/ha and from 3124 to 4446 kg/ha when phosphorous was changed from 0 to 52 kg/ha, respectively at Holeta whereas application of 40 kg P ha $-^{1}$ resulted in higher grain yield (6323 kg ha^{-1}) and 3303 kg ha^{-1} at Lemu-Bilbilo and Bore highlands, respectively. The highest grain yield of 32 kg ha^{-1} was obtained from the application of 92 kg P_2O_5 ha^{-1} at Sekela district while application of 46 kg P_2O_5 ha^{-1} resulted in a substantial increase in seed yield over unfertilized plots on vertisols of Ambo. On the other hand, the results suggest that using starter nitrogen from 0 to 27 kg/ha has marginally increased faba bean yield but, a farther increase of nitrogen has indicated deteriorate of yield at Arsi zone. Proper plant populations play a crucial role in enhancing faba bean production. Planting faba bean at 30 cm × 15 cm spacing gave the highest grain yield in Duna district while it was 30 × 7.5 cm at vertisols of Ambo University research farm. Significantly higher seed yield (4222 kg/ha) was observed in the 40 cm inter-row spacing as compared to 50 cm inter-row spacing, which gave the lowest seed yield per hectare (3138 kg/ha) on fluvisols of Haramaya University. Intercropping and crop rotation are cropping systems that can increase soil fertility and crop yield. Intercropping of faba bean with barley at Debre Birhan increased land equivalent ratio than both crops when planted as sole. An additional income of 18.5% and 40% was gained than planting sole faba bean and wheat, respectively at Kulumsa. Faba bean can fix about 69 kg/ha nitrogen in Northern Ethiopia. Generally, the current review results showed that only limited studies in organic and bio fertilizer, plant density, and cropping systems were done on faba bean in Ethiopia. Hence, studies regarding soil acidity, organic fertilizer, and secondary plus micronutrient impacts on faba bean production and productivity along soil types and weather conditions need great attention in the future in Ethiopia.

Keywords: seed yield, biomass yield, fertilizer, plant population, row spacing, intercropping, crop rotation, soil fertility

1. Introduction

Faba bean (*Vicia faba* L.) is an important legume crop that contains a high protein amounting to 33% and is consumed worldwide as protein source by humans [1]. It is also a crop of considerable importance as a low-cost food rich in carbohydrates [2]. In addition to its great nutrition content, faba bean plays an essential role in crop rotation. It has the ability to fix nitrogen, and provide a significant level of nitrogen from the soil air using a symbiotic relationship with Rhizobium bacteria [3]. Depending on the plant density and the field management, this plant is able to fix nitrogen up to 40 kg ha^{-1} annually [4]. Like the other members of *Fabaceae*, *V. faba* also increases the humus of soil [5].

Faba bean production occupied nearly 2.1 × 10^6 ha worldwide [6]. Its global production is 4.4 million tons [7]. The main faba bean global producers are China (1.64 Mt), Ethiopia (0.92 Mt), Australia (0.34 Mt), France (0.27 Mt), and Sudan (0.16 Mt) [7].

Faba bean is an important pulse crop in terms of area coverage and volume of annual production in Ethiopia [8]. The crop takes the largest share of the area under pulses production [9]. The annual area coverage of the crop in Ethiopia is 492,271.60 hectares with a total production and productivity of 1.04 million tons and 2.1 tons/ha respectively [9]. It is a major staple food crop among pulses and it is mainly grown in the mid and high altitude areas of the country with an elevation ranging from 1800 to 3000 meters above sea level [10]. Some limiting factors of faba bean production are climatic conditions, edaphic factors, disease problems and agronomic practices [11].

According to Central Statistical Agency [12] report, in Ethiopia about 4.34% of the grain crop area of land was covered by faba bean with annual production of about 3.94% of the total grain production and yield of 1.84 t/ha. Despite the importance, the productivity of the crop is far below the potential and is constrained by several limiting factors [13, 14]. Even though the availability of high-yielding varieties, the productivity of faba bean under smallholder farmers is less than 1.89 t ha^{-1} [15]. The low yield of faba bean was related to the vulnerability of the crop to biotic and abiotic stresses [16]. Among the abiotic category, declining soil fertility and low pH (acidity) are the most determinants for the low productivity of most crops [17]. Most of the reports revealed significant improvements in the yield of faba bean due to chemical fertilizers applications [18, 19].

1.1 Socio-economic significance of faba bean

Broad beans are one of the most popular legumes in Ethiopia. It is a crop of manifold merits in the economy of the farming communities in the highlands of Ethiopia. It serves as a source of food and feed and a valuable and cheap source of protein. Faba bean also plays a significant role in soil fertility restoration in crop rotation through the fixation of atmospheric nitrogen [13, 14]. It is tightly coupled with every aspect of Ethiopian life. It is mainly used as an alternative to peas to prepare flour which is used to make a stew used widely in Ethiopian dishes. Its boiled broad bean (*nifro* in Amharic) is also common in Ethiopia. It is also a crop of high economic value [20]. Ethiopia's faba bean export has moved northward since the year 2000 and the major destinations are Sudan, South Africa, Djibouti, Yemen, Russia, and USA, though its share in the countries pulses export is small [21].

1.2 Main constraints for faba bean production or general production constraints

Despite its importance, the productivity of faba bean is far below the potential and is constrained by several limiting factors [14]. It was also mentioned that the productivity of faba bean is far below the expected potential due to low input usage,

natural disasters like a snow storm, depletion of macronutrients from cultivable land, and unavailability of essential nutrients [22]. There are also other limiting factors of faba bean production like climatic conditions, edaphic factors, disease problems, and agronomic practices [11].

2. Research achievements

2.1 Fertilizer study

Soil fertility is an important factor affecting crop productivity in general and faba bean in particular. All plants have their own type and amount of nutrient requirements from the soil. Excess nutrients in the soil cause toxicity to the plant and deficient nutrients cause nutrient deficiency symptom. Nitrogen, phosphorus, and sulfur are among the essential elements determining soil fertility.

2.1.1 Phosphorus

Phosphorous has a great role in the growth and development of crops. It plays a prime role in the growth of roots, nodulation, dry matter production, N fixation, and protein synthesis of leguminous crops [23]. Phosphorous is implicated in speeding up maturity and enhancing the root-shoot growth ratio. It is involved in many metabolism activities [24]. Phosphorous exerts many and varied functions in plant metabolism and hence inadequate phosphate supply to the plant seriously affects numerous metabolic processes. This is the reason why it is called the key to life because it is directly involved in the most life process. Thus, faba bean being a legume it needs phosphorus for better root and nodule development, which is often neglected by farmers. Hence, balanced nutrition of legumes gains significance to harvest better yields, especially under rain-fed cropping conditions, where rainfall quantum and its distribution controls the total crop production system [24].

Phosphorus is among the main limiting nutrients in soil systems in Ethiopia that create high yield gaps [25]. The application of diammonium phosphate to faba bean resulted in either lack of response or negative effects on some on-farm trials in the past in Ethiopia [18]. It was also reported that there was no response to phosphorous fertilizer at Holetta [26]. But, [18] stated that phosphorous fertilization resulted in a significant quadratic response at this location. This study further reported that there was no significant effect on seed yield at Burkitu and Debre Zeit. They reason out that the lack of significant response to the phosphorous application at Debre Zeit is possible since the research field has been fertilized with N and P fertilizers during the past three decades. Seed yield and biomass yield of faba bean was increased from 1338 to 1974 kg/ha and from 3124 to 4446 kg/ha respectively, when phosphorous was changed from 0 to 52 kg/ha at Holeta [27].

Increasing the rate of phosphorus from nil to 40 kg P ha^{-1} changed the seed yield from 1939 to 3303 kg ha^{-1} at Bore highlands, Guji zone [28]. Significantly higher mean dry biomass yield (14,158 kg ha^{-1}) and seed yield (6323 kg ha^{-1}) were produced with the application of 40 kg P ha^{-1} that was at par with 20 kg P ha^{-1} and 30 kg P ha^{-1} at Lemu-Bilbilo. The results also showed that the grain yield of faba bean was significantly increased with P fertilizer application rates over the control whereas the application of 30 kg P ha^{-1} resulted in a higher number of effective tillers $plant^{-1}$ (1.53), which was at par with all other P rates application except the unfertilized plots [29]. The highest grain yield of 3.2 t ha^{-1} was obtained from the application of 92 kg ha^{-1} P_20_5 at the Sekela district of West Gojam [30]. According to [31] fertilization of faba bean with 46 kg P_20_5/ha resulted in a substantial

increase in biological yield (8172 kg/ha) over no fertilizer check (5602 kg/ha haulm yield). Fertilization of faba bean with 46 kg P_20_5/ha resulted in a substantial increase in seed yield (3531 kg/ha) over no fertilizer check (2654 kg/ha seed yield) on vertisols of Ambo University research farm. Harvest index tended to improve with P nutrition (49.7) over no phosphorus (47.4) [31].

On the other hand, the research conducted on phosphorus fertilizer rate at Bore Highlands, Guji Zone revealed that application of 40 kg P ha^{-1} resulted in the highest plant height of faba bean which was significantly higher by 11.8% than the unfertilized and gave the highest nodule dry weight (170.90 mg/plant) and seed yield (3303.0 kg ha^{-1}), but the faba bean plant height difference between 10, 20, 30 and 40 kg P ha^{-1}, as well as seed yield difference between 30 and 40 kg ha^{-1} P rate, were statistically the same (**Table 1**). Increasing the rate of phosphorus application from nil to 10 kg P ha^{-1} did not affect the number of pods produced per plant. However, further increasing to 30 kg P ha^{-1} application rate resulted in significantly higher numbers of pods per $plant^{-1}$ than by plots fertilized with 20 kg ha^{-1}, 10 kg ha^{-1}, and nil rates [28].

Faba bean exhibited a significant response in terms of pod weight/plant with the application of 46 kg P_20_5/ha (24.0 g) compared to 21.7 g obtained with no phosphorus (**Table 2**). Test seed weight has a linear relationship with phosphorus fertilization. Phosphorus fertilization at 46 kg P_20_5/ha significantly improved the test seed weight (520 g) over no phosphorus (492 g) at Ambo University research farm vertisols [31].

The total number of nodules per plant increased significantly in response to increasing the rate of phosphorus application. The application of mineral phosphorus fertilizer at the rate of 40 kg (the highest rate) phosphorous ha^{-1} resulted in the highest number of nodules (94.52) per plant [28].

2.1.2 Nitrogen

Nitrogen is an essential nutrient for plant growth, development, and reproduction. It is so vital because it is a major component of chlorophyll, amino acids, energy-transfer compounds, such as ATP (adenosine triphosphate), and significant component of nucleic acids such as DNA, the genetic material that allows cells (and eventually whole plants) to grow and reproduce. Adequate amounts of nitrogen in the plant are also essential for the absorption of other nutrients [32]. It is involved in cell multiplication, giving rise to the increase in size and length of

P-rate (kg ha^{-1})	Plant Height (cm)	Number of Pods $Plant^{-1}$	Nodule Dry Weight (mg $plant^{-1)}$	Seed yield (kg ha^{-1})
0	104.20b	8.50c	105.50c	1939.00c
10	112.60a	9.40bc	127.80bc	2318.00b
20	113.10a	10.36b	147.50abc	2570.00b
30	114.60a	14.46a	165.70a	3105.00a
40	118.10a	13.08a	170.90a	3303.00a
LSD (5%)	6.69	1.80	23.95	354.13
CV (%)	10.40	28.80	29.10	23.40

Source: [28].

Table 1.
Effect of mineral phosphorus fertilizer application rate on plant height, number of pods $plant^{-1}$, nodule dry weight and seed yield of faba bean during 2015 and 2017 main cropping season at bore.

Phosphorus rate (kg ha^{-1})	Effective tillers plant^{-1}	Dry biomass yield (kg ha^{-1})	Seed yield (kg ha^{-1})
0	1.18b	10970c	5076c
10	1.36ab	12092b	5693b
20	1.49a	13178a	6008ab
30	1.53a	13962a	6248a
40	1.44a	14158a	6323a
LSD (0.05)	0.21	1019	463
CV (%)	15.30	8.2	8.17

Source: [29].

Table 2.
Main effects of phosphorus rates on effective tillers plant^{-1}, dry biomass yield, and seed yield of faba bean in Lemu Bilbilo district of Arsi zone.

leaves and stems and especially the stalks of grains and grasses; increases chlorophyll, giving the leaves their dark green color, plays a part in the manufacture of proteins in the plant, and is part of many compounds in the plant including certain types of basic acids and hormones [33]. Therefore, the application of nitrogen below optimum has a profound influence on crop growth and may lead to a great loss in grain yield [34].

Nitrogen is among the main limiting nutrients in soil systems in Ethiopia that create high yield gaps [25, 31]. Applying starter nitrogen from 0 to 27 kg/ha has slightly increased faba bean yield but, a further increase of nitrogen has indicated a decline of yield. The highest biological yield was recorded at the highest nitrogen level at Arsi zone [35]. Faba bean seed yield increased at Adet, Holeta, and Sheno when nitrogen increased from 0 to 36 kg/ha [18].

2.1.3 Sulfur

Sulfur is another important nutrient required by plants essentially required to form proteins and coenzymes [36]. Sulfur as a protein component is an essential element. Soil sulfate may originate from atmospheric deposition, fertilizer addition, or mineralization of soil organic S, which is the main sulfur fraction. In recent years the importance of appropriate nourishment of plants with sulfur has grown, which is chiefly related to a decrease in the deposition of this element in soils because of a reduction in industrial emissions [37]. The shortage of this component in the soil reduces the yield level and quality of leguminous plants [38, 39]. Sulfur fertilization, moreover, improves the yield quality, increasing the content of protein and sulfur amino acids in seeds [40, 41].

3. Plant population and patterns

Plant density is a major determinant of proper plant development and growth [42]. It has a remarkable capacity to exploit the environment with varying competitive stresses [43]. Both high and low crop densities reduce yield and total revenue. When planting density is too low, each individual plant may perform at its maximum capacity, but there are not enough plants as a whole to reach the optimum yield. If the planting density is too high, plants may compete against each other, known as intra-specific competition. Under those conditions, the performance of individual plants becomes a limiting factor for maximum crop yield [44].

It has been reported that among a various package of improved production technology proper plant population with appropriate adjustment of inter and intra-row spacing play a key role in enhancing faba bean production [45]. Optimum plant density differs from each variety and location since the different location has different soil type, soil moisture, soil fertility, and relative humidity [46]. In line with these findings, the research conducted on plant densities on faba bean varieties at Lemu-Bilbilo district of Arsi zone, Ethiopia indicated that the highest seed yield of faba bean (4649, 4594, and 4162 kg ha^{-1}) was obtained at 90, 70, and 50 plant m^{-2} for Degaga, Moti and Gora varieties, respectively [47]. The authors also stated that the highest total biomass of 9 t ha^{-1} was recorded from the highest plant population (90 m^{-2}), but did not show significant differences to the total biomass obtained from 70, 50, and 25 (control) plants m^{-2}. It was reported that 25 plants population density m^{-2} was economically recommended for Degaga and Moti varieties whereas, 50 plant population density m^{-2} was for Gora variety at the study site and similar agro-ecologies.

On the other hand, [48] reported that the significantly highest seed yield (2495 kg ha^{-1}) of faba bean was obtained at the combination of 30 cm × 15 cm spacing (the lowest and highest inter and intra-row spacing, respectively). The lowest grain yield (1329 kg ha^{-1}) was recorded at 30 cm × 5 cm spacing (**Table 3**). They also reported that significantly the highest dry biomass yield (8738 kg ha^{-1}) was recorded at the combination of 30 cm inter by 5 cm intra-row spacing. This was statistically similar with the dry biomass obtained due to 40 cm by 5 cm inter and intra-row spacing combination, and the lowest dry biomass yield (3812 kg ha^{-1}) was obtained at 50 cm × 15 cm inter and intra-row spacing interaction in the Duna district of Hadiya zone [48].

According to [49] significantly higher seed yield (4222 kg/ha) was observed in the 40 cm inter-row spacing as compared to 50 cm inter-row spacing which gave the lowest seed yield per hectare (3138 kg/ha) at fluvisols of Haramaya University. Seed yield (kg/ha) is significantly affected by inter and intra-row spacing. The higher seed yield was observed in the narrowest as compared to the wide spacing which gave the lowest mean seed yield at vertisols of Haramaya [45]. Another experiment conducted to see the effect of plant spacing on faba bean at Ambo University vertisols research farm revealed plant spacing had a significant effect on seed yield of faba bean [48]. Plots sowing by 30 × 7.5 cm spacing resulted in greater faba bean seed yield (3814.8 kg/ha) than that sowing by 40 × 5.0 cm (3074.1 kg/ha) and 60 × 5.0 cm (2388.9 kg/ha), respectively.

Inter-row spacing (cm)	**Intra-row spacing (cm)**					
	5	10	15	5	10	15
	Seed yield (kg ha^{-1})			**Dry biomass yield (kg ha^{-1})**		
30	1329.0a	2169.0c	2495.0e	8738.0 g	7678.0e	7187.0c
40	1545.0b	2378.0d	1966.0f	8656.0 g	7594.0e	5549.0b
50	1606.0b	2154.0c	1365.0a	8184.0f	6579.0d	3812.0a
LSD (0.05)		99.3			276.4	
CV (%)		7.2			13.8	

Source: [48].

Table 3.
Interaction effect of inter and intra-row spacing on seed and dry biomass yield of faba bean at Duna district of Hadiya zone in 2015.

Further research accompanied on plant spacing at fluvisols of Haramaya University also indicated that significantly the highest numbers of seeds per pod and seed yield per plant were obtained in wider row spacing [48]. At the same location, but different soil types (vertisols) also reported that an increase in the number of seeds per pod with wider plant spacing could be due to less competition for nutrients and water [49]. This is consistent with the results of [45] who stated wider spacing tended to improve the seeds/pod as compared with narrow spacing. These results might be due to the fact that widely spaced plants suffer less from competition than closely spaced plants.

Many literatures report that as plant density decreases (inter and intra-row spacing increases) number of pods/plant increases. For example [45] found a significant increment of the number of pods per plant by increasing inter and intra-row spacing in which the highest number of pods/plant (28.6) was obtained from the widest (50cm × 12cm) inter and intra-row spacing on vertisols at Haramaya University. The authors also state that a decrease in inter and intra-row spacing increases competition which eventually leads to a reduction in the number of pods on the individual plant. An increase in the competition for light and nutrients in high population leads to a decrease in photosynthesis and so more abscission and lower pods per plant.

4. Cropping system

4.1 Intercropping

Intercropping is the agricultural practice of cultivating two or more crops in the same land at the same time [50]. It is intensive management for crop production which aims to match efficiently crop demands to the available growth resources and labor [51]. It is relatively common in tropical and temperate areas because of the effective utilization of water [50], nutrients [52, 53], and solar energy [54]. The most common advantage of intercropping is the production of greater yields on a given piece of land by making more efficient use of the available growth resources. This could be due to different rooting characteristics, canopy structure, height, and nutrient requirements or resource use at different times [55].

In Ethiopia, food production for a rapidly growing population from a continually shrinking farm size is a prime developmental challenge. Researches indicated that inter-cropping is a good way of using land efficiently. A 3 years study of sorghum/groundnut and sorghum/soybean intercropping in Asosa (Ethiopia) showed that sorghum/groundnut intercrop had the highest sorghum yield at all growing seasons. The gross income and land equivalent ratio indicates greater economic benefit with intercropping of groundnut in 1: 1 proportion and simultaneous planting than sole planting [56].

The spatial arrangement of faba bean with barley around Debre Birhan area revealed that a significantly greater land equivalent ratio (LER) was obtained in intercropping than both crops when planted as sole. The 2B:1FB (one row of faba bean intercropped in two rows of barley) was more productive than other planting patterns (1B:1FB and 1B:2FB). All spatial arrangements had the LER values of more than one (LER > 1). It indicated that intercropping had economic advantages in land-use efficiency [57].

Mixed intercropping of wheat with faba bean was compared with sole culture of each species in 2002 and 2003 at Holetta Agricultural Research Center, in the central highlands of Ethiopia, and intercropping increased the land equivalent ratio by +3% to +22% over sole cropping [58]. The authors' findings showed that as faba

bean seed rate in the mixture increased from 12.5 to 62.5% the wheat grain yield was reduced from 3601 kg/ha to 3039 kg ha^{-1} whereas faba bean seed yield was increased from 141 kg ha^{-1} to 667 kg ha^{-1}. However, the maximum total grain yield of 4031 kg ha^{-1} of wheat, gross monetary value of US$ 823, system productivity index of 4629, and crowding coefficient of 4.70 were obtained when wheat at its full seed rate was intercropped with faba bean at a rate of 37.5%. The field research conducted on planting ratio in faba bean and wheat intercropping at Kulumsa showed grain yield of faba bean was significantly affected by planting ratio plus wheat intercropping and additional income of 18.5% and 40% was gained than planting sole faba bean and wheat, respectively [59].

4.2 Crop rotation

Crop rotation is the most among factors significantly increased soil organic matters [60]. Legumes contribute to the maintenance and restoration of soil fertility by fixing N_2 from the atmosphere [61]. The input of fixed N from grain legumes may be a significant contributing factor in relation to sustaining productivity in smallholder systems [62]. The researches findings so far indicated that faba bean can enhance the yield of the following crop and increase the economy of the farmers [63]; can mark residual phosphorus available that otherwise would remain fixed [64] and may indirectly make more phosphorus and potassium available for subsequent crops [65] and the rotational benefit of faba bean to improve the P availability for subsequent crops also is considered to be closely related to the mineralization of its P-rich crop residues rather than to residual effects of root exudates on soil chemistry.

Faba bean improve the structure of poorly structured soil by stabilizing soil aggregates compared to continuous cotton and cereals as pre-crops [66]. Its roots and stubble contributed 44–50 kg N ha^{-1} to the requirements of the following crop in a temperate climate [67]; produce high levels of rhizome deposition which will improve the soil N balance which assists in maintaining soil organic fertility, and appear to provide an important source of N for following crops in the rotation [20].

Other findings revealed that yields of malting barley were greater with some pulse rotations than with continuous barley at Jeldu and Holetta [58]. Mean grain yield advantages of malting barley over the two locations after faba bean, field pea, and rapeseed were greater by 67, 43, and 53%, respectively, than malting barley after barley indicating that the lack of crop rotation has already been manifested in the continuous barley plots. The authors also showed that the highest biomass yield of 7348 (kg ha^{-1}) and protein content (11.3%) of malting barley were recorded from malting barley following faba bean which was 9.5% protein content greater than that of following malt barley.

5. Biological nitrogen fixation

Many studies conducted in Ethiopia and elsewhere in Africa have suggested that biological nitrogen fixation in different legume crops supplies sufficient N for optimum and sustainable crop production [39, 68]. Many studies also confirmed that different legumes have different nodulation and biological nitrogen fixation potentials [69]. Faba bean can fix about 69 kg/ha nitrogen in Northern Ethiopia [70].

5.1 Rhizobium inoculation

Inorganic fertilizer is an immediate supply of nitrogen, but by far the most important source of fixed nitrogen derives from the activity of certain soil bacteria

that absorb atmospheric N_2 gas and convert it into ammonium. According to [71] soil bacteria reduce approximately 20 million tons of atmospheric nitrogen to ammonia. Integration of multipurpose, N-fixing legumes into farming systems commonly improves soil fertility and agricultural productivity through symbiotic associations between leguminous crops and Rhizobium [8]. They also suggested that the contribution of N fixation to soil fertility varies with the types of legumes grown, the characteristics of the soils, and the availability of key micronutrients in the soil to facilitate fixation, and the frequency of growing legumes in the cropping system.

It is widely acknowledged that inoculation of legumes with effective rhizobia can improve yields and provide a substitute for inorganic fertilizers. Research has recognized inoculation with effective and appropriate rhizobial strain is necessary to improve symbiotic nitrogen fixation and optimize faba bean productivity [72]. These authors also revealed that inoculation affects microbial community by increasing desired rhizobia strain population in the rhizosphere and for successful establishment, inoculants strain must be able to survive in the soil environment and take advantage of an ecological niche to be offered by the roots of the host plant.

Since the soil may harbor certain ineffective nodule forming native rhizobia, effective nodule formation largely depends upon the competitiveness of inoculants strain. This upholds that strain competitiveness is key for successful inoculation under field conditions. Therefore, symbiotic performance depends on the abundance of effective rhizobia strain and its competitiveness for nodulation. It is evident that there are diversified faba bean cultivars in Sub-Saharan Africa that are likely to be accompanied by symbiotically effective nitrogen-fixing indigenous Rhizobium strains [72].

Rhizobium inoculation resulted in significantly taller plants (55 cm) compared to not inoculated plants (43 cm). No significant difference in grain yield and biological yield of faba bean were recorded among not inoculated and inoculated faba bean with strain FB-1017 at Arsi Zone [35]. Faba bean grain yield was decreased from 2.65ton/ha to 2.55ton/ha when it was inoculated with rhizobium across locations (Agarfa, Farta, and Sinana) [73].

6. Prospects of agronomic research for enhancing sustainable intensification in Ethiopia

- The influence of secondary and micronutrients on faba bean production was not thoroughly studied in Ethiopia.
- No research has been conducted on faba bean physiology to improve its productivity in Ethiopia.
- Rhizobium inoculation study should be carried out across locations.
- The advantage of crop rotation with faba bean was not studied across locations

7. Conclusion and future outlook

The outcomes of this review revealed that faba bean yield showed an increasing trend as a result of technology improvements by different researchers. Among different fertilizers study phosphorus is a very important nutrient for faba bean production. To know the optimum amount of this nutrient research study should be conducted across locations, soil types and also repeated based on soil test results. Applying a small amount of nitrogen which is different across locations as starter

nitrogen is required for faba bean production and productivity. Intercropping faba bean with cereals can increase income by about 50% over sole cropping component crops. On the other hand, rotating faba bean with cereals increased soil fertility which is can increase the yield of the subsequent crop. A slight decrease of faba bean grain yield was observed when it was inoculated with rhizobium at Agarfa, Farta, and Sinana. In general, it was revealed that there was still a drawback of research done on faba bean yield improvement in Ethiopia. Therefore, further studies on soil acidity, secondary and micronutrients, organic fertilizer study should need focus on across locations, soil type, and weather conditions in Ethiopia.

Conflict of interest

There is no conflict of interest among authors.

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Chapter 15

Callus Induction from Unpollinated Ovary Explants of Beans

Asli Küçükrecep, Dilek Tekdal, İlknur Akça, Selim Çetiner and Rüştü Hatipoğlu

Abstract

Beans one of the essential plant protein sources for human and animal diets. Conventional breeding methods have been used to develop the cultivars of beans with high quality and high yield. However, conventional methods of plant breeding are time-consuming and laborious. Biotechnological methods can accelerate the breeding process in conventional plant breeding. However, the beans are thought to be a recalcitrant crop plant for applying biotechnological methods since plant regeneration under in vitro conditions in beans is not successful. Developing an appropriate method for in vitro bean regeneration remains a significant problem. The objective of this study was to develop a protocol for the culture of unfertilized ovaries of beans. Culture media and genotype are effective on the success of in vitro cultivation. For this reason, 12 genotypes of beans and some nutrient media such as MS and B5 with various 2,4-D/kinetin combinations were tested to obtain callus from unfertilized ovaries. The highest callus induction was obtained with a medium containing 2,4-D (0.5 mg L^{-1}) and Kinetin (2.5 mg L^{-1}). A literature review on beans indicates that no ovary culture has been carried out on tested varieties in this study to date.

Keywords: callus, common bean, 2,4-D, Kinetin, ovary culture

1. Introduction

Due to the increasing awareness of healthy nutrition globally, individuals obtain most of their daily calorie needs from plant-based foods. Legumes, which constitute the primary source of vegetable protein (22%), have an important place in human nutrition as an alternative to meat products. In addition to their rich nutritional values, legumes are also known as soil friendly due to their ability to bind the free nitrogen of the air to the soil [1]. Beans are one of the most grown edible legume plants in the world. Common bean (*Phaseolus vulgaris* L. 2n = 2x = 22) is a diploid species with a wide range of variability of phenotypic characteristics due to its tolerance to a variety of agroecological environments [2–4]. According to 2016 FAO data, the dry bean was grown on an area of 29.392.817 ha worldwide; fresh bean cultivation was carried out on an area of 1.557.233 ha [5]. Turkey is a significant producer of the economically valuable Fabaceae plant family. According to TÜİK data, the most cultivated crop after chickpea and lentil among legumes in Turkey is beans [6]. Although

Turkey could not rank in the top 10 worldwide in dry bean production, it is the third largest green bean producer after China and Indonesia in the world (**Figure 1**) [7].

One of the biggest problems encountered in breeding studies of beans with conventional breeding methods is that the breeding process is long. Different molecular marker systems have been developed to shorten this breeding process. In addition, bean growers often use local bean varieties that are available as a population. These populations used are not genetically and physically pure. This situation causes different problems in bean cultivation: (a) mechanized agriculture is complex because individuals in the population do not show uniform growth and development, and (b) problems occur in both cooking and storage of non-uniform products [8]. It is known that the first breeding studies of legumes in Turkey started in 1965 on fresh beans. The bean plant shows the feature of self-fertilization due to its flower structure, and foreign pollination by insects is also possible. There is a flag (vexillum) leaf on the outside of the flower, a fin (alea) at the bottom, and a boat (carina) in the middle of the flower. The flower has 10 stamens, and these are located in the carina.

It is possible to obtain doubled haploid plants by culturing ovaries under *in vitro* conditions and subsequent chromosome doubling [9]. Although there are many studies on this subject in some plant species, few studies are on obtaining haploid beans [2]. Haploid and doubled haploid plants are currently used in genetic mapping, QTL analysis, mutation breeding, and genomic studies. In addition, homozygosity is achieved in one generation by using doubled plants. Although selfing is possible in the bean plant, it takes a long time to reach homozygous. Crossbreeding can be difficult due to the flower structure. It is known that classical hybridization studies require a high labor force; selfing is required to obtain a pure line and takes a long time, such as 7–9 years. In the dihaploidization method, haploid plants are made doubled haploid as a result of chromosome folding using various chemicals. Each of these 100% homozygous lines obtained is a candidate of a variety.

In this chapter, a protocol for morphogenetic callus induction from ovary samples in beans is defined. This protocol is strongly repeatable for 11 different *P. vulgaris* genotypes and *Phaseolus* sp. (1 genotype).

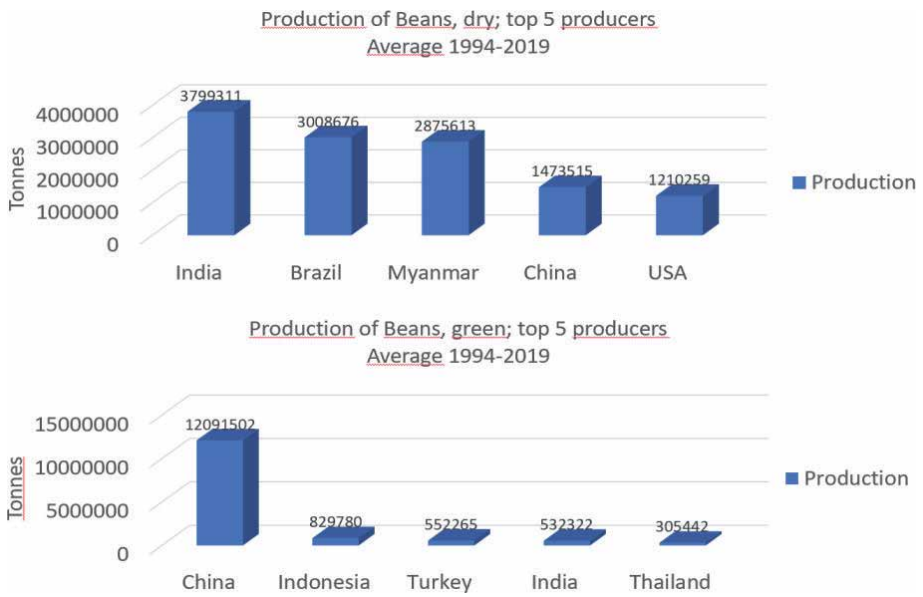

Figure 1.
*Statistical data of dry and green bean production of countries in the world. * From FAOSTAT database, May 2021 [7].*

2. Materials and methods

2.1 Plant materials

A total of 12 bean genotypes, seven genotypes selected from local lines and five commercial varieties, were used within the project's scope. Details on the total 12 bean genotypes are given in **Table 1**.

General views of seeds belonging to 12 bean genotypes are given in **Figure 2**.

2.2 Seed viability detection

The 2,3,5 triphenyl tetrazolium chloride method recommended by ISTA [10] was used for seed viability determination. The seeds of the genotypes tested were soaked in water for 24 h and peeled. Then, seeds were taken into 1 g L^{-1} 2,3,5 triphenyl tetrazolium chloride solution, and viability controls were carried out after 24 h.

2.3 Planting seeds and growing plants

Seeds of bean genotypes were sown in the greenhouse. Considering the weed reproduction situation, the seeds were first sown in viols containing peat and perlite (1:1) and allowed to germinate in order for the seeds to germinate easily. Plantlets

No	Genotype/variety name	Origin and characteristics
1	Akman	It is a variety registered by the Transitional Zone Agricultural Research Institute in 1998. It has a plant height of 60–70 cm. It is a variety with a harvest maturity period of 115–125 days
2	Bitlis-76	Local bean line, it was selfed three times and made homozygously
3	Bitlis-117	Local bean line, it was selfed three times and made homozygously
4	Göksun	It is a variety registered by the Transitional Zone Agricultural Research Institute in 2012. It has a plant height of 90–100 cm. It is a variety with a harvest maturity period of 104–124 days
5	Göynük	It is a variety registered by the Transitional Zone Agricultural Research Institute in 1998. It has a plant height of 45–55 cm. It is a variety with a harvest maturity period of 110–120 days
6	Hakkari-12	Local bean line, it was selfed three times and made homozygously
7	Karacaşehir	It is a variety registered by the Transitional Zone Agricultural Research Institute in 1990. It has a plant height of 50–55 cm. It is a variety with a harvest maturity period of 110–115 days
8	Önceler	It is a variety registered by the Transitional Zone Agricultural Research Institute in 1990. It has a plant height of 40–50 cm. It is a variety with a harvest maturity of 105–110 days
9	Tunceli-1	Local bean line, it was selfed three times and made homozygously
10	Van-59	Local bean line, it was selfed three times and made homozygously
11	Small reddish bean	It was obtained from the growers in the town of Elmalı in the province of Niğde in Turkey
12	Leklek	Local variety, it was obtained from the grower in the Gülnar district of Mersin Province in Turkey

Table 1.
Information on bean genotypes used in the study.

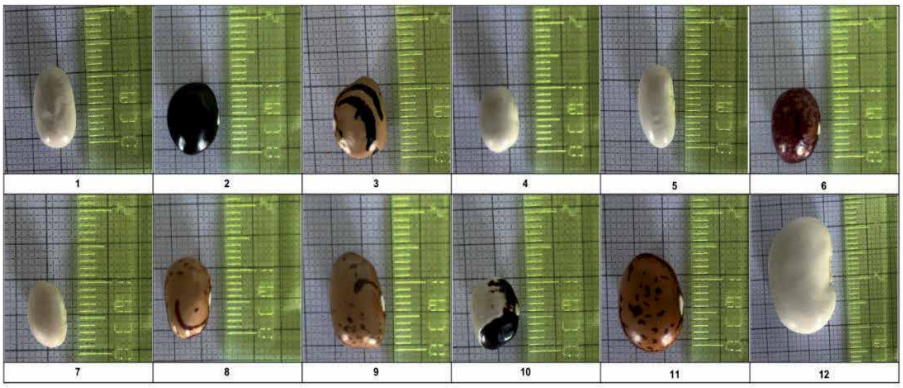

Figure 2.
*General images of seeds belonging to 12 bean genotypes (numbering of genotypes as indicated in **Table 1**).*

were developed in viols before they were transferred to pots. Humic fulvic acid was applied with life water in order to remove the initial stress and increase root activity while the plants were transferred to the pots. Plants growing in viols were transferred in large pots. Plants were planted in pots with 20 cm between the rows and plants and 10 plants in each pot.

2.4 Ovary culture

2.4.1 Surface sterilization

Flower buds of an appropriate size determined for the ovary culture experiment should be kept under tap water for 30 min to remove soil and/or dust residues. After then, it was washed several times with sterile distilled water. Explants taken in a sterile cabinet (Demair, class II A2 MSC 120) are rinsed in 70% EtOH for 1 min. Then, they were washed with pure water. Flower buds were kept in 25% NaOCl (sodium hypochlorite) for 15 min. The surface sterilization process of the samples was completed by washing 4–5 times with sterile distilled water. The unpollinated ovaries in the sterilized flower bud were isolated under a stereomicroscope (Olympus SZ61, Japan) and used as the explant in the tissue culture study.

2.4.2 Culture conditions

The sepals and petals of the flower buds, whose surface sterilization has been completed, were carefully removed. The isolated ovaries were then placed on the different basic media (MS and B5) [11, 12]. Different concentrations of 2,4-D (0, 0.5, 1.0, 2.0 mg L^{-1}) and Kinetin (Kin) (0, 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, and 4.0 mg L^{-1}) and their combinations were added to the basic media (**Table 2**). The experiment was set up with three Petri dishes for each genotype and five ovaries in each petri dish (15 ovaries in total). The samples were kept in styrofoam until callus formation was observed and left to the culture in a climate cabinet (Miprolab, Ankara, Turkey) at 26 ± 1°C.

Calli were transferred to fresh MS media without PGR and MS supplemented with thidiazuron (TDZ; 0.4 mg L^{-1}) and salicylic acid (SA; 20 mg L^{-1}) for plant regeneration, which was previously described as a differentiation medium for *Phaseolus* embryos [13].

Experimental plan for ovary culture			
Application code	**Kin (mg L^{-1})**	**2,4-D (mg L^{-1})**	**Trial plan for each variety/genotype**
MS			
1	0	0	3 Petri dishes (5 ovary explants in each pet)
2	0.5	0.5	3 Petri dishes (5 ovary explants in each pet)
3	0.5	1	3 Petri dishes (5 ovary explants in each pet)
4	0.5	2	3 Petri dishes (5 ovary explants in each pet)
5	1	0.5	3 Petri dishes (5 ovary explants in each pet)
6	1	1	3 Petri dishes (5 ovary explants in each pet)
7	1	2	3 Petri dishes (5 ovary explants in each pet)
8	2	0.5	3 Petri dishes (5 ovary explants in each pet)
9	2	1	3 Petri dishes (5 ovary explants in each pet)
10	2	2	3 Petri dishes (5 ovary explants in each pet)
11	2.5	0.5	3 Petri dishes (5 ovary explants in each pet)
12	2.5	1	3 Petri dishes (5 ovary explants in each pet)
13	2.5	2	3 Petri dishes (5 ovary explants in each pet)
14	3	0.5	3 Petri dishes (5 ovary explants in each pet)
15	3	1	3 Petri dishes (5 ovary explants in each pet)
16	3	2	3 Petri dishes (5 ovary explants in each pet)
17	3	3	3 Petri dishes (5 ovary explants in each pet)
18	3.5	0.5	3 Petri dishes (5 ovary explants in each pet)
19	3.5	1	3 Petri dishes (5 ovary explants in each pet)
20	3.5	2	3 Petri dishes (5 ovary explants in each pet)
21	4	0.5	3 Petri dishes (5 ovary explants in each pet)
22	4	1	3 Petri dishes (5 ovary explants in each pet)
23	4	2	3 Petri dishes (5 ovary explants in each pet)
24	4	4	3 Petri dishes (5 ovary explants in each pet)
B5			
25	0	0	3 Petri dishes (5 ovary explants in each pet)
26	0.5	0.5	3 Petri dishes (5 ovary explants in each pet)
27	0.5	1	3 Petri dishes (5 ovary explants in each pet)
28	0.5	2	3 Petri dishes (5 ovary explants in each pet)
29	1	0.5	3 Petri dishes (5 ovary explants in each pet)
30	1	1	3 Petri dishes (5 ovary explants in each pet)
31	1	2	3 Petri dishes (5 ovary explants in each pet)
32	2	0.5	3 Petri dishes (5 ovary explants in each pet)
33	2	1	3 Petri dishes (5 ovary explants in each pet)
34	2	2	3 Petri dishes (5 ovary explants in each pet)
35	2.5	0.5	3 Petri dishes (5 ovary explants in each pet)
36	2.5	1	3 Petri dishes (5 ovary explants in each pet)
37	2.5	2	3 Petri dishes (5 ovary explants in each pet)

Experimental plan for ovary culture			
Application code	**Kin (mg L^{-1})**	**2,4-D (mg L^{-1})**	**Trial plan for each variety/genotype**
38	3	0.5	3 Petri dishes (5 ovary explants in each pet)
39	3	1	3 Petri dishes (5 ovary explants in each pet)
40	3	2	3 Petri dishes (5 ovary explants in each pet)
41	3	3	3 Petri dishes (5 ovary explants in each pet)
42	3.5	0.5	3 Petri dishes (5 ovary explants in each pet)
43	3.5	1	3 Petri dishes (5 ovary explants in each pet)
44	3.5	2	3 Petri dishes (5 ovary explants in each pet)
45	4	0.5	3 Petri dishes (5 ovary explants in each pet)
46	4	1	3 Petri dishes (5 ovary explants in each pet)
47	4	2	3 Petri dishes (5 ovary explants in each pet)
48	4	4	3 Petri dishes (5 ovary explants in each pet)

Table 2.
Medium variants used in ovary culture.

2.5 Statistical analysis

Variance analysis was applied to the data on the rate of callus/embryo formation (reaction rate) of ovary explants according to the completely randomized design in split plots with three replications by using MSTAT-C Statistical Program.

3. Results and discussion

The first goal of developing a procedure for indirect regeneration of bean genotypes was to develop an optimum medium for morphogenetic calli induction. In this study, flower buds were used as an explant source. Seed germination occurred in all tested genotypes successfully. Unfertilized ovaries of the genotypes were picked on the day of anthesis. Isolated ovary samples were cultured on 48 different media. For callus induction from the explants, MS and B5 media, including different combinations of auxin (2,4-D) and cytokinin (Kin), were tested. Different concentrations and combinations of Kin (0, 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, and 4.0 mg L^{-1}) and 2,4-D (0, 0.5, 1.0, and 2.0 mg L^{-1}) were investigated to optimize callus induction of 12 bean genotypes. Callus was obtained from all ovary samples studied. Non-morphogenic and morphogenic calli were generated in bean ovary cultures inoculated on different agar media. According to the microscope images of the calli developing from the ovary samples, it was observed that the calli mostly developed at the ends of the cultured ovary sample and had a light yellow-brown color scale (**Figures 3–6**).

On the other hand, no regeneration of calli was observed in the samples cultured on the medium free from PGR. The formation of embryos and embryogenic calli was an uncommon occurrence. However, callus was obtained from all 12 varieties tested in this study. Morphogenic calli in the presence of Kin and 2,4-D were characterized by cell proliferation. Nutritional medium with relatively high- and low-growth regulator concentration demonstrated only minor variations in the efficiency of morphogenic calli production. Some factors, such as stress factors and nutrient media composition, are thought to strongly influence the reprogramming

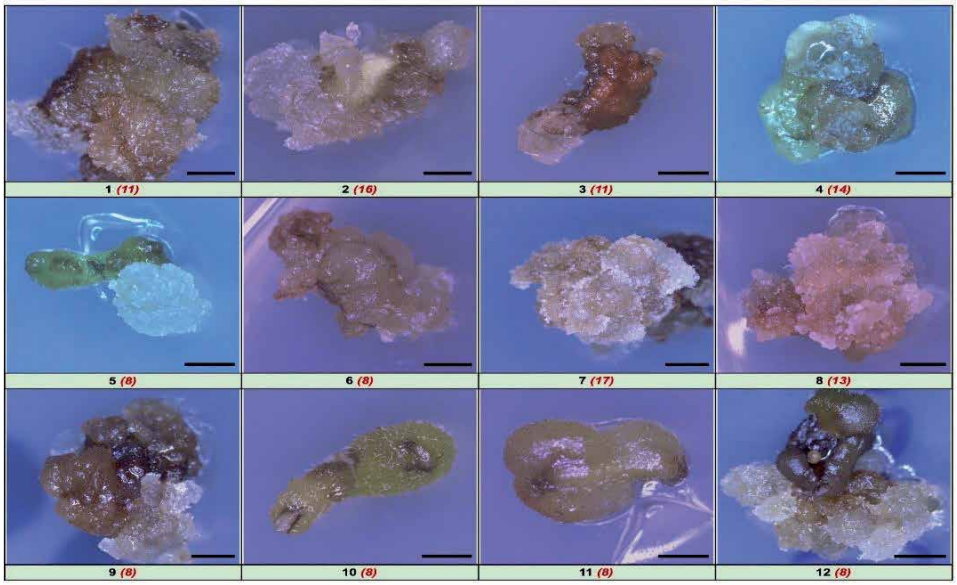

Figure 3.
Stereo-microscope images (Olympus SZ61, Japan) of callus growing from ovaries cultured in MS medium (black numbers indicate the genotype number and the detail is given in ***Table 1****; red numbers indicate the medium in which callus growth was observed; the detail is given in* ***Table 2****; magnification: 1.2×; scale bar 200 μm).*

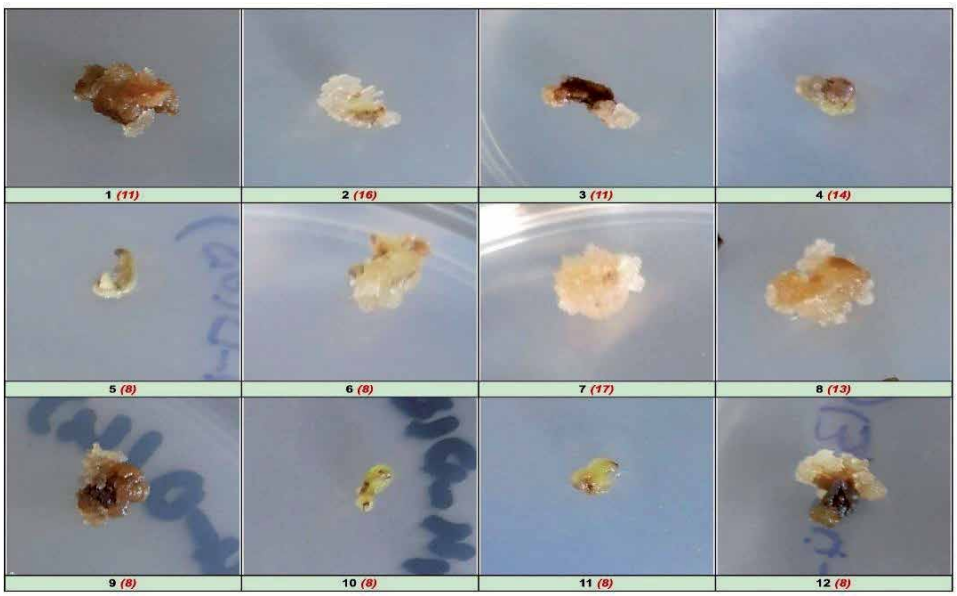

Figure 4.
Calli in petri dishes (black numbers indicate their genotype numbers, and the details are given in ***Table 1****) developed from ovaries cultured in MS medium and given microscopic images in* ***Figure 4****; red numbers indicate the medium in which callus development was observed; the detail is given in* ***Table 2****.*

of bean megaspore into the sporophytic developmental pathway. Kin was used in the presented study since cytokinins act on bud formation and plant cell division [14]. The effect of Kin and 2,4-D concentration on callus proliferation was observed, and calli increased in size and were nodular and compact (**Figures 3–6**).

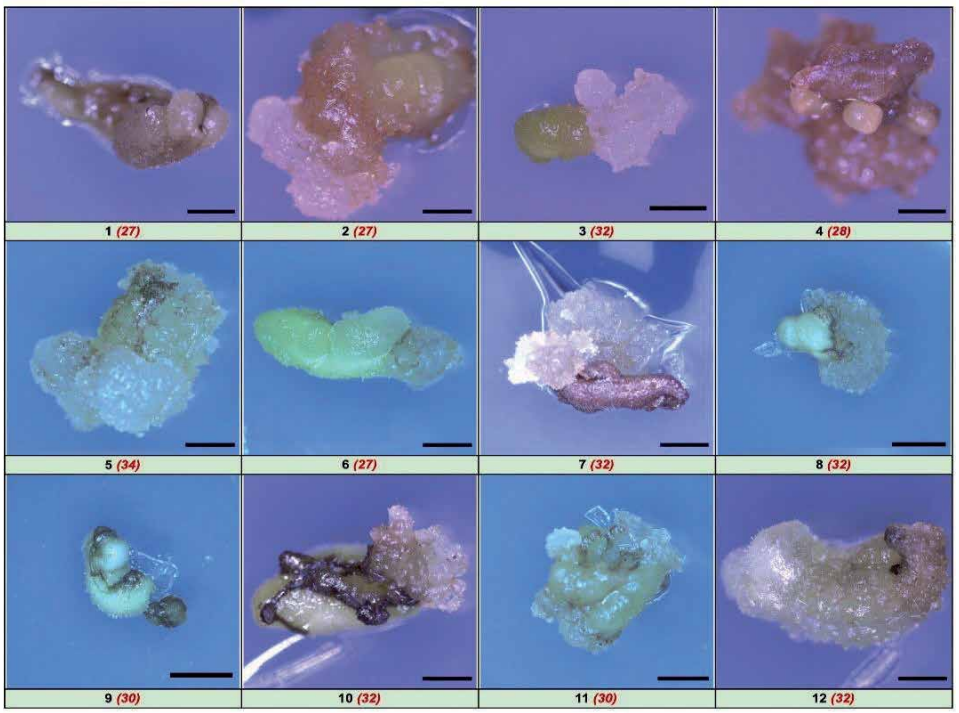

Figure 5.
Stereo-microscope images (Olympus SZ61, Japan) of callus growing from ovaries cultured in B5 medium (black numbers indicate the genotype number and the detail is given in ***Table 1****; red numbers indicate the medium in which callus growth was observed; the detail is given in* ***Table 2****; magnification: 1.2×; scale bar 200 μm).*

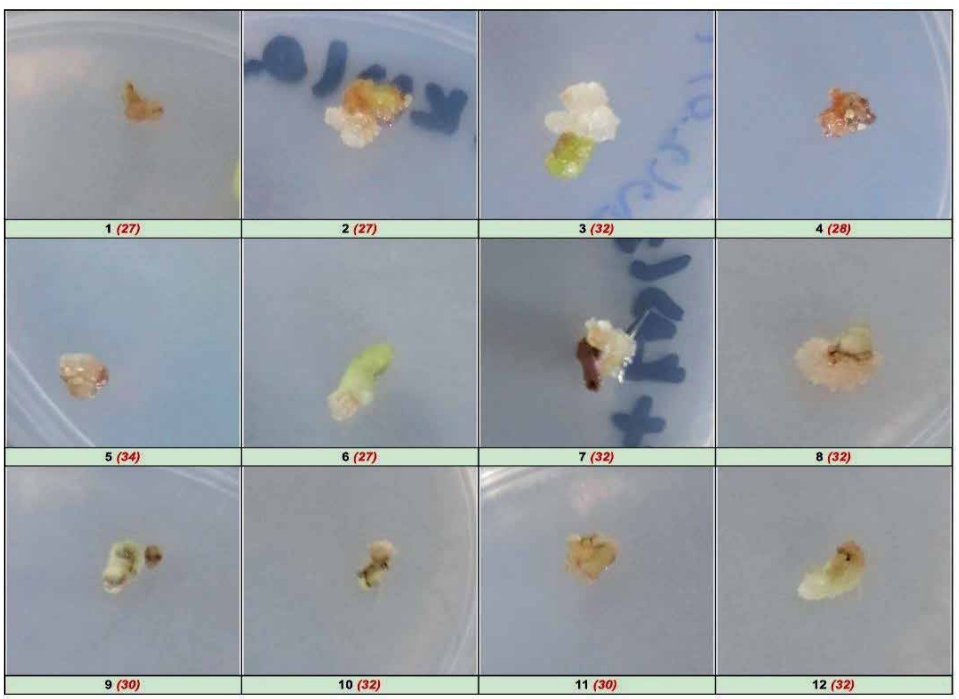

Figure 6.
Calli in petri dishes (black numbers indicate their genotype numbers, and the details are given in ***Table 1****) developed from ovaries cultured in B5 medium and given microscopic images in* ***Figure 6****; red numbers indicate the medium in which callus development was observed, and the detail is given in* ***Table 2****.*

Unpollinated ovaries/ovules or full flowers can be cultured to produce efficient gynogenesis methods that generate many embryos from female gametic cells. When the literature on haploidization studies conducted with the legume family was examined, very few studies were encountered. In a study on the *Cajanus cajan* plant, callus and immature embryos were obtained, but it was stated that callus cells initially had haploid and then a large variety of chromosome complements. Also, mature embryos and haploid plants were not obtained [15]. Grewal et al. [16] mentioned that members of the Fabaceae family are recalcitrant and, therefore, the difficulty of their development in culture.

In vitro regeneration and genetic transformation were difficult for *P. vulgaris* and other members of the *Phaseolus* genus since they are recalcitrant. While many *in vitro* regeneration protocols for *P. vulgaris* have been published, most of them were related to direct organogenesis or shoot production from meristematic cells [3]. Several reports have been on organogenesis in different cultured explants of *P. vulgaris* hypocotyls, cotyledonary nodes, and embryonic axes [3, 17]. However, no study exists on *in vitro* embryogenesis from the unpollinated ovary of *P. vulgaris.* Although plant regeneration is often genotype-specific in tissue culture, callus was successfully obtained from the ovaries of all 12 genotypes in this study. Some plant growth hormones may be stored in the ovary during plant development and may cause a different hormone balance *in vitro* culture with synthetically added hormones. This situation may also differ within each genotype and cause further growth or developmental problems in the culture. Studies indicate that successful shoot formation is observed in different bean explants cultured in nutrient media where TDZ and IAA are used together [18, 19]. In addition, success has been achieved in media containing a combination of TDZ and IAA in different *Phaseolus* species such as *P. acutifolius* A. Gray [20] and *P. polyanthus* Greenman [21]. Morphogenesis (roots) was induced from ovary samples in this study when the low PGR concentrations were used. Translucent embryos were obtained from ovaries when the calli were transferred to the medium with TDZ (0.4 mg L^{-1}) and SA (20 mg L^{-1}) (**Figure 7**). When the calli were transferred into the medium free from the PGR, no development was observed, and the calli began to darken. A previous study

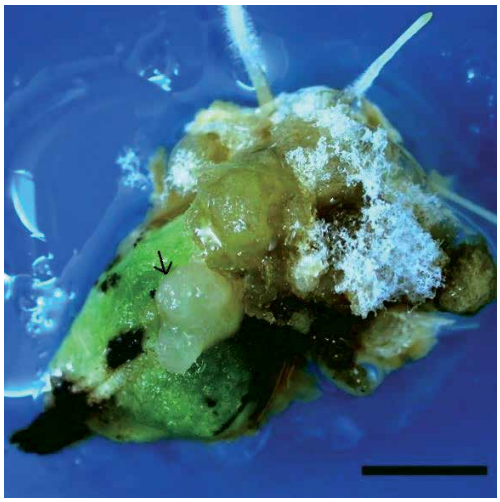

Figure 7.
Development of heart-shaped embryo on MS medium containing TDZ (0.4 mg L^{-1}) and SA (20 mg L^{-1}) in two weeks (black arrow indicates embryogenic formation; scale bar 200 μm).

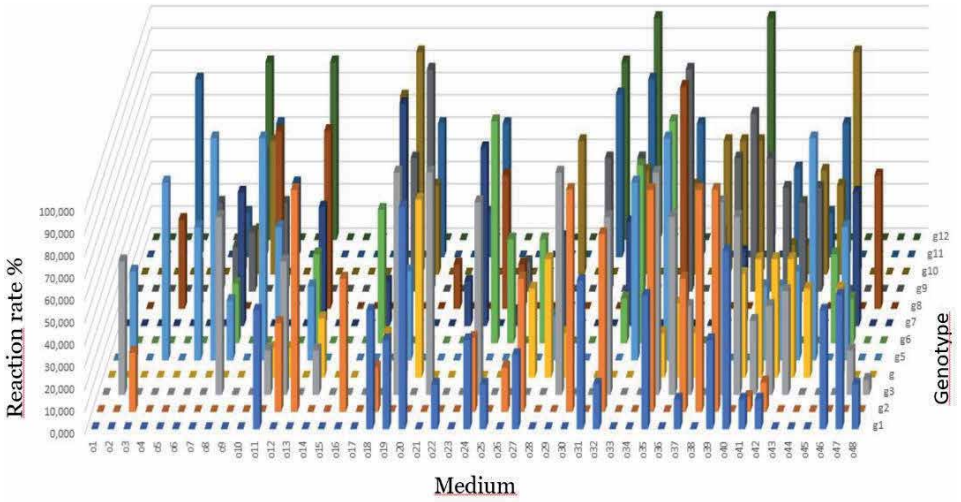

Figure 8.
Callus/embryoid formation rates of ovaries of different genotypes in different nutrient media (%).

of *Cucumis anguria* L. showed that unpollinated ovaries cultured *in vitro* did not enlarge [22].

According to a literature review, it is known that the B5 [12] medium is more effective in the tissue culture of some Fabaceae family members than the MS medium [2, 23]. Ovary samples taken in culture in MS and B5 media specified in **Table 2** were analyzed comparatively for each genotype and each tested medium. The statistically significant interaction of the nutrient medium × genotype revealed that the effect of the nutrient medium on the reaction rate differs depending on the genotypes (**Figure 8**).

The medium, including 2.5 mg L^{--1} Kin, provided a significantly higher reaction rate than all other media studied. The reaction rate of the explants differed significantly depending on the genotypes. According to the analysis results, the best callus yield was obtained from B5 media containing 2.5 mg L^{-1} Kin and 0.5, 1, and 2 mg L^{-1} 2,4-D in the ovary culture experiment. MS medium free from plant growth regulators never triggered callus induction in all tested genotypes, whereas B5 without plant growth regulators resulted in callus induction only in two genotypes (g1 and g6, given in **Table 1**).

In the future, these findings might act as a clue in generating the whole plants *in vitro* conditions. The future applications of these bean genotypes hold a great promise as a management tool for obtaining the plants against climatic conditions.

4. Conclusions

The most successful medium for callus induction in ovary culture of *P. vulgaris* was B5, and the influence of different stages of female gametophyte should be investigated for higher callus induction and plant regeneration in common beans. As a result, the technique we describe has the potential to enhance indirect organogenesis in the future and may serve as the foundation for developing a procedure for *P. vulgaris*. We believe that the research results discussed here contribute to further studies on *in vitro* regeneration of common beans. Understanding the role of growth regulators for selective bean genotypes has greatly aided bean regeneration under controlled conditions.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Appendices and nomenclature

MS	Murashige and Skoog medium
B5	Gamborg's medium
2,4-D	2,4-dichlorophenoxyacetic acid
Kin	Kinetin
P. vulgaris	*Phaseolus vulgaris* L.

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Section 3

Legumes - Microorganisms Interaction

Chapter 16

Legumes and Nodule Associated Bacteria Interaction as Key Factor for Abiotic Stresses Impact Mitigation

Abdelmalik Omar Ahmed Idris
and Elnour Alamin Gibreel Noh

Abstract

Due to climate change, different soil stresses are increasing continuously and they threat the world food security as they limit crop productivity. Therefore, this chapter aims at integrate information about the interaction between legumes and endophytes which will help to: deep understanding of the endophytes-legume relationship, draw attention to the possibilities to exploit this relationship in soil stress mitigation and unraveling what is need to be addressed in the future. The study reviewed the most recent previous scientific works in the field. For legumes tissue colonization, endophytes almost use the same routs which results in their presence in the same niches. Co-inoculation of these bacteria enhances plant growth directly and indirectly. Some endophytes characterized by stress tolerance which interact with legumes and mitigate the adverse effect of soil stresses like salinity, acidity/alkalinity, drought and heavy metal contamination. To reduce stress and enhance plant growth, legume-associated bacteria produce ACC deaminase and other compounds. The interaction process involves induction and expression of many legume-associated bacteria chromosomal and plasmid genes which indicates that this process is a genetic based. So isolation of stress tolerant legume-associated microbes and identification of the gene related to stress tolerance will aid in production of genetic engineered endophytes adaptive to different stresses. It is concluded that all soil stresses can be addressed by application of stress tolerant endophytes to the soil affected with environmental stresses which is sustainable and low cost approach. To maximize the benefit, searching for indigenous stress tolerant endophytes is recommended.

Keywords: Legumes, endophytes, colonization, mechanism, Rhizobium, nodules, stress

1. Introduction

In the last decades the world faced by increasing of food demand due to population increasing. At the same time climate change emerged as a crucial and serious issue which got a global attention [1]. Climate change affects agriculture leading to food insecurity [2]. These problems cannot be resolved unless sustainable

agriculture is practiced, because 36% of the population in the world depends on agriculture for food and as source of economic revenue [3].

Legumes are well-recognized for their impact on the agricultural systems sustainability in addition to their nutritional and health benefits [4]. They are also known for their positive impacts like biological nitrogen fixation, weed suppression, erosion control, improvement of soil health, and eradication of malnutrition in the third-world countries. Therefore, legumes can contribute to meet sustainable food and environmental security objectives [5]. More than that, legumes are also known as "pioneer plants colonizing marginal soils, and as enhancers of the nutritional status in cultivated soils" [6]. All these advantages of legumes make them to be the suitable candidate to address the threatening of climate change which need research approaches to develop crops characterized by the ability to cope with environmental stresses and increasing yield and quality [4]. So using of legumes can lead to sustainable agriculture which "maintains and improves human health, benefits producers and consumers economically, protects the environment, and produces enough food for an increasing world population" [7].

However, sustainable agriculture is faced by abiotic stresses, one of the most important constraints of agricultural production in the world [7]. The most efficient way to face this challenge is using bacteria associated with legumes in the farming systems [8]. These bacteria work together in a team as a community within the root nodule to maintain plant health and survival under harsh conditions and environmental stresses [3, 9]. In addition, the use of these bacteria in agriculture is a low-cost, eco-friendly technology and ethically and socially well accepted [3, 10]. This technology is promising approach due to the increasing recognition that plant tolerance to stress is connected with their associated microbes [11–13]. Among bacteria associated with legumes endophytic bacteria or plant growth promoting bacteria (PGPB) colonize nodules require research focus on exploring their diversity and roles in stress tolerance [3]. So more research on endophytes will enable us to gain insights into the mechanism of colonization and their interactions with plants [3] and best understanding the role they can play in environmental stress mitigation.

Therefore, this chapter aims at review and organize; integrate and evaluate the information about the interaction between legumes and endophytes which will help to: deep understanding of the endophytic bacteria-legume colonization and interaction processes, draw attention to the possibilities to exploit this interaction in soil stress amelioration and unraveling what is need to be addressed in the future studies.

2. The mechanism of legumes nodules colonization by endophytic bacteria

Although root colonization process is very important in nature, till 1987 nothing is known about this process at the molecular level [14] Root colonization is the first step to initiate interaction between the plant and endophytic bacteria. Endophytic bacteria have an affinity with the roots based on several factors including bacterial cross-talk, molecular signaling and quorum sensing (QS) which switch certain genes for using in a variety of plants [15].

The processes of root colonization in *Klebsiella pneumonia*, *Pseudomonas* and *Enterobacter* start by attachment of the bacteria through the fimbriae to root hairs as preferential point, and to the zone of elongation and the root cap mucilage as secondary attachment point without host specificity [16, 17]. For nitrogen-fixing strains it is proposed that type III fimbriae are involved in the adhering to the roots [18]. This mechanism of attachment resembles the adhesion of *Rhizobium japonicum* to soybean roots in which firm attachment was found mediated by pili [19].

The root colonization process probably affected by many factors including "motility, chemotaxis, carbohydrate utilization, and attachment" [17]. Before attachment of endophytes to the plants roots, the plant secretes specific compounds which represent as "chemo-attractant" [20, 21].

For example for attachment of *Pseudomonas* to roots, flagella [14] and other important colonization traits are required like the O-antigen of lipopolysaccharide [22], the ability to synthesize thiamine and high growth rate [23], utilization of organic acids, some amino acids, malic acid and citric acid [20, 21, 24]. However, these traits seem to be characteristic of different *Pseudomonas* species and depend on plant species with which the bacteria associated. This indicates that endophytic colonization is not a passive process, it is an active process controlled by genetic determinants from both partners [25]. It is also reported that cell-surface proteins are involved in the attachment of *Pseudomonas spp*. to plant roots which "include the outer membrane protein OprF of *Pseudomonas fluorescens* OE 28.3" [26] and "an agglutinin isolated from *Pseudomonas putida* a strain Corvallis that mediates agglutination of bacterial cells to a glycoprotein on the plant root" [27].

Following the attraction of endophytes by root exudates and firm attachment, the bacteria distribute along root, the population growth and survival occur, they enter into the root mainly through primary roots and associated lateral roots and tissue wounds, and form micro-colonies [28, 29]. The entry into the root depends "upon the type and availability of nutrients in a tissue, their abundance in the soil and environmental conditions prevailing in that region" [30].

Some routes used by endophyte to colonize plant roots are the same as used by rhizobia in legumes roots colonization which found enter through root hairs and cracks. However, endophytes surpass rhizobia in using more paths to enter plant tissue which make them promising technology as inoculants in sustainable agriculture. This finding supported by the earlier description of endophytes as opportunistic bacteria and "can enter the plant tissue when they find the opportunity either after dissolving the cell wall or through crack entry" [31]. More than that, endophytic associated with legumes nodules were described as "opportunistic bacteria that colonize nodules induced by rhizobia" [32]. Other evidences support the crack entry of endophytes are found in *Klebsiella pneumoniae* 342 which enter the plant after accumulation at lateral roots junctions, which seem reasonable given the nature of this bacterial/host association that does not need the formation of an organized symbiotic structure such as a root nodule as in rhizobia. Before entering the plant, *Klebsiella pneumoniae*342 cells may divide on the rhizosphere or a single cell may enter the plant and then divide in the interior [25].

3. Nodules endophytic bacteria-legumes interaction

After colonization, the endophytes interact with the host plant and beneficial bacteria can significantly affect general plant health and soil quality. Plant growth promotion take place in one of two ways: one way is indirectly by helping plants acquire nutrients through nitrogen fixation, phosphate solubilization or iron chelation, by biocontrol, by outcompeting pathogens for nutrients through siderophore production, or by establishing the plant's systemic resistance. The second way of plant growth promotion is directly by producing phytohormones such as auxin or cytokinin [33] or by producing the enzyme 1-aminocyclopropane-1-carboxylate deaminase, which lowers plant ethylene levels [34]. These processes are achieved by a consortium of different roots or nodules endophytes with eventual coordination with rhizospheric bacteria to help in more nutrient mobilization [30]. For example one of the common plant growth promotion hormones produced by *Pseudomonas*,

Klebsiella and *Enterobacter* spp. is indole acetic acid (IAA) which its production directly associated with plant growth stimulation [35–37]. While both *Pseudomonas* and *Enterobacter* spp. solubilize phosphate and exhibit 1-aminocyclopropane-1-carboxylate-deaminase activity during biotic and abiotic stress and environmental stresses [35, 38]. Some species of endophytes like *Pseudomonas* and *Klebsiella* associated with groundnut nodules were found distinguished by their ability to fix nitrogen [32]. Others like *Enterobacter spp* were found characterized by ammonification and b-1, 3 glucanase activities [35].

The key trait enables interference of endophytes activities with the host plant physiology is production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase by which endophytes might profit from association with the plant because colonization is enhanced, at the same time the plant benefit by stress reduction and increase root growth [12].

However, the process of colonization and interaction between endophytes and different plants is seem to be less complicated and inexpensive regarding energy consumed, if we compared it with colonization and interaction of *Rhizobium*-legumes which includes "pre-infection, root colonization, root adhesion, hair branching, hair curling, infection, nodule initiation, bacterial release, bacteroid development, nodule function, nitrogen fixation, complementary functions, and nodule persistence" [39].

4. Soil stress mitigation

This section shed light on how the interaction between the legumes and their associated endophytes can contribute in addressing the major types of abiotic stress face the plants which include drought, salinity, acidity/alkalinity and heavy metal toxicity [40].

4.1 Using legumes and nodule endophytic bacteria to mitigate soil stress

The application of consortium of proper rhizobia together with plant growth-promoting microorganisms is an effective and environment-friendly approach helps to alleviate different stress conditions such as drought and salinity among others, increase the efficiency of the symbiotic processes and improve the crop yield by different mechanisms of actions under variable conditions [41, 42]. The ability of these bacteria to withstand to high levels of stresses makes them valuable to enhance legume production in harsh environmental conditions [42].

In this regard there are strong evidences that the endophytic bacteria serve host functions like osmolytes [3]. For example Abd-Allah et al. [43] investigated the effect of the endophytic bacterium *Bacillus subtilis* BERA 71 on chickpea plants under saline conditions. They found that application of this endophytic bacterium significantly enhanced the growth of chickpea plants and ameliorate salinity induced oxidative damage. It is also increased macro-nutrients like N, P, K, Ca, and Mg, at the same time decreased sodium accumulation under salinity. Also Barnawal et al. [44] reported that *Arthrobacter protophormiae* strain inhibits the nodule of *Pisum sativum* was found enhanced its growth under high salinity conditions, increasing nodule number and reducing salt stress. Although many investigators have co-inoculated soil isolates and species of *Rhizobium*, fewer studies have co-inoculated nodule associated bacteria and rhizobia [9]. The process of endophyte-*Rhizobium* co-inoculation is promising technology because the association between host and microbiome did not depend solely on N_2-fixing rhizobia, but also required a direct connection between symbiotically linked bacterial communities that resides in the rhizosphere [6].

Co-inoculations of legumes with indigenous rhizobia and salt–tolerant non–rhizobial nodule associated bacteria and rhizosphere bacteria may offer sustainable solution for boosting biological nitrogen fixation and the productivity of legumes in soils affected with different extreme environmental conditions [9, 45]. In the process of co-inoculations, it is not possible to determine exactly which bacterial mechanisms have a more pronounced impact in a given plant–microbe association [46]. However, in multi-microbial interactions local isolates are recommended because of their physiological and genetic adaptation to the environment [41].

Hence to address different soil stress problems, using consortium of locally isolated rhizobia and endophtes is seem to be the most effective and efficient approach than using rhizobium or endphytes alone because in co-inoculation the different plant needs are provided by the different bacteria constitute the inoculum.

4.1.1 Salinity

Soil salinity is one of the major factors destroy environment and limiting the legumes productivity [47]. Soil salinity is increasing continuously due to continuous climate change, and it becomes limiting factor for crop productivity worldwide [3]. It is estimated that more than 6% of land area has affected by salinity [48] and about 10–20% of cultivated and 27–33% of irrigated agricultural lands are afflicted by high salinity [7, 42]. This degradation of the soil results in decreasing the quality and productivity of crops worldwide [42], at the time world population increase which necessitates utilizing lands affected by salinity to meet the food needs [49].

The negative effects of salinity represent in causes osmotic and ionic stresses in plants and constrain the growth. Upon the plant exposed to salinity, osmotic stress occurs immediately because hypertonic conditions outside the cell take place. Ionic stress elevated after several days as a result of the accumulation of Na^+ and Cl^- ion inside the cell. The effects of this osmotic stress are reduction of the "cell turgor pressure, cell elongation and cell division rates" [3]. Other effects include modulation of the cell ion homeostasis which leads to "changes in hormonal status, transpiration, photosynthesis, nutrient translocation" among other metabolic processes [50]. To adapt to the stress, plants have immune system with different physiological mechanisms to induce tolerance. The same role also played by plant-associated microbes [11] which capable to exclude salts and via intracellular accumulate inorganic and/or organic solutes to balance osmotic across the membrane [51]. However, the diversity of microbial properties capable of promoting plant growth makes it difficult to be sure about the importance of particular mechanisms within specific plant–microbe interactions in saline environments [52]. Also to alleviate the effects of salt stress, endophytes play a positive role to adjust cell osmotic, detoxification, regulate phytohormone and nutrient acquisition in plants [3]. The excellent plant growth promoters under stress conditions are endophytes containing ACC deaminase activities due to their ability to block ethylene production at each specific location and "cleaves the ethylene precursor ACC to α-ketobutyrate and ammonia", which metabolized by the bacteria for their growth [53, 54]. These microbes include different genera of *Bacillus*, *Pseudomonas*, *Klebsiella*, *Serratia*, *Arthrobacter*, *Streptomyces*, *Isoptericola*, and *Microbacterium* [55] which show their ACC deaminase properties with high salt concentration [3]. This was verified by Iniguez et al. [56] experiment in which endophytic relationship of the *Klebsiella pneumoniae* strain342 with *Medicago truncatula* was established which indicates that ACC deaminase-producing endophytic bacteria reduce stress ethylene levels in plants and alleviate the damaging effect of this hormone under stress conditions [3]. Microbial volatile organic compounds are among other compounds produced by microbes which play a role in salinity stress conditions due to their

ability to trigger induced systemic resistance in plants [57]. As mentioned before, the symbiotic relationship of rhizobia and legume in presence of non-symbiotic endophytic bacteria, also help in adapting to salinity. For example, *Rhizobium* and *Pseudomonas* when used as co-inoculant promoted mung bean growth under salinity stress by providing auxin and ACC deaminase [58]. This finding indicates that the two bacteria worked in a complementary way, one bacterium provides the plant hormone auxin (probably the *Rhizobium* strain) and the other provides the enzyme ACC deaminase (may be the *Pseudomonas*).

However, still there is no comprehensive review available about exploitation of legume-endophytes relationship to ameliorate salt stress in the soil with concentration in the beneficial effects of endophytes. This necessitates raising scientific community awareness to carry out research in this field to enhance agriculture productivity under saline environments [3].

4.1.2 Acidity/alkalinity

Another problem increased by the impacts of global change is soil acidity or alkalinity which also limits the legumes productivity. During symbiosis process, it is found that rhizobia are more sensitive to acidity than legumes, this means incapability of rhizobia to persist and survive in acidic soils which reduces symbiosis effectiveness and legumes productivity. To overcome this problem, it is important to seek for indigenous acid or alkaline-tolerant rhizobia capable of nitrogen fixation and enhance legumes production under acidic or alkaline conditions [42, 59].

For addressing the problem of acidity or alkalinity of the soil, legumes afford acidity and alkalinity simply can be grown regardless of the growth promoting characteristics and the stress tolerance of their associated bacteria, because some legumes prefer soils pH ranged between light acid to alkaline such as pea, melilot, alfalfa and haricot while clover, lupine and soybean grow well in the acidic soil [60]. Nevertheless, legumes treated with endophytes isolated from acidic or alkaline soils expected to promote their growth in acidic or alkaline soil more than untreated legumes. However, acidic pH (3.8–4.5) was found retarded the development and activity of the bacteria *Rhizobium leguminosarum* and reduces pea yields [60]. At the same time endophytic bacteria like *Klebsiella* isolated from groundnut grown in different regions were found grow at pH ranged between 4 and 8 [61]. Like these endophytes and their leguminous host can be harnessed in co-inoculation process to mitigate acidity or alkalinity of the affected soils.

4.1.3 Drought

Drought is another consequence of climate change and represent major constrain of agriculture. It is estimated that by 2050 drought is expected to cause serious plant growth problems for more than 50% of the arable lands [62]. Among the different environmental stresses, drought is the most destructive factor retarding symbiosis process and rhizobial growth [63].

Legumes and their associated microbes can play role to mitigate the negative effects in the areas affected by drought because microbe live within plant tissues and release various phytochemicals that assist plant to withstand drought stress [1]. The legume associated microbes consortium work in an integrated manner to enhance drought stress tolerance in plants through improve root length and density, root construction to assist in better water and nutrient uptake, enhance soil-water-plant relationships, manipulating phytohormonal signaling, increase

different organic and inorganic solutes, increase the synthesis of osmolytes like proline, increase antioxidant enzymes that scavenge reactive oxygen species (ROS), decrease the regulation of stress-responsive genes and producing drought-tolerant substances like abscisic acid, indole-3-acetic acid, ACC deaminase and volatile organic compounds [1, 8, 52, 57, 64, 65].

Research conducted to study response to drought stress using legumes such as soybean and single endophyte *Pseudomonas simiae* AU showed that inoculation process resulted in expression of their respective genes, induced proline and total soluble sugar content [66]. More drought tolerance characteristics were pronounced when soybean treated with *Bacillus* and *Pseudomonas*, they "improve plant growth, membrane integrity, water status, accumulation of compatible solutes, and osmolytes" [67]. Arshad et al. [68] stated that drought stress on the growth and yield of *Pisum sativum* was significantly decreased by a strain of *Pseudomonas spp*. with ACC deaminase enzyme activity, and concluded that the drought stress induced inhibitory effects of ethylene could be eliminated by application of bacteria containing this enzyme. Likewise, it is reported recently that there is increasing in using rhizobia as biofertilizer to alleviate the effect of drought on legumes growth under stressed environment [63].

It is expected to obtain the best growth conditions in drought affected areas if legumes inoculated with consortium of efficient locally isolated rhizobia and endophytes. The locally isolated strains are more adaptive to the different adverse environmental conditions in the drought areas from where they were isolated, this gives them the advantage to work at maximum rate to mitigate drought.

All the above mentioned advantages of the legume-associated microbes result in positive effects on the overall plant growth which in turn enhance legumes production in the areas affected by drought.

4.1.4 Heavy metal contamination

Heavy metal contamination of the soil is a result of different anthropogenic activities such as mining, modern agricultural practices and industrialization. The deleterious effects of heavy metals discharged from different sources represent in causing potential human risks, accumulation within soils and harm ecosystems, enter food chain, poison plants and seriously affect the beneficial soil microbial compositions and their physiological functions [69]. Soil contamination with heavy metals results in toxic effects on plants [10]. To address this environmental problem, using association of plants with various microorganisms represents a sustainable strategy [40]. However, till now very few studies evaluated the effect of bacterial consortia for heavy metal contamination mitigation [40]. Some studies reported that some bacteria have adapted well to environments polluted with heavy metals and exhibit resistance mechanisms like enhancing the expression of stress related gene, metal bioaccumulation, anti-oxidant activities and alteration of the levels of 1-aminocyclopropane-1-carboxylate (ACC) [10, 70, 71]. Other bacteria adopt different strategies to reduce the toxicity in soil under heavy metal contamination. These strategies include "metal adsorption, bioaccumulation, expulsion of metal outside the cell, biotransformation, release of chelating agents, acidification of adjacent environment, and the ability to change in redox potential" [72].

There are many legumes-associated microbes reported promote plant growth under heavy metal stress like *Trifolium repens* tolerate Fe, Mn, Zn and Cd when associated with *Rhodococcus erythropolis*, *Achromobacter sp*., *Microbacterium sp*. and *Bacillus cereus* [46, 73]. Also *Lupinus luteus* was found grow under high

concentration of Cu, Cd, Pb when associated with *Bradyrhizobium sp*. 750, *Pseudomonas sp*. and *Ochrobactrum cytisi* [74]. In another study *Ochrobactrum* have been used in consortia with nodule-forming bacteria and other plant growth promoting bacteria and *Lupinus luteus* in heavy metal contaminated soils, the result showed increasing of plant biomass and decreasing accumulation of heavy metals [74]. Also when *Vicia faba* cultivated in soil moderately contaminated with Cu and inoculated with consortium of bacteria containing *Rhizobium sp*. CCNWSX0481, *Rhizobium leguminosarum* bv. viciae, *Enterobacter cloacae*, and *Pseudomonas sp*. 2 (2010), significant reduction of accumulated Cu in roots and increase in nodulation, growth and seed yield were observed [75]. These findings strongly indicate that bacterial consortia maximize benefits compared to individual strains in heavy metal stress mitigation [40]. The advantages of using heavy metal-tolerant microbes represent in sustainable and low-cost option to detoxify heavy metal contaminated soils through a process called bioremediation, enhance nitrogen-fixing efficiency, and promote the legumes growth, yields, and grain quality. To realize these benefits, isolation and selection of indigenous metal-tolerant rhizobia are recommended followed by metal resistance genes identification which then can be transferred through genetic engineering to other non-tolerant microbes used in contaminated soils clean up and remediation programs [69].

The legumes-microbes interaction process which results in heavy metal stress mitigation can be useful in coping of legumes with this harsh condition, and at the same time this approach can be useful in bioremediation programs. However, the questions need to be answered through research are: In presence of heavy metal tolerant microbes, Do the legumes able to grow at unlimited concentration of heavy metal or there are limitations? The second question is in case of heavy metal uptake and accumulation in legumes tissue, do legumes able to assimilate the accumulated heavy metals to be useful or at least not harmful? And in which parts of plant more absorbed heavy metals accumulate?

5. The genetic mechanisms involved in nodule endophytic bacteria-legume interaction to mitigate the different stresses

The process of associated bacteria-plant interaction in nature is a complex phenomenon includes biotic, abiotic, and genetic factors. Understanding of this process and the effect of this association is crucial to the agricultural applications [40]. In both plant and associated bacteria, different genes express during the interaction process which start with recognition of the plant and the associated bacteria, passing through colonization and interaction until ending by coping to live in the adverse condition. However, colonizing internal plant tissues differ in endophytes and rhizospheric bacteria due to differences in their genomes [76]. Endophytes protect plants against the inhibitory effects of stresses and at the same time may alter plant gene expression that makes plant less likely to give up to these stresses [77].

Generally, associated microbes have genes responsible for salt stress adaptation [78]. Therefore, during nodule endophytic-legume interaction "ACC-deaminase gene AcdS is expressed and regulated under different stressed environmental conditions" [54]. Significant changes in gene expression take place to mitigate the different environmental stresses. For example in *Sinorhizobium meliloti* 1021 exposed to salt 52 of 137 genes were induced and the remaining 85 were repressed. The long term exposure of this bacterium to salt "activated genes related to polysaccharide biosynthesis and transport of small biomolecules like amino acids, amines, peptides, anions, and alcohols" [79]. Likewise, sudden increase in

salt stress induced genes of unknown functions and repression proteins coding genes. The majority of the regulated genes located in the chromosome and others located on plasmid (pSmbB). This finding suggests the role of *Sinorhizobium meliloti* chromosomal and plasmid genes in the adaptation to salt stress. It is also reported that ribosomal genes and tricarboxylic acid cycle genes are repressed. It is important to show that 25% of genes regulated by salt encode ribosomal proteins [80]. Under osmotic stress, *Sinorhizobium meliloti* regulates the expression of BetS gene which represents a major component of the overall betaine uptake activities in response to salt stress and has a role in Gly-betaine/Pro-betaine transporter [81] involved in salt stress tolerance in *Medicago sativa* [82]. This finding indicates that acdS gene is responsible for salt tolerance and its expression confers host plant the ability to afford salinity. In addition, to overcome salinity stress using of *Sinorhizobium meliloti* would be a useful method [42]. For bacteria induce nodules, genes encoding Nod factors are also included in salt stress. For example in *Rhizobium tropici* CIAT899 46 different Nod factors were identified, of these 14 new Nod factors identified not produced under neutral or acid conditions [47]. Nod factor production increased in the same bacterium when grown under acid conditions [83]. Many other studies used PGPR with leguminous plants confirmed different genes expression under stress conditions. For example in soybean treated with *Pseudomonas simiae* AU, to tolerate drought different genes up-regulated. It is found that different factors involved in the process including "transcription factors (DREB/EREB), osmoprotectants (P5CS, GOLS), and water transporters (PIP and TIP)" [66]. Other studies also reported that stress-related genes may activated to regulate and enhance tolerance toward abiotic stresses through production of Ca^{2+} sensor calcineurin B-like proteins (CBLs) in different legumes such as chickpea [84] and soybean [77]. The definitive targets of these sensors are the abiotic stresses such as drought and salinity [85]. In chickpea the exogenous acdS gene of the salt-sensitive *Mesorhizobium ciceri* strain was found form nodules the same as salt-tolerant strain [86].

For heavy metal tolerance, to enhance the expression of stress response genes or the transcription factors, several signaling pathways activated like reactive oxygen species (ROS) pathway and hormone signaling pathways [85]. *Medicago sativa* produce mitogen-activated protein kinases (MAPKs) when exposed to excess Cu and Cd [87]. In *Medicago truncatula* different concentrations of Hg genes associated with ethylene metabolism and signaling were expressed [88]. From these findings it can be assumed that these genes involved in heavy metal tolerance for *Medicago sativa* and *Medicago truncatula*, and different genes expressed in case of soil contamination with different heavy metals. To address heavy metal stress problem, it is possible to make recombinant bacteria through exploiting different genes including "metal chelators, metal homeostasis, transporters, biodegradative enzymes, metal uptake regulators, and biotic and abiotic stress tolerance" [89].

In spite of the progress of the research in this field, regulatory networks of the interaction of host plant-associated microbes in heavy metal stress are unknown [10] and identification of undiscovered genes involved in endophytism has not been pursued systematically [90]. So efforts should be directed toward identification of different genes of legumes and their associated endophyts involved in the interaction processes, because like these information can benefit in biotechnological applications, recombinant technologies and ensure the efficiency of the interaction between the host legume and its associated bacteria.

The above mentioned findings confirm that the ability of plant growth promoting bacteria to ameliorate stresses is a genetic based, and the genes responsible for these traits induced and expressed once soil stress increased.

6. Strategies to select leguminous plants for future studies related to endophytes and soil stress

The strategy described below can be used as model and applied for legumes although it was suggested by Strobel and Castillo [91] to select plants generally for endophytes isolation. The strategy defined plants distinguished by special characters such as:

i. Plants from unique environmental settings like those characterized by an unusual biology and adopt novel strategies for survival.

ii. Plants have an ethno botanical history.

iii. Endemic plants characterized by an unusual longevity or occupied a certain ancient landmass.

iv. Plants growing in areas of great biodiversity.

7. Future prospective

To understand the endophytes and their interactions with the host legumes, multidisciplinary research include cultivation-independent techniques, the "Omics" fields like genomics, proteomics, metabolomics; and the advancing computational data-mining approaches among others are required. Research focus on isolation and characterization of indigenous rhizobia and endophytes are required combined with studies concentrate in regulatory networks of the interaction of host plant-associated microbes, mechanisms of regulation and expression of already known genes like AcdS gene, and identification of undiscovered genes involved in endophytism can play a crucial role in understanding of this interaction process. Like these studies contribute to obtain the optimum exploitation of legumes and their associated bacteria to mitigate climate change impacts. Also research directed toward using the legumes and their associated endophytes in phytoremidiation programs is highly encouraged to address soil heavy metal contamination which now represents real environmental threat.

8. Conclusions

Legumes and their associated endophytes are one of the key factors in climate change impact mitigation. Bacteria associated with legumes secrete different chemicals and work in social network to alleviate soil stresses and enhance plant growth. The tolerance of these bacteria to different stresses is genetically inherited trait which can be harnessed to produce genetic engineered stress tolerant bacteria used as inoculants in stressed soils. These genetic engineered stress tolerant bacteria will transmit stress tolerance genes through horizontal gene transfer to the indigenous bacteria when applied as inoculants in the stress affected soils, so enrichment of these soils with stress tolerant bacteria will take place eventually. Addressing soil stress problems by using these bacteria is sustainable, eco-friend and cheap approach. To realize the effectiveness and efficiency of this approach, using consortia of locally isolated rhizobia and other endophytes will be more applicable.

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Chapter 17

Legume-Rhizobium Interaction Benefits Implementation in Enhancing Faba bean (*Vicia faba* L.) Crop Yield and Economic Return

Bayou Bunkura Allito

Abstract

This study reports the interaction of rhizobium strains and varieties on yield and yield components of faba bean and the economic feasibility of the inoculant use in faba bean production. The two years field experiments used a split-plot design that involved six elite rhizobium strains as the main plot and three faba bean varieties as sub-plot treatments. Non-inoculated plants with N fertilizer and without fertilizer were included as +N (46 kg ha^{-1}) and −N controls, respectively. Phosphorus (P) was applied as triple super-phosphate at the time of sowing. Data on yield and yield components were collected and statistically analyzed. Partial budget, dominance, and marginal rate of return analysis were conducted to identify profitable rhizobial strain-variety combinations for each study location. Rhizobium strains NSFBR-15, TAL_1035 and NSFBR-12 increased grain and haulm yield of faba bean more than N fertilizer across the study locations. Location, rhizobium strain, and variety interaction influenced yield and yield components of faba bean. Economic analysis document that rhizobium inoculation for symbiotic N fixation is more profitable for supplying N to faba bean than N fertilizer application. Rhizobium strains NSFBR-15, TAL_1035, and NSFBR-12 with all faba bean varieties resulted in the highest revenue with a higher marginal rate of return at all study locations.

Keywords: faba bean, inoculation, nitrogen, strain, yield

1. Introduction

Faba bean (*Vicia faba* L) is the most important grain legume produced in Ethiopia [1]. The crop has high economic value with its edible seed serving as protein complement in the cereal-based Ethiopian diet [2], and contributes to smallholder income earnings [3]. Moreover, it has a great contribution to sustainable soil fertility improvement due to its ability in fixing N through symbiotic association with rhizobia [4] and thus can reduce the cost of inorganic fertilizer use and its negative impact on the environment [5]. Because of its nutritional and

economic values, increasing the production of faba bean in sub-Saharan Africa is very important to meet the demand of the growing population [6, 7].

Despite its high socio-economic importance, the yield of faba bean (1.6 t ha^{-1}) is very low compared with its potential yield (5 t ha^{-1}) [8]. Both biotic and abiotic factors account for the low productivity of faba bean in on-farm growing conditions [9]. Declining soil fertility is a major challenge contributing to decreasing agricultural productivity in sub-Saharan Africa [10]. Available nitrogen (N) often is deficient in soils and limits faba bean productivity in Ethiopia [10]. To get optimum production, N must be adequately available to the plants [11]. Unfortunately, farmers rarely use N fertilizer in faba bean production; instead, the crop is used as a restorer of soil fertility for the subsequent cereal crop [12]. The low use of N fertilizer is because most smallholder farmers have very low financial resources to purchase inorganic fertilizers. It is, therefore, imperative to search for alternatives that can increase crop yields to satisfy the growing protein food demand while maintaining environmental safety and protection [13].

Native rhizobial populations in many soils may not be adequate or effective to symbiotically fix N [14–16]. Effective rhizobial population in the rhizosphere can be increased by inoculation [17] where natural N fixation is not optimal. Thus, there is a need for inoculation with an appropriate rhizobial strain to improve N fixation in faba bean production [18, 19].

Faba bean is one of the most efficient N_2 fixing legumes, which can fulfill most of its N requirement through symbiotic N fixation [20]. However, legume-rhizobia symbiosis is highly specific that, fitness between rhizobium strain and legume variety is very essential for successful nodulation and N fixation [21]. Faba bean usually establishes an effective symbiotic association with *Rhizobium leguminosarum* bv. viciae (Rlv) [22]. However, several studies [4, 23] have revealed that, *R. leguminosarum* bv. viciae varies in legume host-specificity and effectiveness in N fixation. Besides, the adaptability of rhizobial strain in a given soil environment should be considered as an important criterion during inoculant strain selection.

Research in sub-Saharan Africa has mainly focused on developing high-yielding varieties under optimum growing conditions and/or isolation and characterization of native rhizobia in the laboratory and under greenhouse conditions. Although promising faba bean nodulating rhizobia strains can be identified under controlled conditions [24–26], its interaction with the biophysical environment necessitates comprehensive field investigations. Thus, there is a need to identify best performing strain × variety combinations for site-specific inoculant development. This study aimed to (i) investigate the interaction effects of selected rhizobium strains on grain yield and yield component of faba bean varieties under field conditions, and (ii) evaluate the economic benefits of using rhizobial inoculants in faba bean production in southern Ethiopia.

2. Materials and methods

2.1 Description of experimental sites

Four locations were selected in two major faba bean growing agro-ecologies (cool-humid and cool sub-humid) in southern Ethiopia. Two locations, Hankomolicha and Abala-Gase, in cool humid and two locations, Haranfama and Gike-Atoye, in cool sub-humid agro-ecological zones were selected for field experiments. The experimental locations in cool humid and cool sub-humid agro-ecological zones received 1473 and 1093 mm mean annual rainfall (**Table 1**),

Year		Cool humid (location: HK and AG)			Cool sub-humid (location: HR and GA)		
		Rainfall	[a]Max. T	[b]Min. T	Rainfall	[a]Max. T	[b]Min. T
		mm	°C	°C	Mm	°C	°C
2017	June	180	14.1	7.7	128	21.3	12.9
	July	134	16.4	5.6	97	24.3	12.5
	August	182	16.3	6.1	192	22.8	11.6
	September	160	16.5	7.2	104	23.7	13.4
	Annual	1477	17.1	8.1	1303	25.2	15.1
2018	June	63	17.0	9.2	35	25.1	15.3
	July	219	15.6	5.2	161	23.3	11.9
	August	219	14.1	6.5	166	20.9	11.3
	September	206	14.0	7.8	204	19.1	10.9
	Annual	1591	17.4	9.3	1199	24.5	14.4
10 years (2009–2018)	Annual average	1473	15.4	7.1	1093	22.4	11.7

[a]*Maximum temperature.*
[b]*Minimum temperature; HM = Hankomolicha; AG = Abala-Gase; HR = Haranfama; GA = Gike-Atoye.*

Table 1.
Annual average rainfall and the mean maximum and minimum temperatures during the study period and long-term average.

respectively. The distribution of rainfall in both agro-ecologies is bimodal. A minor rainy season occurs from February to April whereas the major rainy season occurs from June to September. In each agro-ecology, experiments were conducted at selected locations during the major rainy season of 2017 and 2018.

2.2 Soil sampling and analysis

Pre-sowing soil samples were collected from each location. Samples were cored to a depth of 20 cm from 20 random locations across each experimental field and composited for the determination of soil chemical and physical properties using standard laboratory methods [27]. The results are shown in **Table 2**. The soil properties were examined to identify whether variability exists which could explain the occurrence and magnitude of treatments response. Such knowledge is important to assist in targeting technologies and to identify the need for further research on soil fertility management options. Textural classes of the surface soil of the study locations varied from clay to loam and soil pH ranged from slightly acidic (6.57) to weakly acidic (5.37–6.02) with the medium organic carbon and total N contents [28]. Cation exchange capacity (CEC) of the soils was in the range of medium to high rating (22.60–32.81 meq/100 g) which is adequate for crop production. Soil available phosphorus contents were low (5.7–12.6 mg kg^{-1}) to medium (12.6 mg kg^{-1}), suggesting that supplementary phosphorus may be required for optimum crop production.

2.3 Sources of strains and seeds

Six elite rhizobial strains (NSFBR-12, NSFBR-15, NSFBR-20, HUFBR-17, TAL_1035, and EAL-110), originally collected by Haremaya University, Holleta

Soil parameters		Study locations			
		Hankomolicha	Abala-Gase	Haramfama	Gike-Atoye
pH (1:2; Soil:H_2O)[a]		6.57	5.37	6.02	5.60
Available P (mg kg^{-1})[b]		12.60	5.70	8.40	6.03
Total nitrogen (%)[c]		0.17	0.17	0.16	0.22
Organic carbon (%)[d]		2.06	2.22	1.75	2.34
CEC (meq/100 g)[e]		29.40	27.56	22.60	32.81
Exchangeable bases $cmol_{(+)}$ kg^{-1}[e]	K	3.14	0.75	2.36	1.25
	Ca	13.40	15.09	12.60	17.73
	Mg	7.22	5.38	6.44	5.20
Exc. acidity ($cmol_{(+)}$ kg^{-1})[f]		0.40	0.48	0.12	0.52
Bulk density (g cm^{-3})[g]		1.24	1.21	1.35	1.25
Textural class[h]		Clay	Clay loam	Loam	Clay

Method:
[a][29]; [b][30]; [c][31]; [d][32]; [e][27]; [f][33]; [g][34]; [h][35].

Table 2.
Initial physical and chemical properties of surface soils (0–20 cm) at the study locations.

Agricultural Research Center, and National Soil Laboratory (NSL) in Ethiopia were used for the study. The inoculum was used at the concentration of approximately 10^9 cells g^{-1} in peat carrier. The purity of strain cultures was assessed in the Soil Microbiology Laboratory at Holleta Agricultural Research and Haremaya University. The sterility of the carrier was checked before mixing with the rhizobial culture. Seeds of three nationally registered faba bean varieties (Dosha (COLL 155/00–3), Moti (EH 95078–6), Gora (EKOl024–1-2) were provided by Holleta Agricultural Research Centers for use in this study.

2.4 Treatments and experimental design

The experimental design was a randomized complete block design (RCBD) in a split-plot arrangement with four replicates nested at four different locations. Main plot treatments consisted of six rhizobium strains (NSFBR-12, NSFBR-15, NSFBR-20, HUFBR-17, TAL_1035 and EAL-110). Non-inoculated plants supplied with and without N fertilizer served as +N and −N controls, respectively. Sub-plot treatments were three faba bean varieties (Moti, Dosha, and Gora).

Land preparation was done manually using a heavy hoe for primary tillage to make the field suitable for planting and divided into blocks and further into individual plots. Sub-plot size was 4 × 4 m (16 m^2). Each variety was planted in 10 rows plot of 4 m length per major plot. The inter-row and intra-row spacing were maintained at 40 and 10 cm, respectively. Spacing between sub-plots and major plots were 1 and 1.5 m, respectively. Peat carrier-based inoculant of each strain was applied at the rate of 10 g kg^{-1} seed [36]. Thus, the required quantity of inoculant was suspended in a 1:1 ratio in a 10% sugar solution in order to ensure that all the applied inoculum stuck to the seed. The thick slurry of the inoculant was gently mixed with dry seed so that all seeds received a thin coating of the inoculant. Inoculation was done just before planting under shade to maintain the viability of rhizobium.

The seed was sown at a depth of about 4 cm. Phosphorus was applied to all plots in the form of triple-superphosphate (TSP) at the recommended rate of 46 kg P_2O_5 at planting. Nitrogen fertilizer was applied two times in equal split doses to non-inoculated +N control treatment, at planting and six weeks after sowing at a recommended rate of 46 kg N ha^{-1}. All other crop management and protection practices were applied uniformly to plots.

2.5 Data collection and analysis

At physiological maturity, 10 plants were randomly sampled per plot from interior rows. Mean plant height was determined by measuring the height of each plant. Pods were counted for all ten plants and the average values were recorded as a number of pods per plant. All pods were picked from sampled plants per plot and the plants were cut at the base and removed from a plot. The straw was cut into small pieces and placed in pre-marked paper bags. The pod samples were sun-dried and threshed manually. The grain and husk were put into separate pre-labeled paper bags. The straw, grain, and husk samples were oven-dried at 70°C for 72 hours and weighed. Harvest index was calculated as a ratio of grain yield to above-ground biomass yield.

At the final harvest, the remaining plant stands were marked leaving the two border rows per plot on both sides and 0.5 m row length on both ends of all plots. Grain yield was determined from an area of 9.6 m^2 on each sub-plot. The pods were picked from all plants which were marked for harvest, and placed in pre-marked separate bags. Harvested pods were sun-dried and threshed manually. The grain was further dried and weighed. The moisture content was measured using a portable moisture tester and later adjusted to 10% standard moisture content. A hundred seeds were counted three times from the total seeds of each plot and weighed to determine the average hundred seed weight per plot.

The data were subjected to Analysis of Variance (AOV) using SAS [37] computer software (SAS Institute Inc.). Combined analysis of variance was done to assess significance among locations, rhizobium strains, faba bean varieties, and interactions among these three factors (location, strain, and variety) for all measured parameters. Mean separation and comparison were done by using Duncan's Multiple Range Test. A Pearson correlation test was conducted to determine the association among treatment means using a $p \leq 0.05$ probability level.

2.6 Economic feasibility analysis

Experimental data were organized in order to elucidate the costs and benefits of each treatment. Additional cost and benefit of each treatment were calculated relative to respective non-inoculated −N control. Extra costs incurred included purchase of inoculants and N fertilizer, inputs application, transportation, and labor. Total variable costs (TVC) comprised all variable costs for particular treatments. The average yield was adjusted 10% downward to reflect the yield expected from the same treatment under farmers' management. Additional benefits comprised revenue from additional faba bean grain yield over the control. Net benefit and benefit-cost ratio were calculated using Eqs. (1–3) as below [38].

$$\text{GFB (in USD)} = \text{AY} \times \text{FP(in USD)} \tag{1}$$

$$\text{NB (in USD)} = \text{GFB (in USD)} - \text{TVC} \tag{2}$$

$$BCR = \frac{NB}{TVC} \quad (3)$$

Where, AY = adjusted yield; FP = field price per unit yield; GFB = Gross field benefit; NB = Net benefit; TVC = total variable cost; BCR = Benefit cost ratio.

In order to select potentially profitable treatments among the 24 treatments, the dominance analysis was employed according to CIMMYT [38]. Treatments were arranged in order of increasing variable costs and considered as dominated if its net benefit was lower than the preceding treatment. Marginal rate of return (MRR%) for each dominant treatment was calculated by using the formula [39].

$$MRR = \frac{\Delta NB}{\Delta TVC} \times 100 \quad (4)$$

Where: MRR = marginal rate of return in percentage, ΔNB = change in net benefits and ΔTVC = change in total variable cost.

The marginal rate of return for dominant treatments is returned that can be obtained per unit of an investment expressed as a percentage. A 100% was considered as the minimum acceptable rate of return for recommendation to farmers [40]. A hundred percent (100%) MRR implies a return of one dollar for every one dollar investment in a given variable input [38].

3. Results

3.1 Effect of inoculation on grain yield of faba bean

Rhizobium strain × faba bean variety interaction effect on grain yield is presented in **Table 3**. Rhizobium strains NSFBR-15 and TAL_1035 resulted in higher grain yields, whereas HUFBR-17, EAL-110, and NSFBR-20 inoculation resulted in lower grain yield relative to 46 kg ha^{-1} (**Table 3**). At Hankomolicha, NSFBR-15 × Moti and TAL_1035 × Gora produced the first and the second highest grain yield, respectively whereas TAL_1035 × Gora and NSFBR-15 × Gora produced the first and the second highest grain yield, respectively at Haramfama. NSFBR-15 × Gora produced the highest grain yield at Gike-Atoye whereas NSFBR-15 × Gora, TAL_1035 × Dosha and NSFBR-15 × Moti produced, the first, the second and the third highest grain yield, respectively at Abala-Gase.

Mean grain yields ranged from 1.89–4.28, 1.64–3.43, 1.79–3.76, and 2.12–3.88 t ha^{-1} at Hankomolicha, Haranfama, Abala-Gase, and Gike-Atoye, respectively (**Table 3**). The highest grain yield (4.28 t ha^{-1}) at Hankomolicha was obtained by Moti variety inoculated with NSFBR-15 which also resulted in the highest grain yields of 3.88 and 3.76 t ha^{-1} at Gike-Atoye and Abala-Gase, respectively for Gora variety. Variety Gora inoculated with TAL_1035 produced the highest grain yield (3.43 t ha^{-1}) at Haranfama. The lowest yields were obtained by non-inoculated −N control plants at all study locations.

There were significant ($p \leq 0.05$) strain × location interaction effects for grain yield of faba bean. The highest mean grain yield among the study locations was obtained at Hankomolicha (3.05 t ha^{-1}) followed by Gike-Atoye (2.97 t ha^{-1}) whereas the least mean grain yield was recorded at Haranfama (2.50 t ha^{-1}) (**Figure 1**). Inoculation with rhizobia strains HUFBR-17, EAL-110, and NSFBR-20 resulted in lower grain yield than their respective location average whereas the grain yields obtained by TAL_1035, NSFBR-15, and

Rhizobium strains	Hankomolicha			Haranfama			Abala-Gase			Gike-Atoye		
	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora
TAL_1035	3.42^{bc}	3.47^{a}	3.93^{a}	2.76^{ab}	2.79^{ab}	3.43^{a}	3.40^{ab}	3.63^{a}	3.15^{bc}	3.37^{a}	3.22^{a}	3.51^{ab}
NSFBR-15	4.28^{a}	3.71^{a}	3.40^{b}	2.81^{a}	3.00^{a}	3.19^{a}	3.54^{a}	3.40^{ab}	3.76^{a}	3.25^{a}	3.23^{a}	3.88^{a}
HUFBR-17	2.66^{ef}	2.87^{b}	2.53^{c}	2.47^{bc}	2.03^{d}	2.09^{c}	2.07^{cd}	2.61^{c}	2.41^{e}	2.79^{bc}	3.00^{ab}	2.43^{de}
NSFBR-12	3.09^{cd}	3.40^{a}	3.54^{ab}	2.74^{ab}	2.54^{bc}	2.86^{b}	3.23^{ab}	3.41^{ab}	3.21^{bc}	3.16^{ab}	2.93^{ab}	3.27^{b}
EAL-110	$3.01d^{e}$	2.27^{c}	2.46^{c}	2.21^{cd}	2.28^{cd}	2.11^{c}	3.25^{ab}	2.59^{c}	2.86^{cd}	3.01^{ab}	2.98^{ab}	2.84^{c}
NSFBR-20	2.42^{f}	2.50^{bc}	2.74^{c}	2.37^{c}	2.06^{d}	1.92^{cd}	2.35^{c}	2.68^{c}	2.55^{de}	3.07^{ab}	2.66^{bc}	2.80^{cd}
+N	3.53^{b}	3.36^{a}	3.62^{ab}	2.81^{a}	2.86^{a}	2.86^{b}	3.03^{b}	3.07^{b}	3.26^{b}	3.19^{ab}	2.85^{ab}	2.79^{cd}
−N	1.89^{g}	2.42^{c}	2.75^{c}	2.03^{d}	2.28^{cd}	1.64^{d}	1.85^{d}	1.79^{d}	1.83^{f}	2.55^{c}	2.39^{c}	2.12^{e}
CV (%)		**8.5**			**8.0**			**8.4**			**8.5**	

Mean values in the same column with a different letter(s) are significantly different at $p \leq 0.05$ *probability level.*

Table 3.
Rhizobium strain × faba bean variety interaction effect on grain yield of faba bean at the study locations.

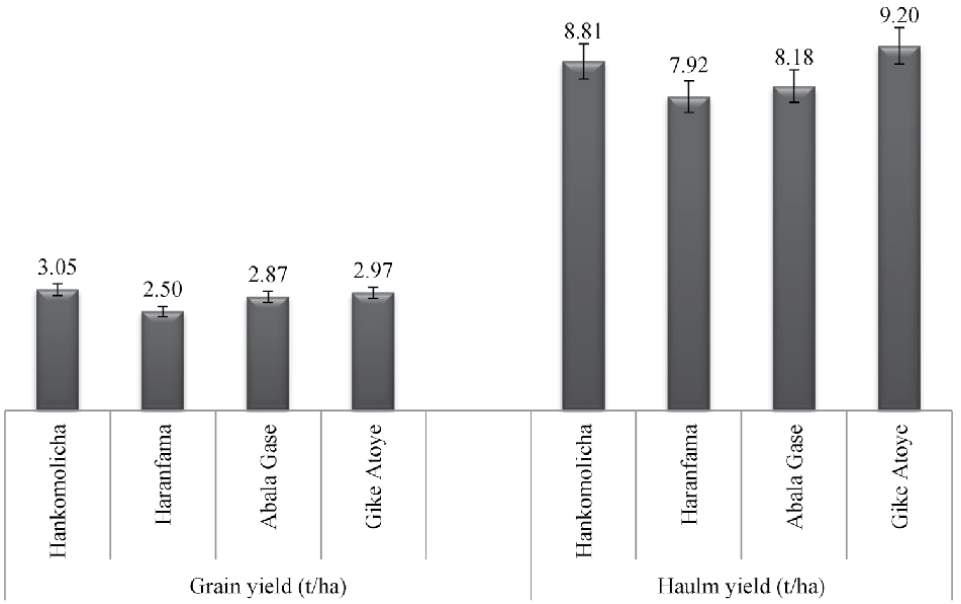

Figure 1.
Mean grain and haulm yield response to rhizobia strain inoculation at the different study locations.

NSFBR-12 inoculation were higher than that of their respective location average (**Table 3** and **Figure 1**).

Grain yield increment due to inoculation ranged from 17.9 to 62.3% over non-inoculated −N control. Inoculation with NSFBR-15, TAL_1035, and NSFBR-12 resulted in 62.3, 56.9, and 46.4% of grain yield increments, respectively; while 46 kg N ha^{-1} resulted in 45.8% grain yield increment over non-inoculated −N control plant (**Figure 2**). Nitrogen fertilizer application (46 kg ha^{-1}) increased grain yields of faba bean by 24.1, 16.6, and 23.5% over inoculation with HUFBR-17, EAL-110, and NSFBR-20, respectively. However, grain yields obtained by NSFBR-15, TAL_1035 and NSFBR-12 inoculation surpassed those obtained by non-inoculated +N controls (**Table 3** and **Figure 2**). Inoculation with NSFBR-15, TAL_1035, and NSFBR-12 showed 11.3, 7.7, and 0.4% increments in grain yield compared to the non-inoculated +N control treatment, respectively.

3.2 Effect of inoculation on haulm yield of faba bean

The effect of rhizobium strains inoculation on haulm (straw + husk) yield is presented in **Table 4**. Haulm yield was significantly ($p \leq 0.01$) affected by location, rhizobium strains inoculation, strain × variety, and strain × variety × location interactions. No significant differences in haulm yields were observed among the faba bean varieties. Rhizobium strains TAL_1035, NSFBR-15, and NSFBR-12 inoculation showed a great positive response in haulm yield compared to non-inoculated −N control (**Table 4**). The mean haulm yields increased by 98.0, 91.0, and 78.7% over non-inoculated −N control treatments when inoculated with NSFBR-15, TAL_1035, and NSFBR0–12, respectively; whereas 46 kg N ha^{-1} enhanced haulm yield by 71.1% over −N control (**Figure 2**). Nitrogen fertilizer application (46 kg N ha^{-1}) also increased haulm yields by 30.9, 20.0, and 26.5% over inoculation with HUFBR-17, EAL-110, and NSFBR-20, respectively. However, haulm yield obtained by NSFBR-15, TAL_1035, and NSFBR-12 inoculation surpassed non-

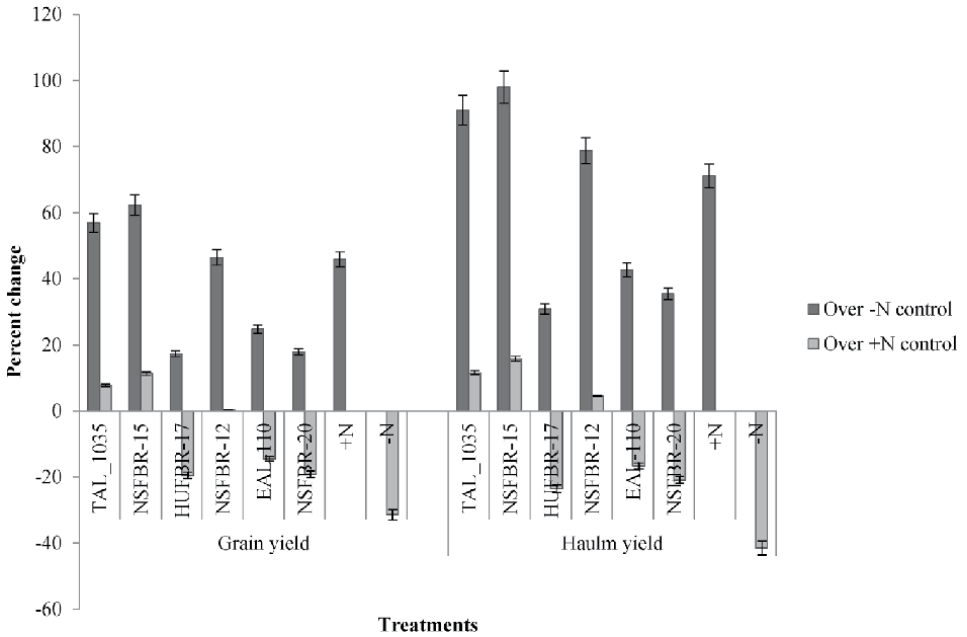

Figure 2.
Percent change in grain and haulm yields of faba bean following rhizobium strains inoculation.

inoculated +N control (**Figure 2**). Rhizobium strains NSFBR-15, TAL_1035, and NSFBR-12 inoculation resulted in 15.8, 11.6, and 4.5% increase in haulm yield over non-inoculated +N control, respectively (**Figure 2**).

Mean haulm yield varied across the study locations (**Table 4** and **Figure 1**). The highest haulm yields at Hankomolicha, Haranfama, Abala-Gase, and Gike-Atoye were 12.20, 10.52, 13.11, and 13.84 t ha^{-1} whereas the lowest haulm yields were 5.47, 5.84, 2.77, and 4.49 t ha^{-1}, respectively. Variety Gora produced the highest haulm yield (12.20 t ha^{-1}) at Hakomolicha when inoculated with NSFBR-12 and 10.52 t ha^{-1} when inoculated with TAL_1035 at Haranfama, 13.11 and 13.84 t ha^{-1} when inoculated with NSFBR-15 at Abala-Gase and Gike-Atoye, respectively. Among the study locations, the highest mean haulm yield was obtained at Gike-Atoye (9.20 t ha^{-1}) followed by Hankomolicha (8.82 t ha^{-1}) (**Figure 1**). Haulm yield increments following NSFBR-15, TAL_1035 and NSFBR-12 inoculation were consistent over the study locations. Haulm yields obtained by NSFBR-15, TAL_1035, and NSFBR-12 inoculation and 46 kg N ha^{-1} was higher than that of their respective location average. In general, the order of rhizobium strains effectiveness on yield and yield components was: NSFBR-15 > TAL_1035 > NSFBR-12 > N fertilizer.

3.3 Inoculation effect on growth and yield components

Location × strain × variety interaction had a significant ($p \leq 0.01$) effect on plant height, pods plant^{-1}, and hundred seed weight of faba bean. Rhizobium strains NSFBR-15, TAL_1035 and NSFBR-12 inoculation significantly increased plant height of faba bean varieties at all study locations relative to non-inoculated −N control (**Table 5**). Variety Gora attained the highest height (171.5 cm) at Gike-Atoye when inoculated with NSFBR-12 while variety Dosha reached the highest height of 168.3 cm at Abala-Gase when inoculated with NSFBR-15. Varieties Moti

Rhizobium strains	Hankomolicha			Haranfama			Abala-Gase			Gike-Atoye		
	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora
	(t/ha)			(t/ha)			(t/ha)			(t/ha)		
TAL_1035	8.97^{b}	10.01^{ab}	11.03^{b}	9.11^{a}	8.80^{ab}	10.52^{a}	11.89^{a}	10.75^{a}	9.53^{bc}	10.75^{a}	11.57^{a}	12.40^{ab}
NSFBR-15	10.41^{a}	10.66^{a}	9.54^{cd}	9.04^{a}	9.50^{a}	9.94^{ab}	10.74^{ab}	11.47^{a}	13.11^{a}	10.78^{a}	10.91^{ab}	13.84^{a}
HUFBR-17	8.44^{b}	8.99^{bc}	7.38^{e}	6.38^{d}	6.81^{de}	6.96^{d}	4.16^{cd}	5.88^{c}	6.83^{d}	8.27^{b}	9.49^{abc}	6.26^{d}
NSFBR-12	10.38^{a}	9.24^{bc}	12.20^{a}	7.99^{b}	8.35^{bc}	8.52^{c}	9.93^{b}	10.82^{a}	8.94^{bc}	10.39^{ab}	9.51^{abc}	11.01^{b}
EAL-110	8.27^{b}	5.82^{d}	7.40^{e}	6.74^{cd}	6.92^{de}	6.51^{de}	10.06^{b}	6.76^{c}	8.11^{cd}	9.07^{ab}	9.37^{bc}	8.59^{c}
NSFBR-20	6.56^{c}	8.40^{c}	8.56^{d}	7.58^{bc}	6.56^{e}	6.11^{de}	5.57^{c}	6.58^{c}	7.19^{d}	9.85^{ab}	8.30^{cd}	7.58^{c}
+N	10.06^{a}	10.03^{ab}	10.38^{bc}	9.04^{a}	9.16^{ab}	9.17^{bc}	9.83^{b}	9.15^{b}	10.02^{b}	9.28^{ab}	7.57^{cd}	8.57^{c}
−N	5.47^{d}	6.48^{d}	6.91^{e}	6.96^{cd}	7.65^{cd}	5.84^{e}	3.01^{d}	2.77^{d}	3.11^{e}	5.99^{c}	6.95^{d}	4.49^{d}
CV (%)	7.8			7.1			12.2			14.5		

Mean values in the same column with a different letter(s) are significantly different at $p \leq 0.05$ *probability level.*

Table 4.
Rhizobium strain × faba bean variety interaction effect on haulm yield of faba bean at the study locations.

Rhizobium strains	Hankomolicha			Haranfama			Abala-Gase			Gike-Atoye		
	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora
	(cm)			(cm)			(cm)			(cm)		
TAL_1035	157^{a}	169^{a}	165^{ab}	156^{ab}	151^{a}	167a	153^{ab}	158^{ab}	165^{a}	168^{a}	154^{ab}	169^{a}
NSFBR-15	168^{a}	160^{a}	165^{ab}	154^{ab}	159^{a}	166^{a}	168^{a}	168^{a}	161^{a}	155^{ab}	170^{a}	169^{a}
HUFBR-17	143^{b}	156^{ab}	141^{cd}	103^{e}	114^{c}	120^{b}	128^{cd}	138^{bc}	122^{b}	152^{ab}	130^{c}	131^{b}
NSFBR-12	161^{a}	165^{a}	165^{ab}	141^{bc}	152^{a}	155^{a}	149^{ab}	164^{a}	161^{a}	166^{a}	159^{ab}	172^{a}
EAL-110	141^{b}	136^{cd}	135^{d}	112^{e}	116^{c}	106^{bc}	145^{bc}	115^{d}	119^{b}	138^{bc}	143^{bc}	132^{b}
NSFBR-20	159^{a}	126^{d}	150^{bc}	132^{cd}	108^{c}	97^{c}	117^{de}	120^{cd}	132^{b}	149^{abc}	129^{c}	120^{bc}
+N	171^{a}	162^{a}	171^{a}	161^{a}	167^{a}	168^{a}	167^{a}	159^{ab}	167^{a}	154^{ab}	150^{abc}	171^{a}
−N	135^{b}	142^{bc}	159^{ab}	117^{de}	134^{b}	90^{c}	106^{e}	117^{d}	132^{b}	127^{c}	143^{bc}	103^{c}
CV (%)	**6.1**			**8.4**			**9.2**			**10.0**		

Mean values in the same column with a different letter(s) are significantly different at $p \leq 0.05$ *probability level.*

Table 5.
Rhizobium strain × faba bean variety interaction effect on plant height of faba bean at the study locations.

and Gora attained the highest height at Hankomolicha (171.3 cm) and Harnafama (167.8 cm), respectively when treated with 46 kg N ha^{-1}.

Rhizobium strains inoculation significantly ($p \leq 0.01$) influenced the number of pods $plant^{-1}$ of faba bean. Inoculation with NSFBR-15, TAL_1035, and NSFBR-12 resulted in a significant increase in the number of pods $plant^{-1}$ relative to non-inoculated −N control at all study locations (**Table 6**). Rhizobium strains TAL_1035, NSFBR-15, and NSFBR-12 resulted in 77.3, 76.9, and 76.4% increment in a number of pods $plant^{-1}$ over non-inoculated −N control, respectively. The 46 kg N ha^{-1} resulted in 66.7% increase in the number of pods $plant^{-1}$ over non-inoculated −N control treatment. The number of pods $plant^{-1}$ significantly varied across the study locations (**Table 6**).

Rhizobium strains NSFBR-15, TAL_1035, and NSFBR-12 resulted in a significant increase in hundred seed weight at all study locations (**Table 7**). The highest hundred seed weights were recorded when variety Moti was inoculated with NSFBR-15 at Hankomolicha (83.7 g) and Abala-Gase (86.1 g) while variety Gora produced the highest hundred seed weight at Haranfama (71.1 g) and Gike-Atoye (78.8 g) when inoculated with TAL_1035 (**Table 7**). The lowest hundred weights were obtained from non-inoculated −N control plants of variety Moti at Hankomolicha (41.4 g) and Abala-Gase (36.5 g), and variety Gora at Haranfama (44.2 g) and Gike-Atoye (46.0 g). Inoculation with rhizobium strains NSFBR-15, TAL_1035, and NSFBR-12 resulted in 43.9, 40.3, and 33.9% increment in seed weight, respectively over non-inoculated −N control.

3.4 Correlation between yield and yield components

Correlation coefficients between the studied characters were computed (**Table 8**). Positive significant ($p \leq 0.01$) correlations were found between grain yield and haulm yield and number of pods $plant^{-1}$ and hundred seed weight. Haulm and grain yields were highly significantly correlated ($R^2 = 0.97$). Grain yield was significantly ($p \leq 0.01$) correlated with pods $plant^{-1}$ ($R^2 = 0.73$) and hundred seed weight ($R^2 = 0.85$).

A significantly positive ($p \leq 0.01$) correlation was also observed between haulm yield and number of pods $plant^{-1}$ ($R^2 = 0.76$) and hundred seed weight ($R^2 = 0.80$) and plant height ($R^2 = 0.84$). Plant height and pods $plant^{-1}$ were also positively correlated. Similarly, a number of pods $plant^{-1}$ and hundred seed weight was positively correlated.

3.5 Economic returns on inoculation

Marginal rate of returns analysis was conducted for dominant treatments (**Table 9**). Net benefits of non-inoculated +N and −N control treatments were dominated at all the study locations while the least net benefits at all locations were obtained from non-inoculated −N control treatment.

Rhizobium strain NSFBR-15 inoculation to variety Moti resulted in the highest net benefit of 2281.8 USD followed by strain TAL_1035 inoculation to variety Gora and strain NFBR-15 inoculation to variety Dosha which gave a total of 2089 and 1971 USD ha^{-1}, respectively at Hankomolicha. The net benefits of all treatments were dominated except HUFBR-17 × Dosha, EAL-110 × Moti, and combinations with strains NSFBR-15, TAL_1035, and NSFBR-12. Net benefit to cost ratio ranged from 4.6 to 4.9 for the dominant treatments whereas MRR ranged from 212.8 to 442.0% (**Table 9**) at Hankomolicha.

Rhizobium strains	Hankomolicha			Haranfama			Abala-Gase			Gike-Atoye		
	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora
		(NPP)			(NPP)			(NPP)			(NPP)	
TAL_1035	21.7^{b}	23.6^{b}	34.9^{a}	15.7^{abc}	16.4^{abc}	26.9^{a}	19.9^{cd}	44.8^{a}	27.2^{c}	27.4^{abc}	$21.1d^{e}$	33.3^{ab}
NSFBR-15	17.0^{bc}	38.3^{a}	23.2^{bc}	19.2^{a}	12.8^{bc}	25.2^{ab}	25.4^{b}	27.6^{b}	40.8^{a}	23.4^{c}	23.4^{cd}	35.8^{a}
HUFBR-17	15.7^{bc}	14.5^{cde}	14.1^{d}	18.6^{ab}	15.2^{abc}	9.73^{e}	18.3^{d}	16.9^{d}	16.5^{e}	24.6^{bc}	23.5^{cd}	16.6^{e}
NSFBR-12	30.6^{a}	21.0^{bc}	19.8^{cd}	20.7^{a}	20.6^{a}	21.0^{abc}	35.8^{a}	24.6^{bc}	23.2^{d}	27.8^{ab}	36.0^{a}	30.1^{bc}
EAL-110	19.8^{b}	9.4^{e}	13.5^{d}	17.9^{ab}	9.8^{c}	12.8^{de}	23.1^{bc}	11.0^{e}	15.7^{e}	25.5^{abc}	17.7^{e}	18.2^{e}
NSFBR-20	12.1^{c}	10.8^{de}	18.5^{cd}	9.1^{c}	18.5^{ab}	15.6^{cde}	14.1^{e}	12.7^{e}	21.6^{d}	14.6^{d}	26.4^{bc}	22.3^{d}
+N	23.3^{b}	18.5^{bcd}	27.8^{b}	20.5^{a}	19.6^{ab}	18.9^{bcd}	27.2^{b}	21.5^{c}	32.5^{b}	29.3^{a}	28.0^{b}	27.0^{c}
−N	9.7^{c}	12.0^{de}	17.9^{cd}	12.2^{bc}	13.6^{abc}	11.6^{e}	11.3^{e}	14.0^{de}	20.9^{d}	17.3^{d}	19.4^{de}	16.5^{e}
CV (%)		**24.8**			**25.9**			**11.7**			**10.8**	

Mean values in the same column with a different letter(s) are significantly different at $p \leq 0.05$ *probability level. NPP = Number of pods plant*$^{-1}$.

Table 6.
Rhizobium strain × faba bean variety interaction effect on the number of pods plant^{-1} *of faba bean at the study locations.*

Rhizobium strains	Hankomolicha			Haranfama			Abala-Gase			Gike-Atoye		
	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora	Moti	Dosha	Gora
	(g)			(g)			(g)			(g)		
TAL_1035	68.5^{bc}	69.1^{a}	80.2^{a}	59.8^{b}	61.2^{a}	71.0^{a}	68.3^{bc}	69.0^{a}	82.1^{a}	65.0^{ab}	66.7^{abc}	78.8^{a}
NSFBR-15	83.7^{a}	72.6^{a}	70.5^{b}	68.4^{a}	62.0^{a}	63.0^{b}	86.1^{a}	73.0^{a}	70.7^{b}	75.5^{a}	67.8^{ab}	69.0^{ab}
HUFBR-17	54.8^{de}	58.4^{b}	54.4^{c}	49.1^{c}	50.3^{b}	56.9^{c}	52.2^{de}	56.4^{b}	51.7^{c}	52.0^{c}	53.5^{d}	61.5^{bc}
NSFBR-12	62.7^{bcd}	67.9^{a}	73.2^{ab}	63.2^{ab}	65.5^{a}	59.7^{c}	61.4^{bcd}	67.6^{a}	73.8^{ab}	69.2^{a}	72.0^{a}	65.0^{b}
EAL-110	61.1^{cd}	47.7^{c}	53.2^{c}	54.4^{c}	51.0^{b}	47.1^{d}	59.5^{cd}	43.8^{c}	50.2^{c}	58.5^{bc}	54.3^{d}	49.5^{d}
NSFBR-20	50.7^{e}	51.8^{bc}	58.4^{c}	50.1^{c}	52.4^{b}	49.3^{d}	47.4^{e}	48.6^{bc}	56.4^{c}	53.3^{c}	56.0^{cd}	52.2^{cd}
+N	70.5^{b}	67.5^{a}	74.8^{ab}	64.7^{ab}	64.4^{a}	64.7^{b}	70.6^{b}	67.1^{a}	75.7^{ab}	71.0^{a}	70.8^{a}	71.0^{ab}
−N	41.4^{f}	50.5^{bc}	58.4^{c}	50.8^{c}	54.8^{b}	44.2^{d}	36.5^{f}	47.1^{bc}	56.4^{c}	54.0^{c}	59.0^{bcd}	46.0^{d}
CV (%)		**8.5**			**6.1**			**11.5**			**11.1**	

Mean values in the same column with a different letter(s) are significantly different at $p \leq 0.05$ *probability level.*

Table 7.
Rhizobium strain × faba bean variety interaction effect on hundred seed weight of faba bean at the study locations.

Variables	Grain yield	Haulm yield		Plant height		
	r	R^2	r	R^2	r	R^2
Haul yield	0.98**	0.97	—	—	—	—
Plant height	0.92**	0.84	0.92**	0.84	—	—
Hundred seed weight	0.92**	0.85	0.90**	0.80	—	—
Pods plant^{-1}	0.85**	0.73	0.87**	0.76	0.83**	0.69

***Significant at 1% level.*

Table 8.
Correlation among grain yield and yield components of faba bean inoculated with different rhizobium strains across the study locations.

Hankomolicha				Haranfama			
Strain × variety	NB ($ ha^{-1})	B:C ratio	MRR	Strain × variety	NB ($ ha^{-1})	B:C ratio	MRR
HUFBR-17 × Dosha	1513	4.6	212.8	HUFBR-17 × Moti	1298	4.4	210.1
NSFBR-12 × Moti	1637	4.6	366.4	TAL_1035 × Moti	1455	4.5	291.3
EAL-110 × Moti	1589	4.6	356.5	NSFBR-12 × Moti	1441	4.5	285.3
TAL_1035 × Moti	1815	4.7	395.4	NSFBR-12 × Dosha	1332	4.5	126.3
TAL_1035 × Dosha	1839	4.7	346.0	TAL_1035 × Dosha	1471	4.6	236.8
NSFBR-15 × Gora	1802	4.7	276.6	NSFBR-15 × Moti	1481	4.6	302.2
NSFBR-12 × Dosha	1801	4.7	336.7	NSFBR-15 × Dosha	1584	4.6	291.6
NSFBR-12 × Gora	1875	4.7	304.2	NSFBR-12 × Gora	1510	4.6	367.7
TAL_1035 × Gora	2089	4.8	362.9	TAL_1035 × Gora	1816	4.7	412.8
NSFBR-15 × Dosha	1971	4.8	373.2	NSFBR-15 × Gora	1685	4.7	397.4
NSFBR-15 × Moti	2282	4.9	442.0				

NB = net benefit (in USD ha^{-1}); MRR = marginal rate of return (in %); B:C = benefit-cost ratio.

Table 9.
Net benefit, benefit to cost ratio, and marginal rate of return for dominant treatments at Hankomolicha and Haranfama.

Variety Gora gave the highest net benefit (1816 USD ha^{-1}) when inoculated with TAL_1035 followed by the same variety (Gora) inoculated with NSFBR-15 (1685 USD ha^{-1}) at Haranfama. The net benefits of all treatments were dominated except HUFBR-17 × Moti, and combinations with strains NSFBR-15, TAL_1035, and NSFBR-12. The net benefit-cost ratio for dominant treatments ranged from 4.4 to 4.7 while MRR ranged from 126.3 to 412.8% (**Table 9**) at Haranfama.

Rhizobium strain NSFBR-15 inoculation to variety Gora and Moti resulted in the first and third highest net benefit of 2000 and 1878 USD ha^{-1}, respectively while strain TAL_1035 inoculation to variety Dosha resulted in the second-highest net benefit (1927 USD ha^{-1}) at Abala-Gase (**Table 9**). Apart from the non-inoculated +N and −N control treatments, the net benefits of all treatments were dominant. The net benefit-cost ratio ranged from 4.3 to 4.8 for the dominant treatments, whereas MRR ranged from 99.6 to 421.6% at Abala-Gase (**Table 10**).

Abala-Gase				Gike-Atoye			
Strain × variety	**NB ($ ha^{-1})**	**B:C ratio**	**MRR**	**Strain × variety**	**NB ($ ha^{-1})**	**B:C ratio**	**MRR**
HUFBR-17 × Moti	1075	4.3	99.6	NSFBR-20 × Dosha	1402	4.5	137.7
HUFBR-17 × Gora	1266	4.4	256.5	HUFBR-17 × Moti	1468	4.6	111.8
NSFBR-20 × Moti	1231	4.4	232.0	HUFBR-17 × Dosha	1585	4.6	265.8
HUFBR-17 × Dosha	1371	4.5	312.2	NSFBR-12 × Dosha	1545	4.6	243.2
EAL-110 × Dosha	1362	4.5	309.0	EAL-110 × Moti	1589	4.6	217.4
NSFBR-20 × Dosha	1409	4.5	323.9	EAL-110 × Dosha	1574	4.6	260.0
NSFBR-20 × Gora	1343	4.5	289.7	EAL-110 × Gora	1499	4.6	288.9
EAL-110 × Gora	1510	4.6	343.4	NSFBR-20 × Moti	1618	4.6	234.4
TAL_1035 × Moti	1802	4.7	398.9	TAL_1035 × Moti	1783	4.7	309.5
TAL_1035 × Dosha	1927	4.7	417.1	TAL_1035 × Dosha	1705	4.7	312.3
TAL_1035 × Gora	1668	4.7	376.8	TAL_1035 × Gora	1864	4.7	382.8
NSFBR-15 × Moti	1878	4.7	407.9	NSFBR-15 × Moti	1722	4.7	286.0
NSFBR-15 × Dosha	1801	4.7	403.2	NSFBR-15 × Dosha	1709	4.7	313.7
NSFBR-12 × Moti	1711	4.7	383.8	NSFBR-12 × Moti	1672	4.7	263.5
NSFBR-12 × Dosha	1807	4.7	402.3	NSFBR-12 × Gora	1732	4.7	358.8
NSFBR-12 × Gora	1700	4.7	381.5	NSFBR-15 × Gora	2061	4.8	411.5
EAL-110 × Moti	1719	4.7	385.4				
NSFBR-15 × Gora	2000	4.8	421.6				

NB = net benefit (in USD ha^{-1}); MRR = marginal rate of return (in %); B:C = benefit-cost ratio.

Table 10.
Net benefit, benefit to cost ratio, and marginal rate of return for dominant treatments at Abala-Gase and Gike-Atoye.

Except for HUFBR-17 × Gora and non-inoculated +N and −N control treatments, the net benefits of all treatments were dominant at Gike-Atoye. The net benefit for dominant treatments (**Table 9**) ranged between 1402 and 2061 USD ha^{-1}. Rhizobium strain NSFBR-15 inoculation to variety Gora resulted in the highest net benefit (2061 USD ha^{-1}) followed by strain TAL_1035 inoculation to variety Gora and Moti which resulted in the second and third highest net benefits of 1864 and 1783 USD ha^{-1}, respectively at Gike-Atoye. The net benefit-cost ratio ranged from 4.5 to 4.8 for the dominant treatments while MRR ranged between 111.8–411.5 USD ha^{-1} at Gike-Atoye (**Table 10**).

4. Discussion

Rhizobium strains NSFBR-15, TAL_1035, and NSFBR-12 significantly ($p \leq 0.01$) increased grain yield of faba bean as compared to non-inoculated −N control at all

the study locations (**Table 3**). Similarly, significant effects of rhizobia inoculation on legume yield have been reported [14, 24, 41]. The grain and haulm yields increment is attributable to the increased supply of fixed N to faba bean plants as a result of inoculation.

There were variations in grain and haulm yields across the study locations (**Figure 1**). Variation in grain and haulm yield across the locations might be related to differences in fertility status of the soils (**Table 2**). Soil N, Ca, CEC and organic C status at Gike-Atoye was relatively higher than that of other study locations, whereas Haranfam had generally lower nutrients and organic carbon status among soils of the study locations, hence, the higher yield in the former following inoculation. Symbiotic N fixation is not active at the early stages of plant growth in low fertile soils [7]. Mineral nutrient deficiency limits legume N fixation, nutrient uptake, and yields of crops [42, 43].

Several studies [14, 24, 44] have shown that rhizobium strains inoculation improved the yield of faba bean. The observed yield difference in inoculated faba bean could be attributed to the variation in plant response to different rhizobium strains inoculation in N fixation. Inoculation with NSFBR-15, TAL_1035, and NSFBR-12 resulted in 62.3, 56.9, and 46.4% grain yield increments, respectively over non-inoculated −N control (**Figure 2**). These results are in line with the findings of Denton et al. [14] and Youseif & Fayrouz [7] who reported 59–81% faba bean yield increment due to different rhizobia strain inoculation. The findings of this current study demonstrated that the increment in grain yield of faba bean depended on rhizobium strain and faba bean genotypes interaction with probably the biochemical characteristics of the soil.

Cultivation of faba bean without N fertilizer is the common practice among small holders in Ethiopia [45]. Application of N fertilizer at rates between 40 and 50 kg N ha^{-1} was reported to increase nodulation, N fixation and yield of faba bean [7, 46] and soybean [47]. In this study, 46 kg N ha^{-1} resulted in a significant haulm yield increase in faba bean over non-inoculated −N control at all the study locations (**Table 4**). The increase in haulm yield due to applied N, in turn, brought about increased grain yield. Previous studies [48, 49] revealed a strong relationship between haulm and grain yield and suggested that increasing biomass is a prerequisite for high grain yield of legumes.

In line with the finding of Albareda et al. [50] and Youseif [47] in soybean and Youseif & Fayrouz [7] in faba bean, this study revealed that response of inoculation varied among rhizobium strains. The three strains (NSFBR-15, TAL_1035, and NSFBR-12) established an effective N fixing association with faba bean, thus producing greater grain yield relative to 46 kg N ha^{-1} (**Figure 2**). This finding is in line with Albareda et al. [50] and Tena et al. [51] who reported that inoculation with effective strains resulted in significantly higher or equal grain yields as compared to non-inoculated +N controls of soybean and lentil, respectively. Youseif & Fayrouz [7] also reported that inoculation with effective rhizobium strains increased the grain yield of faba bean by 35–48% compared to 96 kg N ha^{-1}. The higher yields obtained with NSFBR-15, TAL_1035, and NSFBR-12 inoculation indicate that these strains were more efficient in supplying N to faba bean than inorganic N fertilizer application (46 kg N ha^{-1}). This result showed that inoculation of faba bean with effective rhizobium strain could reduce the need for inorganic fertilizer while achieving higher grain yield.

Rhizobium strains inoculation significantly ($p \leq 0.01$) influenced haulm yield of faba bean (**Table 4**). This finding is in line with Tena et al. [51] who reported that rhizobial strain inoculation increased the straw yield of lentil. Inoculation with NSFBR-15, TAL_1035, and NSFBR-12 resulted in a significant increase in haulm yield compared to non-inoculated control treatments (**Table 4**). In line with this,

Ali et al. [52] reported that, inoculated *Pisum sativum* L. produced significantly higher foliage yield than non-inoculated plants. An increase in haulm yield in response to rhizobium strains inoculation may be attributed to the increased supply of N through N fixation as a result of increased modulation. According to Giller [53], rhizobium strains increase N uptake and stimulate plant biomass production.

Inoculation with NSFBR-15, TAL_1035, and NSFBR-12 resulted in a higher haulm yield of faba bean than non-inoculated +N control (**Table 4**). This shows that the rhizobium strains (NSFBR-15, TAL_1035, and NSFBR-12) were more efficient in supplying N to faba bean than inorganic N fertilizer (46 kg N ha^{-1}) in the study locations. On the other hand, inoculation with HUFBR-17, EAL-110, and NSFBR-20 resulted in lower haulm yield than non-inoculated +N control (**Table 4**). Therefore, HUFBR-17, EAL-110, and NSFBR-20 may not be the best substitute for N fertilizer for maximum haulm yield production. Hence, the study clearly showed that appropriate rhizobium strain inoculation is vital in improving plant growth and increasing haulm yield of faba bean.

Rhizobium strains and strain × variety interaction had highly significant ($p \leq 0.01$) effects on a number of pods $plant^{-1}$, hundred seed weight, and plant height (**Tables 5–7**) of faba bean. Rhizobium strains NSFBR-15, TAL_1035, and NSFBR-12 inoculation had a great positive effect on the number of pods $plant^{-1}$, hundred seed weight, and plant height (**Tables 5–7**) of faba bean as compared to non-inoculated −N control. This is in line with the findings of Solomon et al. [41]; Argaw [24]; Denton et al. [14] who reported significant improvement in yield components in faba bean with rhizobium inoculation. The positive change in the number of pods $plant^{-1}$ and hundred seed weight following NSFBR-15, TAL_1035, and NSFBR-12 inoculation contributed to the increased yield of faba bean.

Plant height was significantly ($p \leq 0.01$) affected by rhizobium inoculation (**Table 5**). In line with this result, Raza et al. [54] and Sajid et al. [55] found that rhizobium inoculation increased plant height of mung bean and groundnut, respectively. The increment in plant height might be due to supplementary N from rhizobium strains inoculation which could promote vegetative growth of the plant. Besides, rhizobium strains may synthesize growth-promoting substances (phytohormones) like auxin as secondary metabolites in inoculated plants. Gamini and Ekanayake [56] reported similar results with different strains of *Bradyrhizobium japonicum* on soybean. There was no significant variation among faba bean varieties for plant height, grain, and haulm yields of faba bean though tested varieties genetically vary in these traits [57].

Rhizobium strains inoculation and N fertilizer application significantly ($p \leq 0.01$) influenced number of pods $plant^{-1}$ of faba bean (**Table 6**). This result disagrees with that of Karasu et al. [58] who reported that inoculation of rhizobia and N fertilizer application did not affect the number of pods $plant^{-1}$. However, Anjum et al. [59] reported that inoculation of rhizobia and N fertilizer application significantly increased the number of pods $plant^{-1}$ in mung beans. The current results of this study, however, confirm that of Malik et al. [60] and Bhuiyan et al. [61] who concluded that the number of pods per plant of soybean and mung bean was significantly increased by inoculating with *Bradyrhizobium*, respectively.

Inoculation with NSFBR-15, TAL_1035, and NSFBR-12 resulted in a higher hundred seed weight as compared to non-inoculated −N control treatment at all the study locations (**Table 7**). In line with this finding, Anjum et al. [59] revealed that hundred seed weight was significantly affected by inoculation in mung bean. Similarly, Aslam et al. [62] reported that hundred seed weight of chickpea was significantly increased by rhizobium inoculation. Zhang et al. [63] and Kazemi et al. [64] also reported that inoculation by rhizobia significantly increased hundred seed weight of soybean. A similar result was obtained by Kyei-Boahen et al. [65]. Higher

seed weight was probably due to the provision of enough assimilate to fill the seeds. The variation in hundred seed weight of faba bean due to inoculation may be related to the differences in symbiotic effectiveness of rhizobium strains on the different faba varieties which could, in turn, have resulted in variation in N fixation and assimilate translocation to the grain. In grain legumes, a hundred seed weight is considered to be an indicator for the seed quality of the crop [66].

There were significant differences among the tested faba bean varieties on a number of pods per plant (**Table 6**) and hundred seed weight (**Table 7**). Significant variation among the faba bean varieties in hundred seed weight might be attributed to genetic divergences in individual varieties in pod production and seed size [57]. They noted that a number of pods plant^{-1} depended on the number of reproductive sites plant^{-1}. The result of this study indicated that tested varieties have different genetic potential in producing pods and seed size. In line with this result, Tagore et al. [67] reported that differences in seed size among chickpea varieties occurred due to differences in genotypes.

Rhizobium strains inoculation had significant effects in increasing yield components and ultimately haulm and grain yields of faba bean. Haulm yield and grain yield were highly correlated (R^2 = 0.97) (**Table 8**) indicating that haulm yield was the most important factor influencing grain yield. High biomass production in grain legumes is a prerequisite for high grain yield [48, 49]. The positive correlation of hundred seed weight (R^2 = 0.85) and the number of pods plant^{-1} (R^2 = 0.73) (**Table 8**) with grain yield indicates the importance of seed size and number of pods plant^{-1} in determining the final yield of faba bean.

Relatively, the lowest net benefit (**Tables 9** and **10**) obtained for the treatments at all the study locations was attributable to the low yields of the non-inoculated −N control treatment. Net benefits from non-inoculated both +N and −N control treatments were dominated at all study locations. A decrease in net benefits for non-inoculated +N control treatments was due to its high variable cost [38]. Whereas, the lowest net benefit for non-inoculated −N control was due to the lowest yield obtained from this treatment at all the study locations. This result indicates that inoculation with efficient rhizobium strain is sustainable and more economical in supplying N to faba bean crop than N fertilizer application (46 kg N ha^{-1}). Thus, the inclusion of appropriate rhizobium strains in faba bean production will be cost-effective in the study locations.

Inoculation with NSFBR-15, TAL_1035, and NSFBR-12 resulted in increased grain yield and profit over the control treatments which eventually resulted in a significantly greater marginal rate of returns at all the study locations (**Tables 9** and **10**). Tairo and Ndakidemi [68] revealed that rhizobia inoculation had a positive significant effect on the nutrition, growth, and economic sustainability of grain legumes. Treatments that have the highest benefit and marginal rate of return greater than the minimum acceptable marginal rate of return can be a tentative recommendation. In this current research, the marginal rates of returns for all dominant treatments were above the minimum acceptable marginal rate of return (100%) [38].

5. Conclusion

This study has shown significant location × strain × variety interaction effects on grain and haulm yields, plant height, number of pods plant$^{-1,}$ and hundred seed weight of faba bean. Results clearly showed that rhizobium inoculation is indispensable for increasing the growth and yield of faba bean in the study locations. The economic analysis showed that efficient rhizobium strains inoculation is more

economical for faba bean production than 46 kg ha^{-1} N fertilizer application. Rhizobium strains NSFBR-15, TAL_1035, and NSFBR-12 were more efficient in supplying N to faba bean as compared with the supply of 46 kg ha^{-1} N fertilizer. Thus, the result suggests the potential use of strains NSFBR-15, TAL_1035, and NSFBR-12 as a powerful alternate source for N in faba bean production in the study locations.

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Chapter 18

A Review on Ecology of Interactions in Soybean Vein Necrosis Orthotospovirus (SVNV): Plants, Vectors, Virus Dispersal and Management Perspectives

Asifa Hameed, Cristina Rosa and Edwin G. Rajotte

Abstract

Soybean vein necrosis orthotospovirus (SVNV, Genus: *Orthotospovirus*, Family: *Tospoviridae,* Order *Bunyavirales*) is a vector and seed transmitted virus that infects soybean in different countries around the world. The purpose of this review paper was to provide information about SVNV, its geographic dispersal, vectors, disease transmission mode, alternative host plants, diagnostic tools and management. SVNV is a negative-sense single-stranded RNA virus reported in all soybean growing states in the USA, Egypt and Canada. SVNV can replicate in plants belonging to six different families, including the Leguminosae member mung bean, which is a major component of the diet of poor people of Asia. The most efficient and abundant SVNV vector species is *Neohydatothrips variabilis* (Beach.) (Sericothripinae: Thripidae). Five other insect species have the potential to transmit the virus, but their rate of transmission is very low. In addition to leaf necrosis, this virus can decrease seed oil content by 0.1% that may lead to a decrease in quality of SVNV infected seed in oilseed markets. In fact, in the infected seeds the quantity of the undesirable linolenic acid, a polyunsaturated fatty acid is increased. Broad presence of SVNV in all soybean growing regions points to the need to manage vector and virus. However, research is needed to determine various management options for the virus and vector including breeding for genetic resistance.

Keywords: soybean, soybean vein necrosis orthotospovirus, soybean thrips, symptoms, alternative hosts

1. Introduction

Soybean is one of the most valuable oil seed, food, forage, biodiesel, feed, and leguminous nitrogen fixer crop which improves soil structure through nodule formation, nitrogen fixation and enhances farmer income along with multiple other benefits [1–4]. Soybean is the second most important broad acre agricultural crop in the US providing high cash benefits to farmers [5]. Soybean was first introduced

in the US for agricultural usage as a forage crop in 1804 [6], probably as part of an interchange of seeds between France and US. However, there is some evidence from Georgia which documents soybean cultivation in 1765. Since 1940, the area under soybean cultivation increased so much that it is now mainly used as an oil seed crop. The expansion of soybean cultivation increased from about 2.7 billion bushels in 2000 to 4.39 billion bushels in 2017 in the US [7]. Brazil, US, and Argentina dominate soybean production around the world [8]. Soybean production has doubled during the last decade because of the increased income benefits to farmers and also because of the availability and diffusion of transgenic soybeans which are glyphosate resistant (first developed in 1998) [9, 10].

Soybean is affected by a plethora of diseases caused by bacteria, fungi and viruses as well as by pests such as insects and mites [11, 12]. The effect of diseases and pests on plants results in the reduction in soybean yield. For example, during 2014, the estimated loss due to diseases was 113 million bushels in 28 states in the US. Of this, losses caused by viruses were 11.6 million bushel [13, 14]. Forty-six viruses are known to infect soybeans [14], and among them eight are economically important viz., alfalfa mosaic virus (AMV), bean pod mottle virus (BPMV), peanut mottle virus (PeMoV), peanut stunt virus (PSV), soybean dwarf virus (SbDV), soybean mosaic virus (SMV), soybean vein necrosis virus (SVNV) and tobacco ringspot virus (TRSV) [13, 15].

2. Species *soybean vein necrosis virus* (tospoviridae: bunyavirales), history and dispersal in different continents of world

In 2008, soybean vein necrosis orthotospovirus (SVNV) was first reported in Tennessee (US). To date, 22 US states have reported the virus presence [16–20], and the incidence of soybean vein necrosis disease in some states has been very high. For instance, in a 3-year survey conducted in the mid-west and mid-south US, it was reported that SVNV was present in 49/50 fields [21]. While this survey highlighted one of the most extreme cases of SVNV presence, in the United States the percent incidence ranged between 10 and 80 depending upon the plant stage and geographic areas. In 2012, the virus was also reported in Canada [22]. The genetic diversity of SVNV was studied from samples taken from different states and showed low variability. In 2013, a comparison of the nucleocapsid protein (NP) coding sequence of SVNV isolates collected from different states was done and it was found that it had 98–100% similarity [16]. At that time, it was proposed that the virus was new and might have been introduced into the US or recently might have been moved to soybeans from other plant hosts [16]. The spread of SVNV is not limited to North America, in fact in 2017, it was reported in Egypt (Middle East) [23] where its incidence was about 67%.

Interestingly, SVNV can spread through seed, an unusual feature for a tospovirus [24], and the US is one of the largest soybean exporters, making seed transmission a concern to importing countries. Until now it is speculated that due to transmission by seed and global soybean trade, seed may be a major source of virus transmission to the entire world [24]. This is because *Neohydatothrips variabilis* (Beach) and other secondary vectors, although dominant in Middle East and North America, are not abundant in other parts of the world such as Asia ([23–26]; **Figure 1**). Furthermore, it is unknown whether the virus is indigenous in importer countries because soybean has an Asian origin, so the disease may already be present in those countries but may have never been reported. Soybean vein necrosis disease symptoms are similar to many others caused by pathogens such as *Cercospora* and by other plant stresses, making its

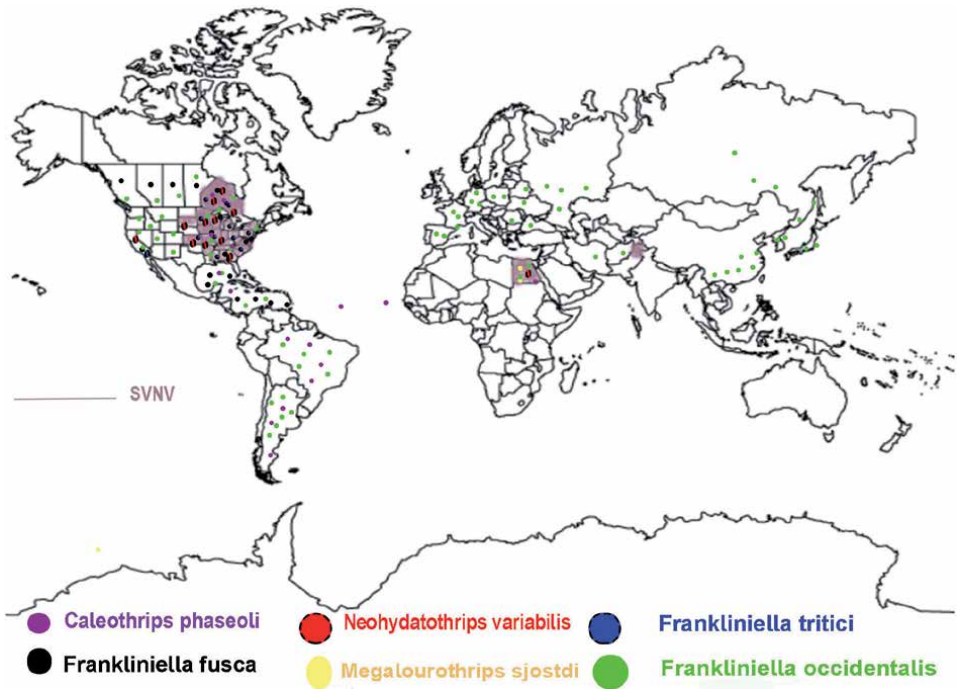

Figure 1.
World map showing thrips species distribution and soybean vein necrosis virus (SVNV) presence in different countries [23, 27–31].

identification a challenge. A comprehensive survey of SVNV and its vectors in different countries is also missing. Until now *Frankliniella fusca* (Hinds), *N. variabilis* (Beach) and *Frankliniella tritici* (Fitch) have been found to be vectors of SVNV in the US [16, 32–34] but in Egypt *Megalurothrips sjostedii* (Trybom), *N. variabilis* (Beach), *F. occidentalis* (Pergande) and *Caliothrips phaseoli* (Hood) transmitted SVNV under experimental conditions [23].

2.1 Symptoms related to infection

Infection by SVNV in soybean is characterized by necrosis of the veins as well as interveinal necrosis, followed by chlorosis of nearby leaf parenchyma [16, 35] (**Figure 2**). In 2013, a clear link between symptomology and virus association was described in soybean, which was confirmed later in various studies [16, 35], but some authors also found non-symptomatic SVNV positive soybeans plants [24], as well as an Asteraceae member, *Dendranthema grandiflorum,* which was virus positive using PCR [21, 35].

SVNV infection in soybean significantly reduces the oil content and may reduce the germination percentage, 100 seed weight (g), protein content percentage, and fiber content percentage [17]. An experiment was conducted to determine the seed transmission in discolored and damaged seeds, It showed that the virus was seed transmitted [24]. Another study conducted on mixed infection of SVNV and BPMV showed that both viruses can be present together as a mixed infection [25]. The seeds of BPMV infected soybean plants were also discolored. Interestingly BPMV is also seed transmitted [36]. It may be possible that both viruses used the same path to invade the seeds either through the developing embryo or any other route; however, research is needed in this context.

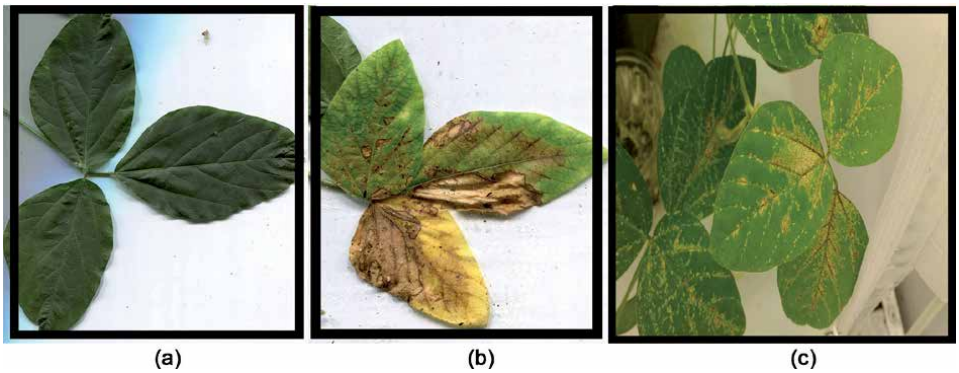

Figure 2.
Symptoms related to infection. a) Uninfected plant leaf. b) Symptomatic plant inoculated with SVNV through mechanical inoculation performed with a syringe. c) SVNV symptomatic plants infected via thrips N. variabilis *transmission.*

However, other studies conducted on the effect of SVNV on soybean yield determined that SVNV does not decrease the yield, but seed quality was affected [37]. Oil concentration was decreased by 0.1% with SVNV infection and linolenic acid, linoleic acid and stearic acid were increased [37]. This means that SVNV infection may result in lower marketability of soybean in high premium markets. In the oil market, a higher price is paid for seed which has lower linolenic acid and higher oleic acid. Bad quality seeds receive lower prices [17].

2.2 Alternative host range plants and their role as inoculum reservoirs

Weeds provide a valuable natural means of virus survival when the soybean is not present. Alternative host plant studies of SVNV showed that the virus can infect chrysanthemum *D. grandiflorum* (Asteraceae), ivy-leaved morning glory *Ipomea hederacea* Jacq (Convolvulaceae), field pumpkin *Cucurbita pepo* (Cucurbitaceae), soybean *Glycine max* (Leguminosae), cowpea *Vigna unguiculata* (Leguminosae), mung bean *Vigna radiata* (Leguminosae), benthamiana *Nicotiana benthamiana* (Solanaceae), wild tobacco *Nicotiana tabacum* (Solanaceae), tobacco *Nicotiana glutinosa* (Solanaceae) in the US [16]. However, in Egypt, ivy morning glory *Convolvulus arvensis* L. *Ipomea hederacea* Jacq (Convolvulaceae), soybean *G. max.* (Leguminosae) pulses *Lupinus sativum* (Leguminosae), mung beans *Vigna radiate* (Leguminosae), cheeseweed *Malva parviflora* L. *Portulaca oleraceae* (Portulaceae), benthamiana *N. benthamiana* (Solanaceae), tobacco *N. tabacum* (Solanaceae) are reported to serve as alternative hosts of SVNV [23]. Kudzu in the southern US States is a known overwintering host plant for the vector and virus [38].

2.3 Seed transmission

Seed transmission of viruses is a very complex phenomenon and is dependent upon the ability of a virus to penetrate the developing embryo as well as various factors including the type of host plant, time of infection of virus, amount of virus and mixed infection (compatibility of two viruses to propagate in the host plant cells at the same time) [39–43]. More than one hundred plant viruses are transmitted through seed [39, 44, 45]. Viruses often become difficult to control when they are transmitted through seed as well [39]. Virus transfer to the seed embryo can take place through different routes such as direct transfer, transfer through pollen, and indirect embryo invasion [39, 46]. Losses due to seed borne viruses increase when a stock of seed harboring virus is planted in a field [47].

There are contrary reports on the transmission of SVNV through seeds. One study conducted by Hajimurad [35] reported that like other orthotopsoviruses SVNV cannot be transmitted through seed but later in a study by Groves [24] found seed transmission and confirmed it through nested PCR and RNAseq. Hajimurad [35] did not find seed transmissibility and found only 1/1955 seeds were positive via ELISA. Hajimurad [35] considered that this observation was an anomaly and that SVNV is not seed transmitted. Another observation in the study by Hajimurad [35] was that all the seeds from the infected mother plants were non-symptomatic (not discolored or mottled, instead the seeds looked normal). However, Groves [24] used mottled and discolored seeds. Recently, a Zhou and Tzanetakis [25] study pointed that the mixed infection of SVNV and BPMV may lead to systemic infection of SVNV in the soybean seedlings. It may be that mixed infection of SVNV with BPMV results in the ability of SVNV to be seed transmitted. This is because it is hypothesized that SVNV uses the movement protein of the BPMV for systemic infection [25]. Although Zhou and Tzanetakis [48] also documented non-seed transmissibility of SVNV in 600 seedlings of field grown SVNV, most of the hybrid soybean seeds commercially available are not seed borne disease free. In SVNV, the seed transmission rate reported by Groves [24] is 6% which is considerable [24]. Until now, no virus belonging to Bunyavirales and Tosopoviridae has been regarded as a seed transmitted virus except SVNV, which gives SVNV a unique position among Tospoviridae [24, 49]. If the seed-transmission of SVNV is real, it would create a big challenge in the commercialization of soybean seeds for planting, especially in countries where SVNV is not present yet.

The avenue of seed transmission opens points for discussion. For example, if SVNV cannot be transmitted through seeds then how did the virus reach to the Middle East? It must be either human movement or thrips long distance migration. Further research is needed to confirm the seed transmissibility or the migration routes.

2.4 Disease diagnostics

SVNV can be diagnosed with commercially available ELISA kits (for instance, Agdia, USA; & Life Technologies India). A Commercially available ELISA kits use synthesized antibodies. SVNV can also be diagnosed using PCR. Various authors have published PCR primers to amplify the different regions of the SVNV genome [16, 21, 50]. The variation in whole genome of SVNV can be measured through sequencing [21].

2.5 Molecular characterization of SVNV

SVNV is a spherical virus with a tri-segmented, negative-sense and ambisense, single-stranded RNA genome, containing 5 open reading frames [21, 51]. A schematic model of the SVNV virion based upon the literature [21, 24] is described in **Figure 3**. The diameter of the SVNV particles ranges between 80 and 100 nm [24]. The 3 genomic segments encode for putative proteins involved in virus replication, in plant defense evasion, virus movement in the plant, virus coating, and vector attachment [21]. The large segment (9010 nt) encodes for the putative RNA-dependent RNA polymerase which is necessary for virus replication [21, 52]. The method of replication has been described in detail for tomato spotted wilt orthotospovirus (TSWV), the type species of this genus [52]. The middle segment (M) is 4955 nt long, ambisense and has two ORFs. ORF 1 encodes for a putative non-structural movement protein (NSm). In TSWV infections, it is assumed that NSm makes tubular structures and is associated with plasmodesmata [53]. ORF 2 encodes

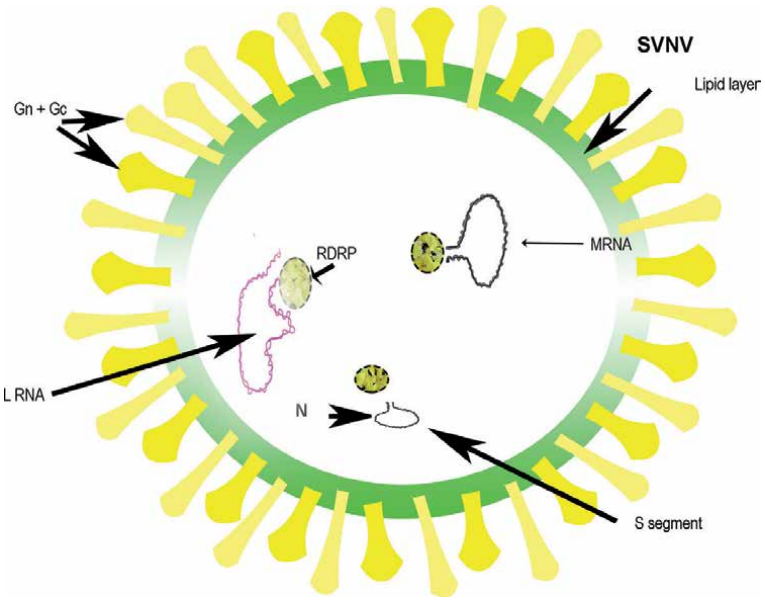

Figure 3.
Model of soybean vein necrosis virus particles showing different RNA segments (small, medium and large) coated by N proteins. Glycoprotein (Gn, Gc) spikes decorating the lipid bilayer. Molecules of RNA dependent RNA polymerase (RdRP) are enclosed in the virus particles.

for two putative glycoproteins, Gn and Gc, and their role in vector attachment has been well documented for TSWV [54]. Gn-Gc's role in the *F. occidentalis* and TSWV interaction showed that membrane mediated endocytosis takes place through interaction of Gc glycoproteins with a 50 kda thrips protein, while the Gn glycoprotein interacts with a 94 kda thrips protein [55]. As a result of this process virions move from the point of attachment in the midgut to the hemocoel and eventually to muscle cells, and from there to the salivary glands. The putative role of Gn-Gc glycoprotein in TSWV attachment was corroborated when antibodies raised against these proteins stopped virus acquisition and transmission [51]. Research on SVNV and *N. variabilis* interaction showed that the virus was present in the principal salivary gland, tubular salivary gland and the efferent duct of infected thrips [34].

The small segment (S) is ambisense, 2603 nt long, and contains two ORFs in opposite orientation [21]. ORF 1 encodes for the nonstructural silencing suppressor protein (NSs) [21]. This protein in TSWV binds dsRNA including miRNAs and siRNAs [52]. The role of NSs in SVNV and vector interaction still needs to be determined. ORF 2 encodes for the structural nucleocapsid protein (N) (31 kda) [21].

2.6 SVNV and vector association

Viruses belonging to Orthotospoviridae are persistent and propagative, which means that after entry into the vector insect, the virus multiplies in the insects and insects remain viruliferous for their entire life [54]. Studies conducted on the virus-vector relationship confirmed that *N. variabilis* (Beach.) is the primary vector of SVNV [16, 48]. The vector can acquire SVNV in the larval stages (L1 and L2) while only adults can transmit the virus [33], as for TSWV and other orthotopsoviruses. In addition, other thrips spp., *F. fusca*, *F. tritici*, *F. occidentalis*, *C. phaseoli* and *Megalurothrips sjostedti* can also transmit the virus [23]. In various experiments, transmission efficiency of vector thrips was evaluated. Keough,

Han [32] reported that *F. tritici*, and *F. fusca* transmission percentage ranges between 5% and 35% respectively. Han, Nalam [34] proved that SVNV-NP was present in the principal salivary gland, efferent duct, tubular salivary gland, and midgut region in the adult viruliferous thrips *F. tritici*, *F. fusca* and *N. variabilis* through immuno-labeling against SVNV NP. The virus was not observed in uninfected thrips species. Acquisition of orthotospoviruses in thrips and further transmission to the salivary gland and dispersal to uninfected plants is a complex process and involves the virus' ability to pass through the epithelial layer of the gut and then penetrate in the muscles and move through the tubular salivary gland to the efferent duct and the principal salivary glands [56, 57]. In *F. occidentalis* the contact between the salivary glands and the gut is closer in the first and second instar stage and later on when the insect grows to the pre-pupal and pupal stage the lack of contact is hypothesized to impede TSWV movement [57]. Although adult thrips can ingest the virus through feeding they cannot acquire the virus because the shift of virus to the salivary gland is not likely at the pupae and adult stages [57]. Also the tropism of virus replication shifts from the larval stages in the midgut epithelium to the salivary gland replication in the adult stages [57]. Moreover, the acquisition access time affects transmission of SVNV viz., transmission was higher after the 12 and 24 hrs acquisition access period (AAP) compared to 6 and 48 hrs AAP (Han, Nalam [34]).

Shazly [23] reported *F. occidentalis*, *C. phaseoli* and *M. sjostdi* can transmit SVNV with transmission efficiencies of 3.4, 6.7 and 3.3% respectively. However, major transmission of SVNV may be attributed mainly to *N. variabilis* as it was abundant in soybean crop in the US and Egypt compared to other species and due to higher transmission efficiency (70%) [23, 32, 33].

The host plant has a role in virus transmission. Shazly [23] stated that *N. variabilis* collected from cowpea can transfer virus 15% less efficiently than thrips collected from soybean. However, soybean thrips collected from mung bean had a transmission efficiency of 12.5%, while thrips collected from weeds such as *Melilotus indicus* and *Melochia corchiforia* can transfer virus with a transmission efficiency of 7.6 and 2.8% respectively [22].

There are complex theories regarding the thrips arrival, migration pattern, oviposition, hibernation and dispersion in the soybean fields (**Figures 1**–**4**) [21, 37]. According to Mueller, Higley [58] soybean thrips overwinter in southern states and annually migrate to northern US States (**Figure 4**). However, Anderson, Irizarry [17], and Zhou and Tzanetakis [48] postulated that due to the high number of thrips in soybean growing season in northern US states, soybean thrips may overwinter on perennial weeds and then during the early summer propagate on cover crops. Cover crops such as buckwheat and vegetables such as melon and winter pea can sustain SVNV and its vectors so they can act as reservoir to maintain inoculum from the overwintered insects and increase their number on the soybean crop [37, 59]. Irizarry, Elmore [59] proposed that alfalfa and other cover crops may act as the host of vectors before soybean planting in Wisconsin and Iowa. Zhou, Aboughanem-Sabanadzovic [38] suspected that Kudzu is a natural reservoir of SVNV and may be a natural shelter for the thrips during south to north movement every year because Kudzu is extensively present in the soybean growing region and interstate regions in the south.

Soybean is not thought to be the original host of SVNV because SVNV isolates collected in various locations on soybeans had more than 98% similarity [16]. However, comparison of the various isolates was done on the basis of the NP gene [16]. It would be interesting to look at the similarity of SVNV isolates in other genomic segments.

The SVNV transmission is complex because different vector species feed on different wild plants, weeds, cover crops and then eventually transfer the virus to the

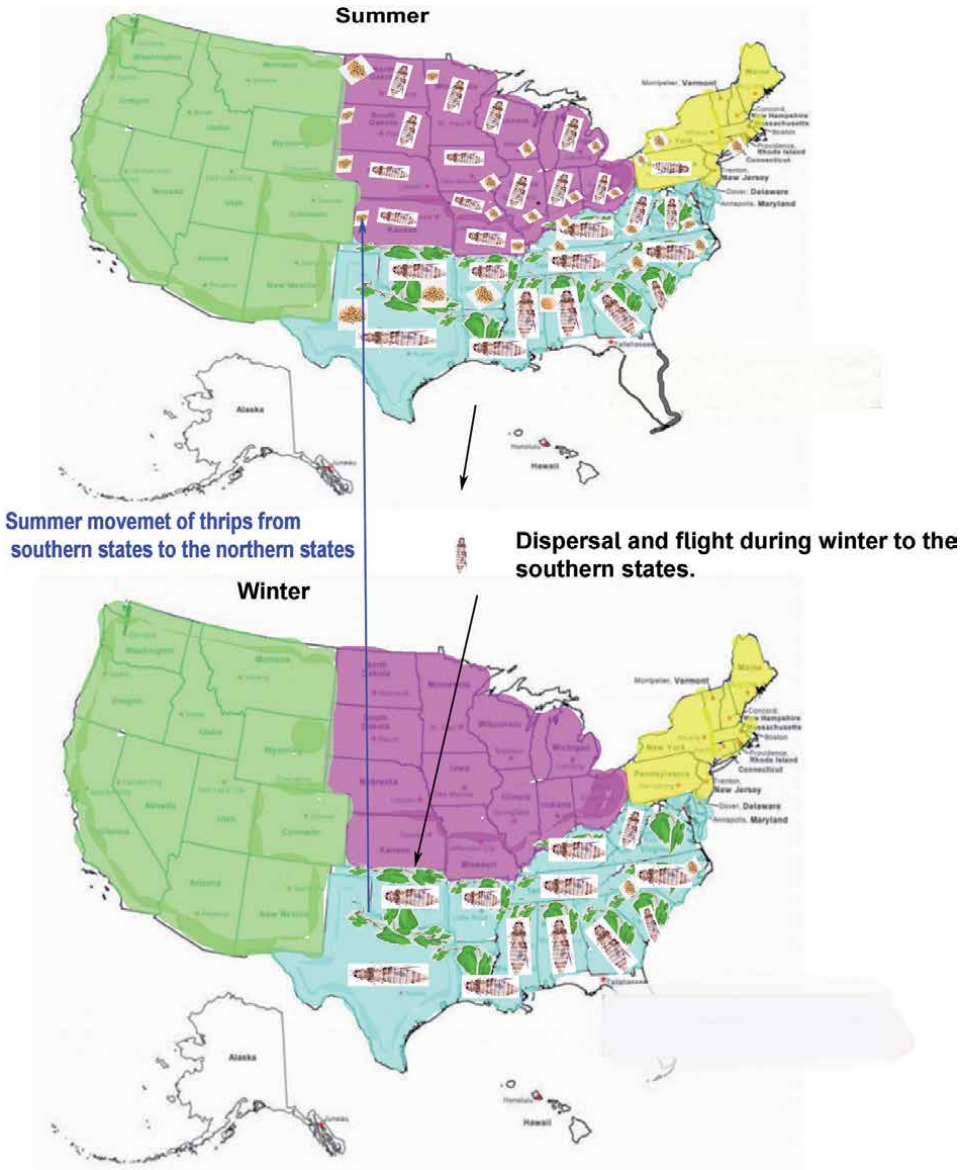

Figure 4.
Migration, dispersal and winter diapause of soybean theories, hypothesis and results. Here the yellow colored states are north eastern states. Light blue states = southern US states, purple = mid-west states, green = western states. This schematic diagram is based upon the Mueller, Higley [58] and Irizarry, Elmore [59] paper. Here the green leaf plant in southern states depict the weeds on which thrips overwinter in south and in the summer they migrate to the soybean crop in the north east and mid-west. However, according to Bloomingdale, Irizarry [60] the thrips do not migrate in the winter and they over winter on the weeds in the mid-west. However, in the northern states due to low temperature and snow the thrips cannot survive under the field conditions.

target crop. Furthermore, the virus can also be transferred to other regions along with infected seeds (**Table 1**) [24].

Seasonally, many plants can support the thrips vector species and virus in various parts of the world until the principal crop is planted. A detailed study is needed in the spring and winter to examine the alternative host plants of vector and virus reservoirs. The detailed list of possible alternative host plants of the vector and their confirmation as the virus reservoir in different parts of the world is described in **Table 1**.

Country	Crop	Productive region/state	Species	Experimental conditions (Field or greenhouse experiment)	Identification technique	Plant hosts	Reference
USA	Alfalfa, buckwheat, and crimson clover, red clover	Iowa	*N. variabilis*	Greenhouse	Insects were slide mounted and identified to the level of species on the plants. Progeny formation was also observed.	Alfalfa, buckwheat, crimson clover and red clover are intermediate host of vector but buckwheat is inoculum reservoir as well. Buckwheat is open end host.	Zhou and Tzanetakis [16]
USA	Smart weed, cucumber, Crab apple, Viburnum, Willow, and Jackson	Iowa, Illinois, Maryland, Virginia	*N. variabilis*	Field collections	Field Collection from the plant hosts and then taxonomic identification after slide mounting until the species level.	Dead end host alternate hosts of vector but virus cannot replicate.	Hood [61]
USA	Hackberry, Elm and clover	Iowa	*N. variabilis*	Field conditions	Taxonomic identification	Presence of virus in these host plants have not been studied yet.	Beach (1896) [62]
USA	Cotton	Alabama, Arkansas, Georgia, Louisinia, Missisippi, Tennsesse	*N. variabilis*	Field conditions	Field capture, slide mounting and identification until the species level.	Dead end host, thrips can feed but the virus can not replicate in cotton.	Cook, Allen [63]
USA	Lima beans and Snap beans	Arkansas	*N. variabilis*	Field sampling	Field capture of thrips from the spring planted crop and slide preparation for identification of thrips species	Replication of virus in Lima beans and snap beans has not been studied so far.	Sweeden and McLeod [64]

Country	Crop	Productive region/state	Species	Experimental conditions (Field or greenhouse experiment)	Identification technique	Plant hosts	Reference
USA	Horse radish	Illinois	*N. variabilis*	Field sampling	Field capture of thrips from the spring planted crop and slide preparation for identification of thrips species	Replication of virus in horse radish has not been studied so far.	Gerdes [65]
USA	Tomato	Virginia	*N. variabilis*	Field sampling	Population sampling	Virus can not replicate and thrips feed on it.	Nault, Speese Iii [66]
USA	Cotton, Peanut and Soybeans	Virginia	*N. variabilis*	Yellow sticky cards	Yellow sticky cards were placed in the fields and thrips were counted after one-week interval. Insects were identified to the level of species.	Soybean is target crop. Whereas the virus cannot replicate in peanut and cotton. Peanut and cotton are dead end host.	Samler [67]
USA	Peach orchards	Georgia	*N. variabilis*	Field collection	Fields were sprayed with insecticide and killing thrips fell down in big sheets of aluminuim and were preserved in ethanol 70%.	Replication of SVNV in peach has not been studied yet.	Yonce, Payne [68]
Hungary (Europe)	Soybeans		*N. variabilis*	Monitoring	Slide preparation	Target crop.	Ábrahám [69]

Country	Crop	Productive region/state	Species	Experimental conditions (Field or greenhouse experiment)	Identification technique	Plant hosts	Reference
Egypt	Groundnut, soybeans, cowpea, mung beans, *Phaseolus vulgaris* (Beans), Egyptian bean, Medic, Yellow sweet clover, Granny vine, ivy morning glory, and Chocolate weed	Cairo	*N. variabilis*	Monitoring	Slide preparation and identification of species.	Virus can replicate in cowpea, mung beans, and ivy morning glory. The other plants are dead end host, thrips can replicate but virus presence has either not been studied or virus do not replicate	Shazly [23]
USA	Mist flower *Conocinium coelestinum (L.)* DC	North Florida	*Frankliniella tritici*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Flowering dog wood *Cornus florida* L.	North Florida	*F. tritici, Frankliniella occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Daisy fleabane *Erigeron annus*	North Florida	*F. tritici*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Dog fennel *Eupatorium eapillifolium (Lam.)*	North Florida	*F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Ivy morning glory	North Florida	*F. tritici*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Dwarf dandelion *Krigina virginica*	North Florida	*F. titici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]

Country	Crop	Productive region/state	Species	Experimental conditions (Field or greenhouse experiment)	Identification technique	Plant hosts	Reference
USA	Lantana	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Hedgeprivet	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Blue toadflax	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Japanese Honeysuckle	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Water primrose	North Florida	*F. tritici*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Crab apple	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Creeping wood sorrel	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Yellow wood sorrel	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]

Country	Crop	Productive region/state	Species	Experimental conditions (Field or greenhouse experiment)	Identification technique	Plant hosts	Reference
USA	Parthenium weed	North Florida	*F. tritici*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Chickasaw pulum	North Florida	*F. fusca, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Wild cherry	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	False dandelion	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Wild radish	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Rose	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Sand black berry	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Sassafras	North Florida	*F. tritici*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]

Country	Crop	Productive region/state	Species	Experimental conditions (Field or greenhouse experiment)	Identification technique	Plant hosts	Reference
USA	Arrow leaf sida	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Large hop clover	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Crimson clover	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Venus looking glass	North Florida	*F. tritici, F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Moss verbena	North Florida	*F. occidentalis, F. fusca*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Brazilian verbena	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Common vetch	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]
USA	Chinese wisteria	North Florida	*F. tritici, F. occidentalis*	Field collection	Slide preparation and identification of species.	SVNV infection status as the inoculum reservoir is not confirmed	Chellemi, Funderburk [70]

Table 1.
Alternative host plants of vector species in different parts of the world.

2.7 Life cycle of *N. variabilis* (Beach)

Soybean thrips lay eggs inside the leaf parenchymatous tissues near the leaf vein using a barbed ovipositor (**Figure 5**). A female lays about 70–90 eggs in her lifetime. Eggs hatch into first instar larvae having red eyes. These first instar larvae are transparent and feed on the leaf. The second instar larvae are pale yellow. The first instar duration is 3–4 days. Second and third instar duration is 2–3 days each. Fourth 4th instar duration is 2–4 days. Total adult male duration is 17–19 days and female duration is 20–23 days. Virus infection increased female survival [62]. Males are haploid. The mode of asexual reproduction is Arrehenotoky unlike *T. tabaci* L. where the mode of reproduction is deuterotoky.

2.8 Management of SVNV and vector

The importance of SVNV seems to be increasing. Several years ago, it was largely unknown, but recent studies have raised concerns about its severity. Management of seed and vector borne viruses requires complex knowledge of vector ecology, type of virus transmission (circulative, semi persistent, persistent), mode of virus introduction in the field (primary or secondary spread), the method of perception of the volatile compounds by insect sensillae, insect response to the plant released stressed volatile compounds, complex interaction between herbivores occupying same niche and threshold level of disease and vector as well [71]. Management considerations include:

1. The first step is always to start with clean seed. Planting damaged and discolored seeds may increase the chance of virus. Planting with mycorrhizae will

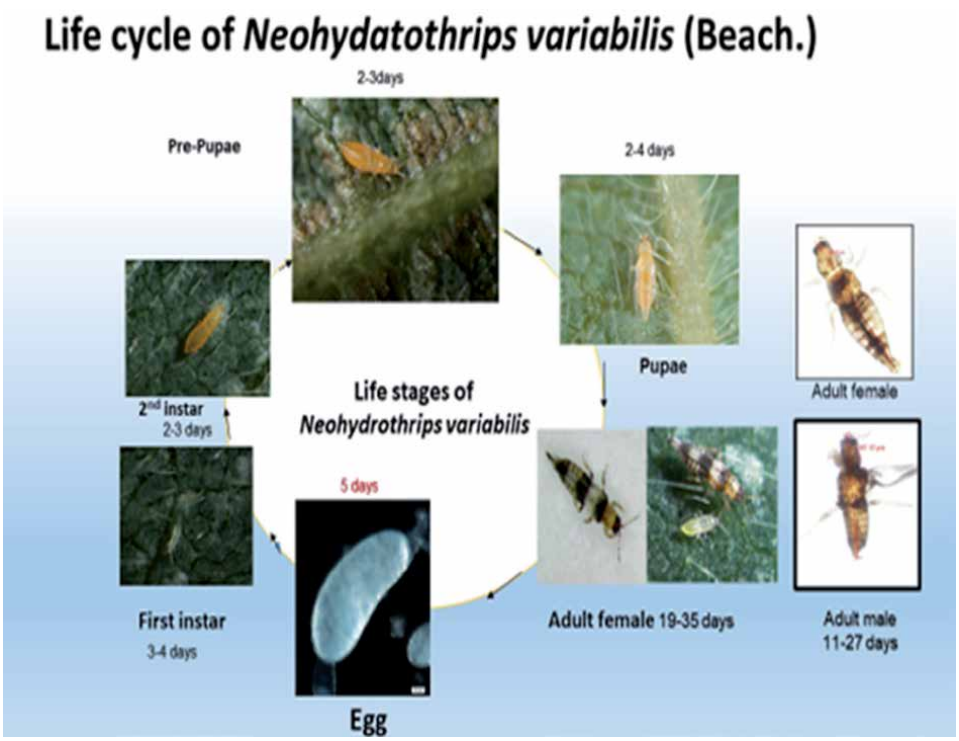

Figure 5.
Life cycle of N. variabilis. *The colored photographs were taken through the Olympus microscope 5RTV with colored CCD camera attached.*

increase plant vigor, canopy establishment, plant height, number and weight of nodules, number and weight of pods, total grain yield [72] and plant would be able to combat viruses and vector [72].

2. Monitoring can provide an estimate of thrips types present on soybean and nearby crops. Monitoring can be done using the beating sheet method or counting the number of adult thrips on the upper most leaves and preserving the specimens in 70% ethanol. Irwin, Yeargan [73] demonstrated that *N. variabilis* were higher in number at uppermost leaves. So, for estimation of ETL (Economic Threshold Level) of thrips population, thrips should be sampled from upper most leaves. The suspected infected leaves can be sent to a disease diagnostic lab which can confirm the presence of SVNV. However, presence of thrips on plants does not mean that they are causing enough damage to justify the application of insecticides. Yellow sticky traps/blue sticky traps, yellow or black water traps can also help to determine the kind of thrips species present in soybean fields. Insect samples can be sent to taxonomists at USDA for species identification.

3. Irizarry [37], Shazly [23] and Zhou and Tzanetakis [16] found that soybean vein necrosis virus can propagate in crimson clover, tobacco, mung beans, alfalfa, chrysanthemum, ivy morning glory, squash, black eyed pea, blind weed, peas, cheese weed, common purslane and melon. Plantation of soybeans near weeds and alternative host of soybean vein necrosis virus may increase the inoculum of SVNV in soybean plants. Control of weeds may decrease the virus prevalence. Planting of glyphosate resistant seeds may suppress the weeds and hence can increase the yield through reduction in competition between soybean and weeds. However, weeds or host plants during the overwintering season should be rogued. Culling and removal of the infected reservoir plants and weeds may suppress the SVNV inoculum.

4. Moreover, the winter pea, red clover and ivy morning glory can sustain adults of thrips and immature. Since winter pea, red clover and ivy morning glory can sustain the virus and vector, avoiding plantation of these crops near soybean at least 15 m apart may help to reduce pest numbers.

5. Nature is rich with biocontrol agents which suppress the thrips population. *Chrysopa* larvae, *Geocorus*, *Orius*, predatory thrips, parasitic nematodes and predatory mites can suppress pest numbers [74, 75]. In our insect rearing facility, we observed high reductions in pest numbers, when *Cucumeris* mites were present. *Cucumeris* mites can be exploited to control vector numbers in field conditions.

6. Unlike other plant pathogens, orthotospoviruses are not spread by shearing or pruning. Hence pruning or cutting the infected parts of plants would not help to reduce inoculum.

7. Pesticides can be used against vectors for management of the vector population. However, increased application of insecticides may lead to insecticide resistance, as it has been already reported in *F. occidentalis* populations. Cyantriniliprole (Minceto Pro or any formulation) is quite effective in reducing thrips number [67].

8. In the case of *N. variabilis* we did not observe pupation in soil for P2 (pupae) and P1 (pre-pupae) stage. Vance [76] reported that soybean thrips under

experimental conditions can pupate on leaves but in nature they pupate in the soil if it is available. We grew soybean thrips on plants and always found prepupae and pupal stage on leaves [62]. Some other thrips species do not move into soil and hence they can pupate on leaves, so we assume from our studies that fumigation of soil would not help reducing pest numbers however, this may help in greenhouse conditions to reduce *F. tritici* and *F. fusca* numbers.

9. For thrips control insecticide treated seeds, provide protection for about 40 days. Also, in northern US states thrips arrive in the month of July and hence symptoms appear in August. But in southern states thrips colonized soybean in May and symptoms were observed in June. This may point to the movement of the vector from South to North [77]. Losses are higher in southern states as compared to Northern US states, however research is still needed to understand comparative losses in southern and northern states. Irizarry [37] estimated losses in between soybean growing states but their studies did not compare infected and uninfected plants, but only compared less symptomatic and higher symptomatic plants due to lack of control plants. Still more studies are needed in field conditions to determine the impact of virus on yield and quality. Application of thiamethoxam, imidacloprid, acetamiprid, lambda cyhalothrin, & chlorpyrifos can provide effective control of thrips populations. In northern US states, thrips populations do not reach to higher numbers because of low temperature, rainfall, and overwintering period but in the south the population grows rapidly and hence pesticide applications may be required.

10. In the US, a high SVNV incidence in soybean crops was reported, and yield losses on full-season crops were marginal but in double-cropped beans the losses were substantial [17, 37]. Since planting takes place later, thrips colonized on normal cultivated soybeans shift at flowering stage to the double-cropped beans when the plants are often very small, only about 12–24 inches tall. Populations of thrips are very high on double-cropped beans and yield is remarkably decreased [17]. On double-cropped beans insecticide application along with yellow sticky card placement, and *Cucumeris* release may help to reduce the pest losses.

3. Future research suggestions

- Acibenzolar S methyl, or other organic compounds that like salicylic acid induce plant resistance. Application of this product can reduce bacterial and fungal diseases. Also, this will induce salicylic acid in plants which may reduce SVNV incidence through promotion of plant resistance through a phyto hormone pathway. However, all research related to Acibenzolar S methyl has been conducted with Acibenzolar S methyl and TSWV interaction but has not been done with soybean plants and SVNV. Further research on time of Acibenzolar S methyl application before thrips attack through spray may determine if induction of salicyclic acid can reduce SVNV.

- In TSWV and thrips interaction, Gn-Gc glycoproteins have a specific role in the receptor-mediated endocytosis and movement of virions from insect gut to the salivary gland. Although Han, Nalam [78] showed the virus presence in salivary gland of *N. variabilis*, movement of virus within vector has not been determined. Future Research on specific thrips protein which bind with

Gn and Gc glycoproteins may help to understand putative role of these viral proteins in thrips cells.

- Non-Structural silencing suppressor proteins (NSs) in TSWV and thrips interaction are hypothesized to overcome thrips inner immune processes. Elucidating the putative role of SVNV NSs protein in *N. variabilis* may help to understand wide range of adaptability of virus to multiple vectors and increase in fitness of the thrips.

- In our experiments we found plant cultivars responded differently to vector colonization and hence virus titer was variable on different cultivars [62], similar results have been reported by Zhou et al., 2019. Possibly in nature there are certain processes involved which govern host plant resistance against vector virus. These mechanisms in relation to SVNV isolates may decrease SVNV incidence in farmer's fields. However, SVNV resistant varieties may also be developed through strategizing against virus and vector.

- In our work on SVNV in Pakistan we found that symptomatic SVNV infected plants were present within one month after plantation of seed [62]. In US we did not find symptomatic plants until August while crop is planted in May [62]. This may be due to insecticide treated seeds, Thrips cannot colonize plants early in the season in US but in Pakistan herbicide resistant and insecticide treated seed is not available. Hence farmers and scientists use untreated seeds which may be reason behind higher disease incidence in Pakistan as compared to Northeastern US but studies regarding global warming and its relation to viral epidemics and insects' abundance may help to better understand and forecast the disease incidence in future.

- The work on virus evolution would provide information about the origin of the virus. Up to the present, we have the characterization of SVNV from US and Egypt [62]. More information on sequence comparison may help to resolve this mystery of evolution of this virus. This is because soybean is native to Asia but now US, Brazil and Argentina dominate the world production, but since the virus can be transmitted through seed, may be this virus could have arrived along with seeds from Asia to US and inhabited here generation after generations until sequenced for first time in 2008 in Tennessee [21].

- Management of SVNV requires a broad knowledge of thrips natural history as well as knowledge of the biology of the virus inside the plant host and the vector. Until now research has been done on virus characterization and the vector/virus relationship, but research is needed to understand the resistance mechanisms in plants against SVNV. According to our research experiments we did not find any cultivar which is resistant to the virus although some varieties were less preferred and some were highly preferred by thrips resulting in lower and higher incidence of SVNV [62]. But soybean (*G. max*) was derived from *Glycine soja* about 9000 years ago. Interestingly, *G. soja* is still cultivated in Russia, Korea and East Asia (including China, India and Pakistan) since ancient times. It may be possible that these wild ancestors possess resistance against SVNV, as the case of *Solanum peruvianum* against TSWV. In this case the dominant or recessive resistant genes may be identified and virus incidence can be reduced through genetic engineering and utilization of gene silencing in plants.

- Various kinds of microbes induce resistance in plants against orthotospoviruses. One example is *Pseudomonas fluorescens. P. fluorescens* application to tomato induce polyphenoloxidase, B-1,3 glucanase, and chitinase. Plants growth and performance is enhanced, TSWV concentration is reduced (reference). However, the role of these microbes and SVNV has not been studied. May be in southern US states where SVNV incidence is high, application of these microbes before sowing may increase crop productivity and decrease SVNV. Further research in this field may explore opportunities of ecofriendly way of reducing disease incidence through enhancing planting vigor and promoting induced resistance.

- The diet of poor people in developing countries mostly consists of proteins derived from legumes. Mung beans, mash beans, & tofu are the food sources of the poor. Soybean vein necrosis virus decreases the oil content of seeds which decreases the profit margin of oil seed firms and hence the product become more expensive as well. The cost of production can be lowered through introduction of virus resistant cultivars and hence more high-quality food can be provided to poor of the world.

- Disruption of the binding of the virus to its vector through transgenic cultivar development has been a pursuit of IPM specialists against viruses and vectors. In TSWV and *F. occidentalis* interaction, Gn glycoproteins promotes virus penetration of the thrips epithelial cells by membrane mediated endocytosis. Gn rich transgenic soybeans can be developed and their response to virus transmissibility by the thrips vector may be monitored under lab conditions and then it can be used in the field for vector and virus management.

4. Conclusion

Soybean vein necrosis virus is an important seed and vector transmitted virus present in middle East, US and Canada. This virus can decrease the oil content percentage. SVNV can be transmitted through different species of thrips. Among them *N. variabilis* is an important vector. SVNV has also been reported in various species of weeds where it can over winter. In the US, Kudzu is an important interstate virus reservoir for migrating thrips. Although various species of thrips can transmit SVNV, the rate of transmission of *N. variabilis* is considerably higher. SVNV is a negative sense single stranded RNA virus that can replicate in thrips and plants. Management of SVNV must be strategized as the vector and virus colonization on double beans can lower plant yield. Hence monitoring of thrips population using yellow sticky cards, and application of new chemistry insecticides should be done on late planted soybeans to reduce the pest pressure on double cropped soybeans. Future research is needed to understand the mechanism of propagation of SVNV in plant seeds, development of resistant varieties, exploring the role of Gn rich transgenic soybeans, and of gene silencing, a method that could be used to control SVNV.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Chapter 19

Symptoms of Damage to Soybean Varieties Due to Major Pest Attacks in South Sulawesi, Indonesia

Abdul Fattah, Idaryani Djamaluddin, Asriyanti Ilyas, Muslimin Muslimin, Andi Nurhayu and dan Muhammad Yasin

Abstract

South Sulawesi Province is one of the centers for soybean development in Indonesia. The varieties that are widely planted by farmers in South Sulawesi include Anjasmoro, Argomulyo, Grobogan, Gema, Dering-1, and Burangrang. These varieties have different levels of seed yield and damage levels. This paper aims to provide an overview and information about the types of soybean varieties, the level of pest damage, and the types of pests that cause damage to soybean varieties developed by farmers in South Sulawesi Province. The method used is to collect various information in the form of secondary data and primary data from research results related to soybean varieties, types of pests that damage soybean plants and the level of damage caused by soybean pests in South Sulawes. The results obtained provide information that the highest level of leaf damage caused by *Spodoptera litura* F. occurred in the Anjasmoro variety 10.94–32.69% followed by Argomulyo 10.16–26.17% and Grobogan 8.61–24.81%. The highest level of pod damage due to pod sucking was found in Burangrang varieties, namely 13.20%, Gema 12.51%, Dering 10.5%, Argomulyo 9.40%, Grobogan 8.50%, and Anjasmoro 7.70%. The level of fruit damage caused by the fruit borer *Etiella zinckenella* T., the highest occurred in Detam-1 15.71%, Ring 14.50%, Burangrang 10.60%, Gema 10.0%, Argomulyo 8.20%, Grobogan 7.10%, and Anjasmoro 6.70%. The rate of soybean yield loss caused by *S. litura* F. was the highest at Anjasmoro 8.97%–11.29%, then Grobogan 7.88–12.80%, and Argomulyo 6.77–14.90%. Meanwhile, the percentage of seed yield loss caused by the attack of the pest *Nezara viridula* L. ranged from 10.0–41.0% for all varieties. Likewise with *Riptortus linearis* F., the percentage of soybean seed loss caused ranged from 15 to 79% for all varieties.

Keywords: Soybean, varieties, symptoms, damage, main pests

1. Introduction

Soybean has a strategic position as a source of vegetable protein and functional food that has been affordable to all levels of society. Soy products such as tempe, tahu, soy milk, soy sauce, chips and so on are needed every day of the year. To meet the demand for raw materials for the processing industry, Indonesia needs around

2.2 million tons of soybean raw materials per year. Meanwhile, domestic soybean production is currently only able to meet 30–40% of national needs [1].

The national soybean productivity achieved by farmers in Indonesia only reaches 1.80 t / ha, while the potential national soybean productivity can reach 2.5 t/ha [2]. One of the factors causing low soybean productivity is the high pest attack. Pest attacks on soybean plants can reduce yields up to 80%, even puso if no control measures are taken [1]. According to Oerke [3], the loss of soybean yields due to pest attacks can reach 26–29%.

In the tropics, there are about 60 types of insects that can cause significant leaf damage in soybeans [4]. Meanwhile in India, there are about 150 species of insects that can cause serious damage to soybeans from planting to harvest [5].

Pests on soybean plants are classified into pests that destroy leaves and pests that destroy pods. Pests that destroy soybean leaves include whitefly (*Bemisia tabaci* G.), aphids (*Aphis glycines*), red mites (*Tetranychus cinnabarinus*), soybean green leafhoppers (*Empoasca* spp.), Armyworms (*S. litura*), jengkal caterpillars (*Chrysodeizis chalcites*), rollers. Leaf (*Omiodes indicata*), and soy beetle (*Phaedonia inclusa*). In principle, leaf damage caused by pests can interfere with the photosynthesis process [6]. Meanwhile, pests that destroy soybean pods include pod suckers *Nezara viridula, Reptortus linearis*, and *Piezodorus rubrofasciatus*. For pod borer, among others, *Etiella zinckenella* and *Heliothis armigera* [7].

S. litura F. armyworms is one of the important pests on soybeans in the world, including in Indonesia. In India, *S. litura* F is one of the important pests of soybeans [8]. A part from soybeans, in India, *S. litura* F. is also an important pest of tobacco with a damage rate of around 25–50% [9]. In Asia, *S. litura* F. is also an important pest and is a polyphagous which can attack about 122 species from 44 plant families [10]. In Bangladesh, about 15–20% of the total soybean production has decreased due to *S. litura* F attacks [11]. In Brazil, *S. Litura* F. can destroy soybean leaves by about 35% [12]. Rao et al. [13], *S. litura* F. can cause about 35–50% yield loss in tobacco. In cotton, in India, *S. litura* can result in 25.8–100% yield loss [14].

In Indonesia, armyworms, *S. litura* F. are important pests that eat soybean leaves compared to other pests such as jengkal caterpillars (*Chrysodeixis chalcites*), *Heliothis armigera*, leaf-rolling caterpillars (*Lamprosema indica*). Armyworms, *S. Litura* F. is a type of polypagus pest that attacks various types of plants, including soybeans. This is according to Santi and Krisnawati [15], in Indonesia, *S. Litura* F. is an important pest on soybeans with a leaf damage rate of around 70%. According to Adie et al. [16], soybean yield losses due to armyworm attack can reach 80% in Japan, 90% in America, and 23–45% in Indonesia. Meanwhile, according to Marwoto and Suharsono [17], the yield loss due to *S. litura* F. armyworm attacks in Indonesia can reach 80%.

R. linearis F. is an important pest of soybean in South Sulawesi. Yield losses due to pod sucking pests were 79% [18]. Both nymphs and imago suck the seed fluid by sticking their stylet which causes damage to the pods. The degree of damage due to *R. linearis* F. varies, depending on the stage of development of pods and seeds. The attack in the seed filling phase will cause the seeds to turn black and rot, at the ripening phase the pods will wrinkle the seeds and in the old pods before harvesting will cause the seeds to become hollow [18].

2. Soybean varieties developed by farmers in South Sulawesi

2.1 Anjasmoro variety

The Anjasmoro variety has a purple hypocotyl color, purple epicotyl color, white stem coat color, purple flower color, yellow seed coat color, light brown ripe pods,

and yellowish brownish hilarity of seeds. This variety also has oval leaf shape, wide leaf size, deterministic growth type, flowering age 35–39 days, pod ripe age 82–92 days, plant height 64–68 cm, number of branches 2–5 branches, has a large seed size (weight of 100 seeds 14.8–15.3 g). The seeds contain 41.8–42.1% protein, 17.2–18.6% fat content, and are not resistant to falling. Anjasmoro variety is moderate to leaf rust, and the pods do not break easily [19]. Meanwhile, according to Hendrival et al. [6], Anjasmoro variety has 83.38 pods, 24.69 empty pods, 173.27 seeds per plant and 3.81–9.39% *S. litura* F. attack.

2.2 Argomulyo variety

Argomulyo variety has purple hypocotyl, brown fur color, purple flower color, yellow seed coat, bright white hilarity of seeds, deterministic growth type, flowering age 35 days, age at harvest 80–82 days, plant height 40 cm, number of branches per plant 3–4 stems from the main stem, has a large seed size (weight 100 seeds 16.0 g), has a seed yield of 1.5–2.0 t ha $^{-1}$, has a protein content of 39.4%, contains fat, 20.8%, has a fall resistance property [19]. In addition, the Argomulyo variety is tolerant of leaf rust disease and this variety is suitable for soy milk as raw material. Meanwhile, according to Poniman et al. [20], the Argomulyo variety had the number of pods filled with 79.00, the weight of 100 seeds was 15.38 g, and the percentage of pod damage caused by pod borer attack was 13.11%.

2.3 Grobogan variety

According to the description of the soybean variety [19], the Grobogan variety has a determinitic growth type, purple hypocotyl color, purple epicotyl color, brown stem coat color, purple flower color, dark brown pod color, lanceolate leaf shape and hilarity brown seed color, plant height 50–60 cm, flowering age 30–32 days, mature pods 76 days, have large seed size (weight 100 seeds, 18 g), potential seed yields 3.40 t/ha, and an average seed yield of 2.77 t ha^{-1}. The seeds have a fat content of 18.4% and a protein content of 43.9%. It is well adapted to several different growing environmental conditions, has pods that are not easily broken, and at harvest 95–100% of the leaves are shed (**Figure 1**).

2.4 Burangrang variety

The Burangrang variety has purple hypocotyls, yellowish brown fur, purple flowers, yellow seeds, bright hilium seeds, oblong leaves, pointed tips, deterministic growth type, number of branches 1–2 branches, flowering age 35 days, pod age cook 80–82 days, plant height 60–70 cm, large seeds (weight of 100 seeds 16 g), seed yields range from 1.6–2.5 t ha^{-1}, have 39% protein content, 20% fat content, not

Figure 1.
Appearance of Grobogan (a), Argomulyo (b), and Anjasmoro (c) varieties. Source: Fattah et al. [21].

easy to fall down, tolerant of leaf rust disease. This variety is suitable for soy milk, tempe, and tahu [19].

2.5 Dering varieties

Dering variety has a deterministic growth type, flowering age 35 days after planting and 81 days after planting, plant height 57 cm, brown fur, oval leaf shape, purple hypocotyl color, purple epicotyl color, purple flower color, brown pod skin color., yellow seed coat color, dark yellow hilium seed color, white cotyledon color, resistant to falling, the number of branches 3–6 stems per plant [19]. Meanwhile, according to Poniman et al. [20], the Dering variety has medium seed size (100 seeds 10.7 g weight), the potential yield of seeds is 2.80 t ha^{-1}, the average seed yield is 2.0 t ha^{-1}, the seeds contain 34.2% protein and 17.1% fat content. Furthermore, it was said that the variety was resistant to pod borer (*E. zinckenella* T) and susceptible to armyworms (*S. Litura* F), resistant to leaf rust disease ((*Phakospscora pachithyzi* Syd) and tolerant to drought during the reproductive phase.

2.6 Gema varieties

According to Poniman et al. [20], the Gema variety has a deterministic type of growth with light brown coat color, purple cotyledon color, purple hypocotyl color, green epicotyl color, and white cotyledon color. Furthermore, it is said that this Gema variety has a plant height of 55 cm, has a medium seed size (100 seeds weight 11.90 g), a flowering age of 35 days, a harvest age of 73 days, a potential yield of 3.06 t ha^{-1}, an average seed yield. 2.47 t ha^{-1}, brown pod color, purple flower color, round seed shape, light yellow seed coat color, and brown hilium color. The seeds have a protein content of 39.07% and a fat content of 19.11%. The Gema variety is sensitive to leaf virus (CMMV) and moderate to rust disease [19]. In addition, these varieties are also somewhat susceptible to pod suckers, somewhat resistant to pod borer, and moderate to armyworm pests (**Figure 2**) [19].

2.7 Deja-2 varieties

The Deja-2 variety has a deterministic growth type, ± 37 days of flowering, ± 80 days of maturity, purple hypocotyl color, purple epicotyl color, green leaf color, purple flower color, brown coat color, light brown pod skin color, seed coat color. Yellow, yellow cotyledon color, brown hilum color, oval leaf shape, medium leaf size, 3 branches per plant, the number of pods per plant ±38 pods, ± 52.3 cm plant height, lying with resistance to collapse, pod breaking with the pods are not easily broken, the size of the seeds is large, the weight of 100 seeds is ±14.8 grams, the shape of the seeds is oval, the potential yield is 2.75 t ha^{-1}, the average yield is ±2.38 t ha^{-1}, the protein content is ±37.9%, fat content ±17.2%, susceptible to

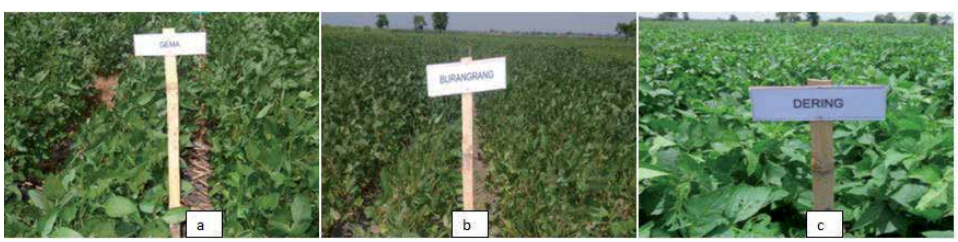

Figure 2.
Appearance of Gema (a), Burangrang (b), and Dering (c) varieties. Source: Fattah et al. [21].

Figure 3.
Dena-1 (a), Deja-1 (b), and Dega-1 (c) varieties. Source: Fattah et al. [21].

armyworm pests, mildly resistant to pod borer, somewhat resistant to pod suckers, and somewhat resistant to leaf rust disease (**Figure 3**) [19].

2.8 Dena-1

According to the description of the soybean variety [19], the Dena-1 variety has a deterministic growth type, purple flower color, purple fur color, purple hypocotyl color, green epicotyl color, and yellow-yellowish pod skin color. Flowering age 33 days, pod ripe age 78 days, oval leaf shape, number of branches 12 branches per plant, growth type determinant, flowering age ± 33 days, maturity ±78 days, hypocotyl purple color, green epicotyl color, green leaf color, purple flower color, brown fur color, yellowish brown pod skin color, yellow seed coat color, green cotyledon color, brown hilum color, oval leaf shape, medium leaf size, branching 3 branches per plant, number of pods planted ±29, plant height ± 59.0 cm, slightly resistant to falling apart, pods breaking easily, large seed size, weight of 100 seeds ±14.3 grams, oval seed shape, potential yield of 2.9 t ha^{-1}, average yield ±1.7 t ha^{-1}, protein content ±36.7% DM, fat content ±18.8% DM, resistance to pests, resistance to leaf rust disease, susceptible to pod sucker *R. linearis* F. and armyworm pest *S. litura* F., and tolerant up to 50% shade. According to Poniman et al. [20], the Dena-1 variety weighed 100 seeds 13.95 g, the number of pods per plant was 44.25, and was resistant to pod borer attack.

2.9 Dega-1

Has a deterministic growth type, ± 29 days of flowering, ± 71 days of maturity (69–73 days), purple hypocotyl, purple epicotyl color, green leaf color, purple flower color, brown coat color, light brown pod skin color, yellow seed coat, purple cotyledons, brown hilum color, oval leaves, medium-sized leaves, branching from 1 to 3 branches/plant), number of pods per plant ±29 pods, plant height ± 53 cm, resistant to falling, resistant to breaking pods, have a large seed size, weigh 100 seeds 22.98 g, have a potential yield of 3.98 t ha^{-1}, have a protein content of 37.78% DM, a fat content of 17.29%, are resistant to leaf rust disease [19]. According to Poniman et al. [20], the Dega-1 variety had 27.75 pods per plant, 100 seeds 21.38 g weight, and was somewhat resistant to pod borer attack.

2.10 Varieties of Detam-1

The Detam-1 variety has a deterministic growth type, hypocotyl purple color, green epicotyl color, purple flower color, light brown hair color, dark brown pod skin color, black seed coat color, and yellow cotyledon color, slightly round leaf shape, and brightness of shiny seed coat. This variety also has a plant height of

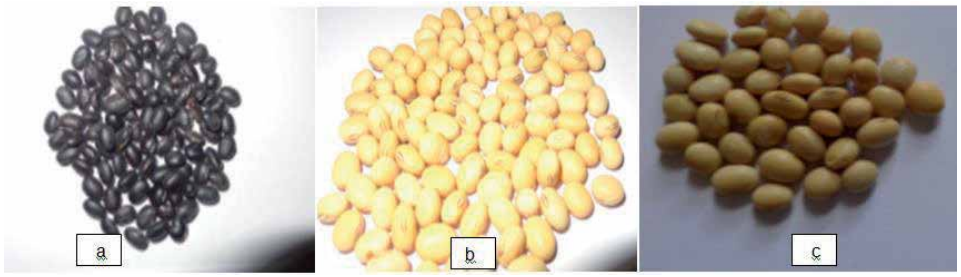

Figure 4.
Seed color of the Detam-2 (a), Anjasmoro (b), and Argomulyo (c) varieties. Source: Fattah et al. [22].

58 cm, a flowering age of 35 days, a pod ripe age of 84 days, has a large seed size (100 seeds weight 14.84 g), has a potential yield of 3.45 t ha^{-1} and an average yield of 2 seeds. 2. 51 t ha^{-1}, the seeds have a protein content of 45.36% and a fat content of 33.06%. The nature of resistance to pests, sensitive to armyworms and somewhat resistant to pod suckers and other properties are somewhat sensitive to drought (**Figure 4**) [19].

3. Armyworm life cycle, level of damage, percentage of yield loss, and economic threshold (ET) in armyworm pests

3.1 Life cycle of *S. litura* F

3.1.1 Egg phase

Adult insects (imago) lay eggs in clusters containing about 350 eggs and covered in fine hairs. The total eggs laid by one female insect in one life cycle are around 2000–3000 eggs [23]. Meanwhile, according to Schreiner [24], *S. litura* F. imago lay eggs in groups of about 200–300 under the leaves covered with brown hairs from the female body. Furthermore, it is said that the total eggs laid by one female insect in one life cycle are about 2,000 eggs.

The eggs that almost hatch, turn brown in color and enlarge like fish eggs (**Figure 5b**). According to Kalshoven [23], the almost hatched eggs turn brown and get bigger. Then hatch into larvae 3–5 days. Meanwhile, Ahmad et al. [26], the eggs hatched 3 days after being laid by the female *S. litura* imago. Furthermore, Kranz et al. [27], suggested that the eggs are laid in groups of 50–300 eggs

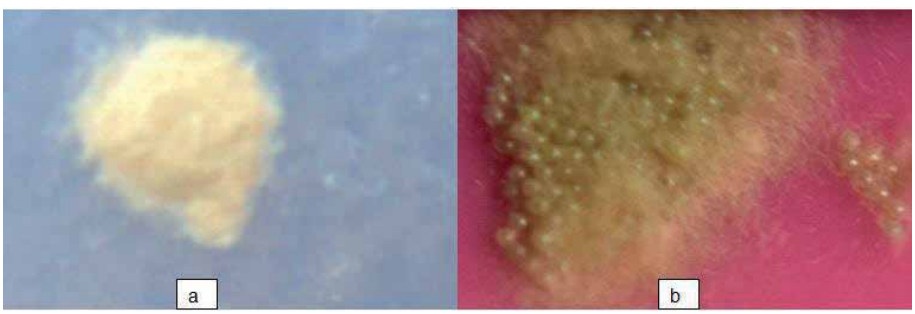

Figure 5.
Eggs in groups covered with hairs from female imgo (a) and eggs that are ready to hatch (b). Source: Fattah, Ilyas [25].

Figure 6.
Instar-1 larvae (newly hatched) (a), and instar-4 larvae (b). Source: Fattah [27].

under the leaf surface and hatch for 3–4 days, and one adult insect can produce 1,500–2,500 eggs.

3.1.2 Larva phase of S. Litura F

The newly hatched larvae feed from the leaves occupied by the eggs in groups (**Figure 6a**), then spread by using threads that come out of their mouths and are used to move from plant to plant. Armyworm larvae have different colors. The newly hatched larvae are light green, the sides are dark brown or brownish black and the last instar larvae have dark black necklaces (crescent moons) on the fourth and tenth abdominal segments. On the dorsal lateral side there is a yellow stripe, the larval stage consisting of 5 instars which lasts 20–46 days [23].

3.1.3 Pupa phase

The last instar larvae enter the soil, then become inactive larvae (Pra pupa) (**Figure 7a**). Then it turns into a pupa (without a cocoon (**Figure 7b**). The pupa is in the ground with a depth of 0–3 cm [28]. The pupa is reddish-brown, weighing about 0.341 g per pupa [29]. The pupal stage ranges from 8 to 11 days [17].

3.1.4 Imago phase

Pupa in the soil will change to the next phase to become butterfly insects (Imago) (**Figure 8**). The life cycle of *S. Litura* F. from egg to imago is about 30–60 days [17]. Meanwhile, Javar et al. [29], the life cycle of *S. litura* is approximately 29–35 days.

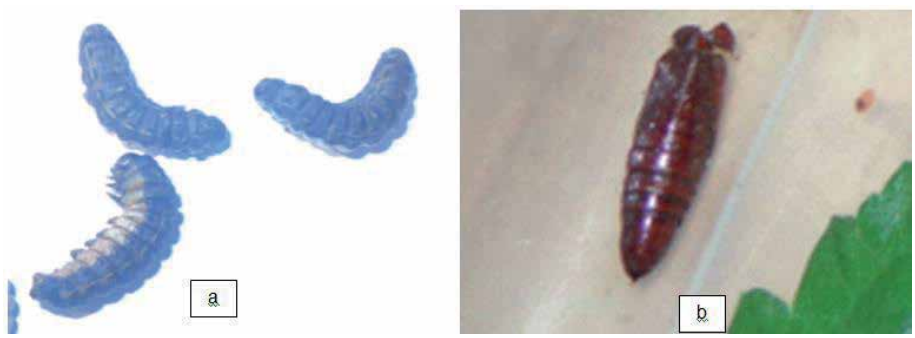

Figure 7.
Prepupa phase (a) and pupa phase (b) of S. Litura F. source: Fattah, Ilyas [25].

Figure 8.
Imago (female) S. litura F. (a) and imago (male) of S. litura F. (b). Source: Fattah, Ilyas [25].

3.2 The level of leaf damage due to attack by armyworm pests on soybeans

The young larvae (instar-1 and instar-2) damage the leaves by leaving remnants on the upper (transparent) epidermis and leaf bones. The rates of armyworm infestation differ between plant types and between varieties. In susceptible plants provide better growth for pests. Conversely, resistant varieties will give poor growth and development of armyworm pests. The results of research by Shahout et al. [30], of several types of plants tested on *S. litura*, the development of the larvae was shorter in the feeding of mustard greens (15.55 d), cotton (15.73 d), and potato (15.82 d) than the diet from cowpeas (19.55 d). Likewise, the response of soybean varieties to the level of *S. litura* F. attacks will vary in each region. This is indicated by the results of research conducted by Fattah and Hamka [31] in Panincong, Soppeng Regency, showing that the intensity of armyworm attack on the Mahameru variety was 17.26%, Kaba 13.5%, Anjasmoro 10.94%, Sinabung 12.16%., Detam-1 12.53%, Wilis 14.41%, Detam-2 15.34%, Burangrang 12.11%, Argomulyo 10.16, and Grobogan 8.61%. Meanwhile, the results of research conducted by Rahman and Fattah [32] in Simbang, Maros Regency showed that the intensity of attacks on Grobogan was 11.60%, Anjasmoro 11.20%, Argomulyo 12.71%, Detam-1 15.21%, Wilis 15, 51%, Gema 13.30%. The results of research by Hendrival et al. [6], the intensity of *S. litura* attacks at plant age 1–2 WAP on the Kipas Merah variety was lower (2.36% -5.02%) than the Anjasmoro variety (3.81% -9.39%). Fattah et al. [33], the highest level of soybean leaf damage due to *S. litura* F. attack was in Anjasmoro variety 31.65% and the lowest was Grobogan 23.96%.

Damage and yield loss due to armyworm attack is determined by the level of the pest population, the stage of insect development, the phase of plant growth, and the type of soybean varieties. Pest attacks on susceptible varieties will cause very significant losses. Leaf defoliation due to armyworm attack when it occurs during the full flowering phase and pod formation phase will result in greater yield losses than attacks in the full pod filling phase (**Figure 9**) [17].

Symptoms of damage to leaves due to *S. litura* F. pests in each soybean variety have different levels. According to Fattah et al. [34], the symptoms of leaf damage due to *S. litura* pests on Anjasmoro varieties ranged from 20.19 to 28.61%, Argomulyo 14.68–26.17%, and Grobogan around 13.28–18.00%. Fattah et al. [36], the highest *S. litura* F., attack rate was in Anjasmoro (26.68–32.69%) and the lowest was in Grobogan (17.07–24.81%). One of the factors that influence the differences in the level of leaf damage is the number and length of trichomes possessed by each variety. The greater the number of trichomes in soybean leaves, the lower the symptom level of the attack. Likewise, the length of the trichomes, the longer the trichomes on soybean leaves, the lower the level of leaf damage. This is evident from the results of research by Fattah [35], Grobogan variety has the lowest symptoms of leaf damage because it has the highest

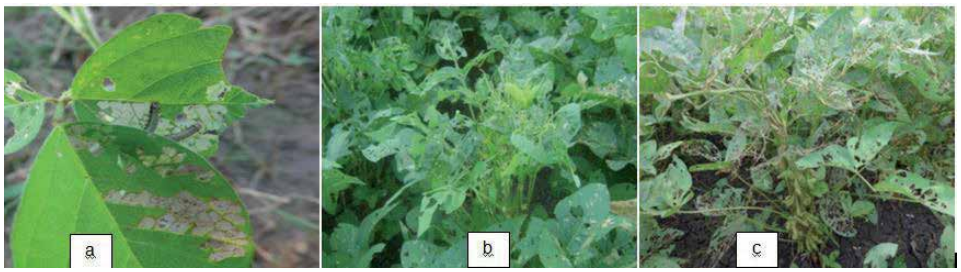

Figure 9.
Symptoms of leaf damage due to S. litura F. pests in the vegetative phase (30 days) (a), the vegetative phase before flowering (b), and the generative phase (c). Source: Fattah [27].

number of trichomes and lengths of trichomes (58.80 per cm2 and 1.90 mm) compared to Anjasmoro variety, only 28.95 per cm2 and 1.66 mm.

The level of damage to soybean leaves due to *S. litura* F. pests is not only determined by the type of variety that the farmer uses, but also by the population density of *S. litura* F. in the field. This is in accordance with Fattah et al. [36], the level of damage to soybean leaves at 35 days after planting due to *S. litura* F., pests was the highest at a population density of 6 larvae per plant (38.64–43.52%), 4 larvae 33.43–36.33%), and 2 larvae per cropping (25.82–27.88%). The same thing in the results of Fattah's study [35], the level of damage to soybean leaves at the age of 25 days after planting due to *S. litura* F. pests was the highest at a population density of 6 larvae per crop (32.01–34.50%), 4 larvae per cropping (22.00–28.70%), and 2 larvae per crop (19.17–26.74%).

3.3 Yield loss due to attack by armyworm pests on soybeans

The rate of loss of soybean seeds due to *S. litura* F. pests was different for each variety depending on the level of damage. This is consistent with Fattah [35], the highest rate of seed loss due to *S. litura* F. armyworm attack was in Anjasmoro (8.97%) and the lowest was in Argomulyo (6.77%). Meanwhile, Fattah et al. [36], the rate of loss of soybean seeds due to *S. litura* F. pests was the highest in Argomulyo variety (13.57%) in the vegetative phase and 14.93% in the generative phase.

The difference in the level of loss of soybean seeds due to *S. litura* F. pests, apart from being influenced by the variety, is also influenced by the level of larval population density. The higher the population density of *S. litura* F larvae per plant, the higher the yield loss. According to Fattah et al. [36], the level of soybean yield loss due to *S. litura* F. armyworm attack was the highest at a population density of 6 larvae per plant (38.64–43.63%) in the vegetative phase and 38.35–41.98%) in the generative phase. While the lowest was in the population density of 2 larvae per plant (25.82–30.96%) in the vegetative phase and 24.39–30.96%) in the generative phase. Meanwhile, Fattah [35] stated that the highest rate of soybean seed loss due to *S. litura* F. attack was at a population density of 6 larvae per plant (23.47%) and the lowest was at a population density of 2 larvae per plant (13.94%).

3.4 Economic threshold (ET) on *S. litura* F

The national economic threshold set by the Government in the use of insecticides for the control of *S. litura* F. armyworms on soybeans is if 1 instar-3 larvae per clump is found at the age of the plant 20 days after planting or if an attack intensity is found around 12.5% at age the same [17]. This is different from the results of Fattah's research [35], by using three varieties namely Anjasmoro, Argomulyo and

Grobogan with the calculation of the costs incurred by farmers (cost) during one growing season with 2 insecticide applications per week, so the total cost of farmers' expenses is around IDR 2,340,000 per ha and loss of seed yield per larva per plant is 96 kg. Based on this data, it was obtained the economic threshold (ET) of *S. litura* F. armyworms of 3.0 3 instar larvae per plant [35]. The difference in the economic threshold is influenced by several factors, including the types of soybean varieties planted by farmers (recommended) which are different from those that were in the past. Some of the factors that differ between recent varieties and ancient varieties are morphological factors including physical resistance, seed yield, plant height, number of pods, number of branches, and chemical resistance characteristics.

Based on the results of Fattah's research [35] from the results of data analysis, it was found that the average yield loss in Anjasmoro variety was around 130 kg, the total cost (Cost) was IDR 2,340,000 per ha, then the economic threshold (AE) for Anjasmoro was 2.25 tails. Larvae per plant or 2.0 larvae per plant. Furthermore, the economic threshold (ET) was found in the Argomulyo variety, if the average yield loss per hectare was 105 kg, then the economic threshold (ET) for Argomulyo variety was 2.78 larvae per plant or 3.0 larvae per plant. The economic threshold (ET) for Grobogan variety if the average yield loss is 91 kg per ha, then the economic threshold value is 3.21 larvae per plant or 3.0 larvae per plant [35].

According to Fattah [35] the economic threshold (ET) value of Anjasmoro variety (2 larvae plant^{-1}) is lower than Argomulyo (3.0 larva plant^{-1}) and Grobogan (3.0 larva plant^{-1}), this is due to the variety Anjasmoro is more sensitive to armyworm attacks than Argomulyo and Grobogan. This is consistent with Fattah and Hamka [31], the attack rate of *S. litura* F. armyworms in Anjasmoro variety (10.16%) was higher than Grobogan (8.60%).

4. Pod sucking pests *N. viridula* L

The pod sucker *N. Viridula* L. is the main pest on soybeans in Indonesia, including in South Sulawesi. According to Marwoto et al. [37] Mature green ladybugs begin arriving at the plant near the flowering phase. Furthermore, it was said that the eggs were laid in groups, with an average of 80 eggs, on the lower leaf surface, the upper leaf surface, pods and plant stems. The egg's cup-like shape is yellow and turns brick red when it hatches. The eggs hatch after 5–7 days. Young ladybugs (nymphs-1) live in groups on the egg shell. To become an adult insect, the nymphs of 5 instar-5 will experience a change in color and size. The body length of nymph-1 to nymph-5 is 1.2 mm, respectively; 2.0 mm; 3.6 mm; 6.9 mm, and 10.2 mm. Young instar-4 ladybugs begin to spread to surrounding plants. In the morning, ladybugs usually stay on the upper leaf surface, but during the day will descend to the pods to feed and take shelter. Young and adult ladybugs damage the pods and seeds by poking their stylet on the pod shells and into the seeds and then sucking the seed juices. The damage caused by these green ladybugs causes a decrease in yield and seed quality. Host plants other than soybeans are rice, beans, Crotalaria, potatoes, sesame, maize, tobacco, chilies, and Tephrosia. According to Kalshoven [23], *N. Viridula* L. has a green color which is commonly called the green ladybug, lays the eggs in groups of 10–90 eggs on the leaves. Its life cycle from egg to adult is around 4–8 weeks, the total life cycle is around 60–80 days. This pest has a host of legumes, maize, cotton, and rice.

According to Manurung et al. [38], the level of pod sucking pest *N. viridula* L. attack on soybeans was 51.66%. Bayu and Tengkano [39], the rate of yield loss due to pod sucking pest *N. Viridula* L. can reach 10.0–41.0%. Sari and Suharsono [40], the level of damage to soybean pods due to pod sucking pests of *N. viridula* L on Burangrang varieties was 32.50%, Kaba 31.50%, and Wilis 36.83%. According to

Rahman and Fattah [32], the level of pod damage caused by pod sucker *N. viridula* L was the highest in Burangrang variety (13.20%), followed by Gema (12.50%), Dering (10.50%), Argomulyo varieties. (9.40%), Detam-1 (9.0%), Grobogan 8.50% and Anjasmoro (7.70%).

5. The pod borer *E. zinckenella* T

The pod borer *E. zinckenella* is an important pest on soybeans. This is in accordance with Sidabutar et al. [41], the pod borer *E. zinckenella* T. is one of the important pests of soybean in Indonesia. The same thing was expressed by Apriyanto et al. [42], the pod borer is an important pest of soybeans. Furthermore, it was said that in addition to attacking soybean plants, *E. zinckenella* T. also attacked other legumes and could cause pod damage levels of up to 100% without the use of insecticides. According to Marwoto et al. [7], adult insects *E. zinckenella* T. lay eggs in groups of 4–15 eggs under leaves, flower petals or on pods. Eggs hatch 3–4 days after being laid, instar 1 and 2 bore the pod shells, bore the seeds and live in the seeds. Furthermore, it is said that the last instar larva has a size of 13–15 mm with a width of 2–3 mm. This last instar turns into a pupa 8–10 mm long and 2 mm wide which forms in the soil. The pupae will turn into moths after 9–15 days.

The level of damage to pods due to *E. zinckenella* T. pests was different for each soybean variety. This is evident from the results of research by Rahman and Fattah [32], the level of damage to soybean pods due to pod borer *E. zinckenella* T. attacks was the highest in Detam-1 (15.71%), then followed by Dering (14.5%), Kaba (11.30%, Burangrang (10.60%), Gema 10.0, Detam-2 (9.20%), Tidar (9.10%) Argomulyo 8.20%, Grobogan 7.10%, and Anjasmoro 6, 70%. According to Baliadi et al. [43] argued that female imago disinterest in laying eggs on host plants plays an important role in the resistance of soybean varieties to pod borer. Furthermore, it is said that trichomes have a negative effect on the number of eggs laid, but have a role important in the mechanism of resistance to pod borer. The average density of trichomes in pods of Wilis variety was 10 mm^{-1}, lower than those in the IAC-100 and IAC-80-596-2 lines, respectively 25 and 22 mm^{-1} so that the genotype it is more resistant than Wilis. Bayu et al. [44] suggest that wa genotypes IAC 100 and G100H had the lowest pod and sed attack rates and were categorized as resistant to *E. zinckenella* T. attack. Furthermore, it was said that this happened because the *E. zinckenella* T. imago did not like laying eggs in both genotypes because they had hard pod skin and dense trichomes. Furthermore, it is said that in addition to the two genotypes having non-preferential characteristics, it is also suspected that the two genotypes have secondary compounds which are not preferred by *E. zinckenella* T. imago as a place to lay eggs. According to Poniman et al. [20], the Argomulyo variety had the highest trichomes (24.75) compared to Demas (8.0), Dega-1 (15.50), Dena-1 (15.00), Dering 16.0) and Gema (21.50) so that Argomulyo has the lowest population of *E. zinckenella* T. pod borer larvae (8.0 larvae).

6. Pod-sucking pests *R. linearis* F

The *R. linearis* pest is an important pest in Indonesia. According to Marwoto [45], one of the important factors in soybean is *R. linearis* F. and can cause pod damage by about 15–20% when pods are formed and filled. Furthermore, Prayogo and Suharsono [18] suggested that the level of damage to the seeds was also influenced by the location and number of punctures in the seeds, while the attack of *R. linearis* F. in the pod formation phase caused the pods to dry and fall and in the pod growth phase

and seed development it caused pods and seeds to collapse later pods dry up and eventually fall off. Furthermore, Asadi [46], the loss of soybean seed due to *R. linearis* F. attack can reach 79% depending on the type of genotype or variety. Furthermore, it was said that the genotypes GM425 Si and TGM131–1-1-1B had the lowest *R. linearis* F. attack rates, respectively 11% and 14%, pods were attacked by 19% and 20%, respectively, and seeds were attacked by 11% and 14%, respectively. The attack of pod pods on soybeans on farmers' land is largely determined by the type of variety the farmer is growing. According to Sarjan and Sa'i [47], the attack rate of *R. linearis* F. pod suckers varied greatly from the lowest, namely Burangrang 17.69–22.35%, then followed by Anjasmoro 26.31–29.07%, Grobogan 31.92–37.88%, Argomulyo 35.83–38.32%, Panderman 42.63–72.87%, Kaba 44.79–85.77%, and 54.89–86.87%. Furthermore, it was said that the low rate of pod sucker attack on Burangrang was one of the causes was the high length of pod trichomes in these varieties. The following is the length of the pod trichomes of each variety from highest to lowest Burangrang 1.54–1.59 mm^2, Anjasmoro 1.26–1.29 mm^2, Panderman 1.13–1.28 mm^2, Argomulyo 1.20–1.24 mm^2, Grobogan 1.20–1.26 mm^2, Kaba 1.22–1.26 mm^2, and Tanggamus 1.18–1.24 mm^2 [47]. According to Sunarno [48], the IAC-100 variety had a higher number and length of trichomes 1.90 mm and 13.1 per mm2, respectively, having a lower number of stylet punctures per seed 5.48 and 6, respectively. 33 imago phases, while the MGL 2979 variety had a low length and number of trichomes, respectively 1.0 mm and 4.90 per mm2, having a higher number of stylet punctures per seed 12.62 nymph phases and 9.31 phases, respectively. Imago.

7. Conclusion

South Sulawesi Province is one of the centers for soybean development in Indonesia. Farmers develop new high yielding varieties such as Anjasmoro, Argomulyo, Grobogan, Dering, Gema, Deja-2, Dena-1 Dega-1, Detap-1, and Detam-1. The level of leaf damage caused by *S. litura* F and pod damage caused by *N. viridula* L. and *E. zinckenella* T. varies greatly depending on the level of resistance of each variety. The level of leaf damage caused by *S. litura* F was the highest in Anjasmoro (10.94–32.69%) and the lowest was in the Grobogan variety (8.61–24.81%). The level of pod damage due to the attack of *N. viridula* L. was the highest in the Burangrang variety (13.20%) and the lowest in Anjasmoro (7.70%). The level of pod damage caused by *E. zinckenella* T. was the highest in Detam-1 (15.71%) and the lowest in Anjasmoro (6.70%). The rate of yield loss due to S. litura F. was the highest in the Anjasmoro variety (8.97%) and the lowest was in Argomulyo (6.77%). The results of this paper are expected to be a reference or guideline for farmers in South Sulawesi in choosing superior soybean varieties for growing crops.

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Conflict of interest

All authors claim to have no conflicts of interest.

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Chapter 20

Synthetic Communities of Bacterial Endophytes to Improve the Quality and Yield of Legume Crops

Mariela I. Monteoliva, Lucio Valetti, Tania Taurian, Clara S. Crociara and María Carla Guzzo

Abstract

Plant-associated microbiomes confer fitness advantages to the plant host by growth promotion through different mechanisms including nutrient uptake, phytohormones production, resistance to pathogens, and stress tolerance. These effects of the potentially beneficial microbes have been used in a diversity of biotechnological approaches to improve crop performance applying individual bacterial cultures. However, healthy plants host a diversity of microorganisms (microbiota). Next-generation sequencing technologies have offered insights into the relative abundances of different phylogenetic groups in a community and the metabolic and physiological potential of its members. In the last decade, researchers have started to explore the possibilities to use temporal and functional combinations of those bacteria in the form of synthetic communities. In this chapter, we review the benefits of using endophytic bacteria in legumes, the available methodological approaches to study the effects of bacterial communities, and the most recent findings using synthetic communities to improve the performance of legume crops.

Keywords: sustainable agriculture, abiotic and biotic stresses protection, food security, endophytic bacteria, synthetic communities

1. Introduction

Plants constitute vast and diverse niches for endophytic organisms, and there is not a single plant species devoid of them. The most up-to-date definition for endophytes defines them as the microorganisms isolated from surface-sterilized plant tissues, which do not cause any noticeable harm to their host plants [1, 2]. The most abundant and common microbes living as endophytes are bacteria and fungi [3]. Endophytic bacteria are present in any kind of plant, from ferns and bryophytes to mono and dicotyledonous species [4]. In nature, mainly the intercellular spaces of the plant host are colonized by the endophytic bacteria [1, 5, 6]. But, endophytes have been also found in intracellular spaces of grapevine, barley, tobacco, Arabidopsis, and pine [7], suggesting that legumes may also have intracellular endophytes.

The endophytic bacterial communities make significant contributions to growth promotion and plant health in mutualistic (even symbiotic) relationships. The plant host protects the bacteria from the environment, while the endophytic community provides several benefits to the host. The benefits for the plant may include nutrient assimilation (such as nitrogen, phosphorus, or iron), growth stimulation, defense against pathogens, and/or protection against environmental stresses [8, 9]. Some of these effects might be altered when the plant is under stress [10].

The use of these natural symbionts/mutualists offers an opportunity to maximize legume crop productivity while reducing the environmental impacts of agriculture. For decades, most of the studies (and agricultural applications) have been about the effects of individual strains of bacteria, but recently with the bloom in bioinformatics and sequencing technology development, the knowledge about the plant microbiota has burst, and the potential to use and manipulate complex bacterial communities has started to be the target of a large research community.

2. Plant endophytic microbiome

In natural environments, the intracellular spaces of legumes are inhabited by numerous microorganisms, such as virus, fungi, nematodes, and bacteria. Here we focus on bacterial endophytes that benefit the plant in some way. Those bacteria colonize the host by several mechanisms, such as natural opening or injures and proliferate within the host. There is a huge taxonomic and functional diversity of endophytic bacteria, adapted to the microenvironments that the plant host provides. That diversity will be shaped by the microbial community members, the plant host, and the environmental conditions.

2.1 Colonization and distribution within the host plant

Colonization mechanisms vary with the type of interaction between the host and the bacteria and the life cycle of the microbe. Overall, most of the endophytic bacteria enter the plant through the roots. Since the microbial diversity decreases from the root to the leaves, it has been proposed that most of the microbes colonize the plant through the roots and proliferate to aboveground tissues [11] (**Figure 1**). Endophytic bacteria are usually "recruited" by plant host root exudates, such as organic acids, amino acids, and proteins [12, 13]. Once the bacteria are close to the root surface, they enter through lateral root emergence areas or other openings, caused by wounds or mechanical injuries. In the early stages, most of the endophytes are first observed in root hairs and subsequently in the root cortex [14]. However, endophytes can also colonize the leaves through the stomata, injuries in the epidermis, or introduced by vectors. In leaves, bacterial endophytes have been observed in the intercellular spaces of mesophyll, substomatal areas, and xylem tissues [15, 16].

In addition, the habit of the microbe conditions its colonization strategy. For example, obligate endophytes, which depend on the plant metabolic activity for their survival, are usually transmitted to the seed (vertical transmission) and spread inside the plant or through the action of a vector. On the contrary, most of the facultative endophytes, which have a free life in the soil and colonize the plant during some stage of their life cycle, colonize the plant through occasional wounds [17].

The colonization process itself alters host plant physiology (in a process called "niche construction" from the microbe's point of view) by defense alterations or direct shift of the host metabolism [18]. Those microenvironment changes can affect the local microbiome structure and functions, by altering relationships

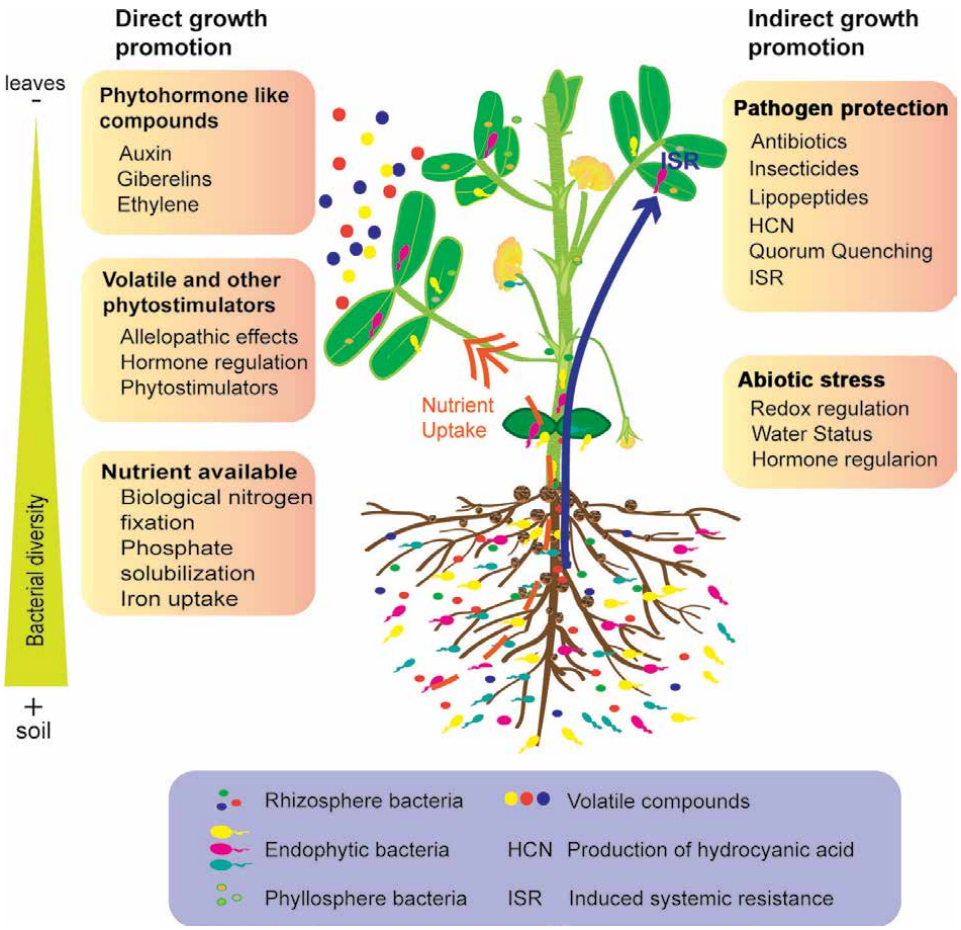

Figure 1.
Diversity gradient of bacterial endophytic microbiota and growth promotion mechanisms to legumes. Legumes are surrounded and interact with bacteria in the soil and air (epiphytic bacteria in the rhizosphere and phyllosphere) and in the inter- and intra-cellular spaces (endosphere). Those bacteria can be saprophytic, pathogenic, or beneficial for the plant. The beneficial bacteria can promote plant growth by direct and indirect mechanisms. Direct mechanisms include phytohormone, volatiles, and other compounds production and facilitation of nutrient assimilation. Indirect mechanisms include pathogen and abiotic stress protection. ISR, induced systemic resistance.

among bacterial species and within the host. Furthermore, under particular conditions, part of the response of the plant will stimulate or recruit specific endophytes, which may contribute to survival or tolerance of that condition [19, 20]. It was proved in tomato cultivars that the transplant of the rhizosphere from a resistant to a susceptible cultivar suppressed *Ralstonia solanacearum* disease symptoms. They found a highly abundant flavobacterial genome in the resistant cultivar rhizosphere, and the isolated flavobacteria suppressed disease symptoms in the susceptible cultivar in pots [21]. In legumes, it was reported that *Fusarium*-resistant common bean cultivars showed a higher abundance of Pseudomonadaceae, Bacillaceae, Solibacteraceae, and Cytophagaceae families [22], but no further inquiries have been reported.

Another aspect affecting the colonization process of the endophytic bacteria is the host defenses. Endophytes live in the same environment as many plant pathogens and share close similarities with them. Microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) are conserved and necessary for microbial survival, but plants have evolved multiple receptors to recognize them and induce

the plant immune system. Then, the colonization of endophytic bacteria triggers plant defenses, and the process needs to be avoided or blocked by the beneficial endophytes to be able to colonize and proliferate within the host [2, 23, 24]. It is not well understood yet how the beneficial bacteria overcome the defenses, but a few mechanisms have been unraveled, including the blockage of MAMPs and defense signaling [25]. The beneficial bacteria *Bacillus subtilis* avoid a strong defensive response in the host by blocking the detection of their own flagellin by the secretion of the flagellin-binding peptide subtilomycin [25, 26]. Another mechanism is the secretion of bacterial antioxidant enzymes, such as superoxide dismutases and glutathione-S-transferases to detoxify the reactive oxygen species that signals the plant defense [27]. An alternative mechanism is the suppression of salicylic acid (SA)-mediated defense signaling. *Sinorhizobium fredii* HH103 with defective type III secretion system (T3SS) is unable to suppress SA-dependent defenses and subsequently fails to promote nodulation on the host [28], indicating that the suppression of the SA-dependent defense is critical for endophyte colonization. Some of those mechanisms have not been reported in legumes, but if those bacteria are colonizing legumes, similar mechanisms might be in action.

The establishment of the endophytic bacterial community in the legume host is a complex and dynamic process that has been studied mostly in fragments and simplified systems (usually one bacterial strain in one host under one or a few conditions), and it must be further understood to take the best advantages of their potential benefits for legume agriculture.

2.2 Endophytic bacterial diversity

There is an enormous diversity of bacterial endophytes in legumes, considering that the rhizobia are also endophytes. The interaction of rhizobia and legumes has been studied for more than a century [29]. Since then, many rhizobial endophytic bacteria were isolated from different legumes, particularly root and nodule tissue. These bacteria can establish a symbiotic interaction, induce the formation of new organs in roots and stems called nodules, and fix atmospheric nitrogen. In addition, the so-called "new rhizobia" (or noncanonical rhizobial genera) of Alfa- and Beta-Proteobacteria has been reported in the last decades. They can form nodules and fix nitrogen and mainly belong to *Microvirga* spp. and *Burkholderia* spp., respectively [30]. Other non-nitrogen-fixer endophytes are present in nodules and sometimes improve nodule formation [31–33]. For instance, Hoque et al. [34] isolated rhizobia and non-rhizobia endophytes from two wild *Acacia* species from Australia, and nodules were produced by species of the genera *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Burkholderia*, *Phyllobacterium*, and *Devosia*, much more than expected. In addition, rhizobial species were isolated from other plant tissues apart from nodules [3].

Overall, from a large number of bacterial genera present in legumes, the most frequent ones (excluding rhizobia) are *Agrobacterium*, *Bacillus*, *Enterobacter*, and *Pseudomonas*, followed by *Acinetobacter*, *Arthrobacter*, *Curtobacterium*, *Devosia*, *Dyella*, *Herbaspirillum*, *Klebsiella Micromonospora*, *Microbacterium*, *Mycobacterium*, *Ochrobactrum*, *Paenibacillus*, *Pantoea, Rhodopseudomonas, Serratia, Staphylococcus*, and *Sphingomonas* ([3, 9, 21], and reference therein) (**Tables 1** and **2**).

2.3 Factors affecting diversity

The composition, diversity, and abundance of the endophytic microbiome are influenced by the soil microbial pool; the plant host identity and status (genotype, development, and physiology); agricultural practices; and climate and environmental conditions (such as temperature, water supply, and nutrients) [8, 16, 71].

Legume species	Organ	Treat.	Method	Most abundant bacterial	Functions	Ref.
Peanut *Arachis hypogaea*	Seed germs, sprout, cot.	Develop.	*16S*	*Synechococcus; Halothiobacillus, Paracoccus, Agrobacterium, Gallionella; Mycobacterium, Rhodococcus, Burkholderia, Erwinia, Hyphomonas, Devosia*	N.D.	[35, 36]
	Root	Monocrop vs. crop rotation	MG, MT	*Bordetella, Burkholderia, Ktedonobacter, Ktedonobacter racemifer, Opitutus terrae, Thermomicrobium roseum, Chloroflexus aggregans, Thermosediminibacter oceani, Dehalogenimonas lykanthroporepellens*	N, S, P metabolisms, oxidative stress resistance, antibiotics, siderophores, IAA synthesis genes	[37]
Chickpea *Cicer arietinum*	Roots, nodule	BT-transgene	*16S*	*Calothrix, Rickettsia, Mesorhizobium, Methylobacillus, Arthrobacter, Bacillus, Streptomyces, Saccharopolyspora, Rhodococcus, Ramlibacter, Propionivibrio, Janthinobacterium, Kaistobacter, Sphingomonas, Ammoniphilus, Rubrobacter. Actinocatenispora, Pseudaminobacter, Burkholderia Shinella.*	N.D.	[38]
Rosewood *Dalbergia odorífera*	Nodule	Seedlings, rhizobial inoculation	*16S*	*Bradyrhizobium, Chloroplast norank, Lactococcus, Mycobacterium, Bacillus, Rhizobium, Mesorhizobium, Burkholderia*	N.D.	[39]
Soybean *Glycine max*	Nodule	Salty soils	*16S*	*Ensifer, Enterobacter, Stenotrophomonas, Chryseobacterium*	N.D.	[40]
	Root	Soil type	*16S*	*Klebsiella, Pseudomonas, Stenotrophomonas, Rhizobium, Acinetobacter, Chryseobacterium, Acidovorax, Achromobacter, Agrobacterium, Burkholderia*	IAA, BNF, P solubilization, ACC-DA	[41]
		Strigolactone-related genes	*16S*	*Microbacteriaceae, Rhizobiaceae, Bdellovibrionaceae*	N.S.	[42]
	Root, nodule, soil	Develop., soil type	*16S*	Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes	N.D.	[43]
		Develop.	*16S*	*Bacillus, Bradyrhizobium*, *Rhizobium*	N.D.	[6]

Legume species	Organ	Treat.	Method	Most abundant bacterial	Functions	Ref.
Alfalfa *Medicago sativa*	Nodule	Synthetic community	*16S*	*Brevibacillus, Paenibacillus, Pantoea, Pseudomonas*	Antibiotics	[44]
		—	*16S, nodC, nodA, nifH genes*	*Sinorhizobium, Rhizobium, Bacillus Shinella, Pseudomonas, Variovorax, Novosphingobium, Methylibium, Bradyrhizobium, Mycobacterium*	N.D.	[45]
Medicago truncatula	Leaf, nodule, root	Genotype, soil	*16S*, MG	*Pseudomonas, Niastella, cyanobacteria Phormidium. Thioalkalibacter, Neorhizobium, Ohtaekwangia, Nodules: Ensifer, Rhizobium, Bradyrhizobium, Rhizobacter, Shewanella*	N.D.	[46]
Pea *Pisum sativum*	Root, nodule	Develop.	*16S*	*Rizhobium Mezorizhobium, Pseudomonas*	BNF	[47]
Black mung bean *Vigna mungo*	Nodule	—	Full-length *16S*	Ferrmicutes. *B. subtilis*, *Paenibacillus taichungensis*	P solubilization, IAA, siderophore, ammonia, HCN, ACC-DA	[48]
			18S, 16S	*Candida glabrata, C. tropicalis*	IAA, ACC-DA, siderophores, ammonia, polyamines synthesis	[49]
Mung bean *V. radiata*	Nodule	—	*16S*	*Bacillus aryabhattai, Bacillus megaterium and B. cereus*	IAA	[50]
Cowpea *Vigna unguiculata*	Nodule	—	*16S*	*Rhizobium, Paraburkholderia, Enterobacter, Strenotrophomonas Pseudomonas*	BNF	[51]
Red clover Trifolium pratense	Root	—	*16S*	*Rhizobia, Pantoea, Sphingomonas, Novosphingobium, Pelomonas*	N.D.	[52]
Lens culinaris, *P. sativum (plus canola and wheat)*	Root	Species, soil type	*16S*	*Pseudomonas, Arthrobacter,* unclassified genera of *Enterobacteriaceae, Comamonadaceae*	N.D.	[53]
A. hypogaea, G. max, V. radiata, V. unguiculata, V. mungo	Nodule	—	*16S*	*Enterobacter cloacae, E. ludwigii, Chryseobacterium indologenes, Klebsiella pneumoniae, Klebsiella variicola, Pseudomonas aeruginosa.*	BNF, P solubilization, siderophores, IAA, ACC deaminase (*nifH* gene)	[54]

Legume species	Organ	Treat.	Method	Most abundant bacterial	Functions	Ref.
Vicia villosa, T. repens, T. pretense, M. sativa	Seed	—	*16S*, MG	*Acinetobacter, Sphingomonas, Lactobacillus, Bacillus, Pantoea, Salmonella*	Energy, amino acid and carbohydrate metabolisms, cell growth and death programs, transport, genes	[55]

ACC, 1-aminocyclopropane-1-carboxylate; ACC-DA, ACC deaminase activity; IAA, indole-acetic acid; BNF, biological nitrogen fixation; Develop., developmental stages; MG, meta-genomics; MT, meta-transcriptomics; N.D. not determined; N.S., not significant; Treat, treatment or factor affecting microbiome.

Table 1.
Culture-independent studies of the endophytic bacterial microbiome in legume crops.

Legume species	Organ	Treat.	Met.	Most abundant bacterial	Function	Ref.
Peanut *Arachis hypogaea*	Nodule	Genotype	*16S*	*Rhizobium phaseoli, Bacillus tequilensis, B. altitidinus, B. tequilensis, B. siamensis, B. subtilis, Pantoea dispersa, Paenibacillus illinoisensis, Kosakonia oryzendophytica, Rhizobium mayense, P. dispersa*	IAA; ACC-DA; P, Zn, and Si solubilization, siderophore	[56]
	Seed	—	*16S*	*Pseudomonas* spp.	IAA, P solubilization, siderophores, cellulase, protease, control of *S. rolfsii*	[57]
Chickpea *Cicer arietinum*	Root	Soil type	*16S*	*Enterobacteriaceae, Pseudomonadaceae, Xanthomonadaceae, Bacillus, Stenotrophomonas, Pseudomonas, Enterobacter*	N.D.	[58]
	Root, nodule	—	*16S*	*Mcrobiospora, Streptomyces, Micromonospora, Actinomadura*	N.D.	[59]
		—	*16S*	*Enterobacter, Rhizobium, Stenotrophomonas, Pseudomonas, Burkholderia, Bacillus, Brevibacillus*	IAA, siderophores	[60]
Soybean *Glycine max*	Nodule	Antifungal activity	*16S*	*Enterobacter, Acinetobacter, Pseudomonas, Ochrobactrum, Bacillus*	BNF, IAA, siderophore	[61]
	Leaf, stem, root	RR-transgene	*16S*	Enterobacter ludwigii and Variovorax paradoxus	IAA, P solubilization	[62]
	Leaf, stem, root, nodule	—	*16S nifH*	Pseudomonas aeruginosa and Bradirizhobium	IAA, P and Zn solubilization, siderophore, ACC-DA, cell wall degrading enzymes, pathogenicity	[63]

Legume species	Organ	Treat.	Met.	Most abundant bacterial	Function	Ref.
Lentil *Lens culinaris*	Nodule	—	*16S*	*Pseudomonas stutzer, Lysinibacillus pakistanensis*,	N.D.	[64]
Common bean *Phaseolus vulgaris*	Roots	—	*16S*	*Bacillus velezensis Bacillus amyloliquefaciens Bacillus halotolerans,Bacillus mojavensis,Bacillus methylotrophicus, Bacillus subtilus Pseudomonas frederiksbergensis Pseudomonas lini, Agrobacterium fabrum Glutamicibacter halophytocola.*	IAA, P solubilization, siderophores, HCN, xylanase chitinase, lipopeptide genes, antifungal activity	[65]
Cowpea *Vinga unguiculata*	Nodule	—	*16S*	*Rhizobium, Paraburkholderia Enterobacter, Strenotrophomonas* and *Pseudomonas*	BNF	[51]
C. arietinum, Pisum sativum	Nodule, root	—	*16S*, RFLP	*Pantoea agglomerans, Bacillus cereus,B. sonorensis, B. subtilis, Pseudomonas chlororaphis, Ornithinibacillus* sp., *Ochromobacterium* sp.,	IAA, P solubilization, siderophores, ammonia, organic acids, HCN, biocontrol	[66]
Crotalaria spp., *Indigofera* spp. *Erythrina brucei*	Nodule	Genotype	*16S*	*Achromobacter, Agrobacterium, Burkholderia, Cronobacter, Enterobacter, Mesorhizobium, Novosphingobium, Pantoea, Pseudomonas, Rahnella, Rhizobium, Serratia, and Variovorax. Bacillus, Paenibacillus, Planomicrobium,* and *Rhodococcus*.	N.D.	[67]
V. mungo, V. radiata	Stem	—	16S	*Enterobacter, Bacillus, Pantoea, Pseudomonas, Acromobacter, Ocrobacterium*	BNF, IAA, P solubilization, siderophores, antifungal activity	[68]
P. sativum, *V. faba*	Nodule	—	*16S, nodC*	*Rhizobium leguminosarum, R. indigoferae, R. hidalgonense, R. sophorae, R. laguerrea, R. acidisoli, R. anhuiense,*	IAA, P solubilization, siderophores	[69]
A. hypogaea, G. max, *V. radiata*, *V. unguiculata*, *V. mungo*	Root nodule	-	16S *nifH*	*Enterobacter cloacae, Chryseobacterium indologenes, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter ludwigiiy, Klebsiella variicola*	BNF, P solubilization, AIA, siderophores, ACC-DA	[54]

Legume species	Organ	Treat.	Met.	Most abundant bacterial	Function	Ref.
Trifolium, Lupinus, Ornithopus, Scorpiurus, Medicago, Trifolium, Vicia	Root	Field sites	16S	*Microbacterium, Chryseobacterium, Bacillus, Paenibacilus, Staphylococcus,Pantoea, Erwinia, Achromobacter, Lelliotia, Enterobacter, Acinetobacter, Janthinobacterium, Pseudomonas, Stenothrophomonas, Serratia, Rahnella*	IAA, P solubilization, siderophore, cellulase	[70]
Anthyllis, Colutea, Cytisus, Lathyrus, Lotus, Lupinus, Medicago, Melilotus, Ononis, Ornithopus, Robinia, Trifolium, Vicia, Wisteria	Nodule	Ecoregions (Belgium)	16S	*Bacillus, Paenibacillus, Arthrobacter, Microbacterium, Rhodococcus, Sphingomonas, Cohnella, Pseudomonas, Herbaspirillum, Pantoea, Corynebacterium, Chryseobacterium, Sphingomonas and Xanthomonas*	N.D.	[31]

ACC, 1-aminocyclopropane-1-carboxylate; ACC-DA, ACC deaminase activity; IAA, indole-acetic acid; BNF, biological nitrogen fixation; Develop., developmental stages; MG, meta-genomics; MT, meta-transcriptomics; N.D. not determined; N.S., not significant; Morph & Bioch., morphological and biochemical characterization, Treat, treatment or factor affecting microbiome.

Table 2.
Culture-dependent studies of the endophytic bacterial microbiota in legume crops.

Comparisons among plant species (canola, wheat, pea, and lentil) in different locations and soil types pointed to the genotype influence as the highest effect determining endophyte diversity ([72] in **Table 1**). However, when considering close *Medicago* genotypes (intraspecies comparison), the host genotype effect was not significant (1% of contribution to the total variance), but both soil and plant genotypes were significant for the root microbiota diversity [53]. In the case of the leaf microbiome, the soil reduces its relative importance, since some bacteria colonize it from underground organs, but others enter through stomata or vectors [46]. Broadly, the soil limits the available microbial pool, while the host genotype is a relevant barrier for colonization. Agricultural practices could directly affect the microbiome by chemical applications or through changes in the host physiology. The effects of biotic and abiotic factors shaping the endophytic bacteria communities in plants were reviewed by Papik et al. [73]. In addition, the actual diversity could be masked by the method used to describe it (such as culture-dependent or -independent, see Section 2.4) [16].

2.4 How to study microbiome diversity

Natural communities of endophytic bacteria are conventionally studied using culture-dependent and -independent methods [74]. Culture-dependent methods

imply the extraction of the microbes and their growth in synthetic media. Those strategies allow to isolate the microbe and further study them *in vitro* and in manipulative experiments, but they strongly underestimate the number of bacteria (and the diversity of the community), as cultivable bacteria usually represent only 0.001–1% of the actual bacteria in a sample [16, 75]. Recently, Hartman et al. [52] isolated 200 bacteria strains that represent ~20% of the most abundant genera in *Trifolium* roots, which was one-quarter of the ~3500 detected OTUs in a manageable effort to increase the cultivated endophytic bacteria from a legume (**Table 1**).

On the other side, culture-independent methods mostly rely on the extraction of bacterial genetic material from plant tissues. The genomic DNA can then be analyzed using a range of molecular fingerprinting techniques such as Amplified rDNA Restriction Analysis, Gradient Gel Electrophoresis, and Terminal Restriction Fragment Length Polymorphism (RFLP) [16]. In recent years, DNA fingerprinting techniques have been set aside by more advanced molecular techniques. Those new methods involve DNA extraction from the entire bacterial population to sequence a specific phylogenetic marker, such as the 16S rRNA gene, or the whole genome [76]. In addition, using RNA instead of DNA, it is possible to detect active functional diversity, which provides information about the transcriptionally active functions, as well as the massive analysis of proteins (peptides) or metabolites (by high throughput analysis of "omics"). The latter two do not provide taxonomic information but a functional one.

The sequence-based methods allow a deeper analysis of the endophytic diversity than traditional fingerprinting, although some of the species with low abundance might be still missed. To minimize those losses, it is important to sequence with high depth and carry out rarefaction analysis (to check that the OTU versus the diversity or richness reaches the plateau). Other technical considerations for sequencing analysis are discussed in detail by Lucaciu et al. [77].

The bacterial diversity of the microbiome can be described taxonomically and functionally by different approaches. The most traditional strategy is the taxonomic description of the diversity, which identifies the species present in the microbiome and quantifies their abundance by genome or specific gene sequencing. From that data, researchers have started to uncover what is known as the "core microbiome" [78], which is defined as the group of species present in one plant across different genotypes, environments, developmental stages, etc. Depending on the scale of the analysis, a higher or lower number of species are shared among them. For instance, if dicot and monocot species are compared, the number of shared species will be lower than if two cultivars of the same species are compared in the same environment. A core endophytic microbiome of roots of red clover (*Trifolium pratense*) includes 70% of Rhizobia, and it was dominated by the genera *Pantoea*, *Sphingomonas*, *Novosphingobium*, and *Pelomonas* [52] (**Table 1**). *Glycine* spp. nodules showed a majority of *Ensifer* genera, followed by *Enterobacter*, *Stenotrophomonas*, and *Chryseobacterium* (>0.5%), and some nonrhizobial bacteria only in soybean (*Glycine max*), including *Enterobacter cloacae* (3.62%), *Stenotrophomonas* sp. CanR-75 (2.79%), and *Stenotrophomonas maltophilia* (2.41%) [40] (**Table 1**). Overall, little is known about the core endophytic microbiome in legumes, although some core rhizospheric microbiomes have been described (e.g., [79]).

In addition to the core microbiome, the "keystone" species have been described [80]. Keystones are highly connected species that largely change the structure and function of the microbiome when removed. They may be predicted by co-occurrence networks (by correlation analysis) and are defined as those whose abundance highly correlates with most of the other species [81]. Those correlations can be positive or negative (i.e., two species are always together or the presence of one excludes the other), and the interaction between each other may be indirect (for instance,

mediated by a change in the host) [82]. It has been predicted that when the keystone species is missing, the abundance and proportion of the community change, and occasionally, one species may extremely proliferate over the others. Knowing which are the keystone species for one host is critical to effectively design any agricultural management strategy to protect a healthy microbial community and improve the fitness of the crop.

A second strategy to characterize the microbiome is the functional description, based on the metabolic functions present in the microorganisms. According to the previous model (with a core microbiome and keystone species), the communities in the microbiome are built to occupy functional niches [81]. This means that one species might be (at least partially) replaced by another one, which provides the same function to the community and/or the host. Those key functions of a particular species are given by a set of genes that allow the microbe to effectively interact and benefit the rest of the microbial community and the plant host under specific conditions. These functional traits can be screened and studied by any "omic" analysis and then grouped by the presence of specific metabolic functions (see [83–85] in **Table 1**). For instance, the most important genes differentially detected in the rhizosphere of pea (*Pisum sativum*) under different tillage and fertilization treatments were genes coding ABC transporters and secretion systems, transcription factors, peptidases, methane metabolism, quorum sensing, and bacterial motility proteins [85]. To understand which services the microbial community provides and may favor the host plant, the functional analysis may be more useful than a taxonomic-only approach. However, both are necessary and provide valuable information about the microbiomes.

3. Benefits of endophytic microbiota to the host plant

Once within the plant, endophytes might provide several benefits. We grouped them into three different kinds: direct growth promotion, protection against pathogens, and protection against abiotic stress (**Figure 1**).

Direct promotion occurs when endophytes stimulate shoot and/or root growth by increasing the availability of limiting nutrients or producing compounds that directly stimulate growth. On the other hand, indirect promotion occurs when the endophytes can protect the plant against diseases, pests, or environmental stress, indirectly improving the host performance [86]. The molecular mechanisms and pathways are not exclusive for each direct or indirect growth promotion effect. A single endophytic bacterial strain may have more than one of these plant-growth-promoting traits (e.g. [37, 41, 48, 49, 55] in **Table 1**, and [56, 57, 63, 65, 66, 68] in **Table 2**).

3.1 Increase of nutrient availability

The main mineral nutrients required for plant growth are nitrogen, phosphorus, and iron. There are numerous plant-growth-promoting microorganisms able to increase their availability, and some mechanisms have been determined.

3.1.1 Biological nitrogen fixation (BNF)

Nitrogen is crucial for plant growth and health. Approximately 30–50% of the N in crop fields results from BNF by soil microorganisms. The ability to fix atmospheric nitrogen (N_2) is present in various bacterial species that are either free-living or endophytically associated with plant roots. BNF is the most and long-term studied

plant-growth-promoting effect of soil microorganisms in legumes [87, 88]. Other plant growth promoter bacteria genera, different from rhizobia, are also able to enhance the acquisition of N by legumes. Anzuay et al. [89] and Taurian et al. [90] observed that endophytic bacteria belonging to *Serratia, Acinetobacter, Bacillus*, and *Enterococcus* enhanced peanut (*Arachis hypogaea*) N content. Dey et al. [91] reported that the increase in the number of nodules in plants inoculated with growth promoter bacteria could be attributed to the enhancement of root growth and root length. This enhancement provides more sites for nodulation by rhizobial strains in the soil. Furthermore, since BFN is a highly demanding ATP process, phosphorus is a critical nutrient for legumes.

3.1.2 Phosphate solubilization and mineralization

Even in phosphorus-rich soils (such as phosphate-fertilized soils), most of this element is in insoluble forms, and only a small proportion (~0.1%) is available to plants [92]. The solubilization of phosphates in the rhizosphere is one of the most common modes of action of growth promoter microbes that enhance nutrient availability to plants [93]. Phosphate-mineralizing and phosphate-solubilizing bacteria (PMB/PSB) secrete phosphatases and organic acids to convert insoluble phosphates (organic and inorganic) into soluble monobasic and dibasic ions [93]. Among legume endophytes, there are several phosphate-solubilizing bacteria able to promote plant growth, and some studies demonstrated that plant growth promotion was directly correlated with the increase of P in the plant tissues [89]. Soybean and peanut endophytes solubilize mineral phosphate [90]. In addition, several studies described endophytic bacteria with phosphate-solubilizing/-mineralizing ability that increase legume growth [89, 90, 94, 95]. The inoculation of pea with phosphate-solubilizing *Pseudomonas* spp. isolated from this legume, enhanced the plant biomass [96]. *Pantoea* spp. isolated from root nodules of peanut showed a strong phosphate-solubilizing activity [97]. However, the inoculation of phosphate-solubilizing bacteria isolated from peanuts did not promote growth when they were inoculated in the rapeseed culture [98]. These results point to a specific plant-bacteria interaction that directly affects the ability to promote growth or the efficiency of the mechanism.

The main phosphate-solubilizing mechanism in Gram-negative bacteria involves the bacterial PQQ cofactor, described as essential in P nutrition and plant growth. Mutation in the *pqqH* gene from *Pseudomonas fluorescens* caused the loss of the phosphate-solubilizing phenotype and plant growth promotion ability on tomato plants [99]. In legumes, Ahmed and Shahab [100] observed that a non-producing-PQQ bacteria (which lost the phosphate solubilization ability) showed a decrease in the growth promotion of bean plants. On the contrary, Ludueña et al. [101] determined that in the non-producing PQQ strain *Serratia* sp. promoted the growth of peanut at a similar level to the wild type, indicating that PQQ is not essential for growth promotion.

3.1.3 Iron uptake

Iron is essential for all living organisms, and its bioavailability in the soil is limited. Siderophores are small molecular compounds, secreted by microbes, which chelate iron in the soil and generate soluble complexes that can be absorbed by plants [97]. Microbial siderophores' secretion directly stimulates plant growth by increasing the availability of iron in the soil surrounding the roots [102]. Plants lacking soil bacteria suffered from iron deficiency [103]. Therefore, this mechanism helps plants to thrive in low-iron soils. The inoculation of black mung bean (*Vigna radiata*) with the siderophore-producing endophyte, *Pseudomonas* sp. GRP3,

reduced iron deficiency and chlorotic symptoms and increased the content of chlorophyll a and b [104]. Furthermore, since diazotrophic organisms require Fe^{+2} and Mo^{+2} factors for the functioning and synthesis of nitrogenase, iron solubilization by microbes also improved nitrogen fixation in legumes [105]. Native peanut isolates produce siderophores together with other plant-growth-promoting traits, increasing peanut growth and performance [106].

3.2 Phytostimulators

Endophytic bacteria directly promote plant growth by the production of phytohormones, such as auxin or cytokinin, or by lowering the plant ethylene (ET) levels. By these mechanisms, bacterial endophytes can also accelerate seedling emergence and promote plant establishment under adverse conditions.

3.2.1 Phytohormone-like molecule production

The production of phytohormones-like compounds is considered an important trait of endophytes that positively affects the growth and development of many plants including legumes [8, 10, 107]. Thus, changes in plant growth frequently reflect alterations in phytohormone levels induced by endophytes [3]. But, even when production of these compounds by growth promoter microbes has been demonstrated, that effect cannot be unequivocally attributed to them.

The five main phytohormones produced by bacteria are auxins, cytokinin, gibberellins, ET, and abscisic acid (ABA). It has been postulated that genes encoding biosynthesis of the auxins, cytokinin, and gibberellins are often present in the metagenome of plant endophytic bacterial communities [108]; however, it has not been yet explored in legumes using any omics approach (ET and ABA are discussed in Section 3.4.3).

Among these growth regulators, auxins are the most studied. These compounds affect plant growth by inducing cell enlargement and division, root development, apical dominance, increase growth rate, photo- and geo-tropism [109]. The production of auxin-like compounds increases seed production and germination along with increased shoot growth and tillering. Within these compounds, indole-acetic acid (IAA) is the most frequent and indeed most studied phytohormone in growth promoter bacteria. IAA produced by endophytic bacteria is one of the most relevant and studied effector molecules in growth promotion, pathogen defense, and plant-microbe interactions [104]. For instance, rhizobia from soybean, pea, and faba bean nodules not only fix nitrogen and produce siderophores, but also auxins (see Refs. [54, 110] in **Tables 1** and **2**, and [61]). IAA can be synthesized directly by plant-associated microbes, and ~ 80% of the rhizosphere bacteria may produce IAA [69, 111]. For instance, it could be produced by *Alcaligenes, Azospirillum, Pseudomonas, Pantoea, Rhizobium*, and *Enterobacter* in the presence of L-tryptophan as a precursor, although there are other pathways and a variety of auxins, such as indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPA), or tryptophol (TOL), which are also produced by growth promoter bacteria [112].

Cytokinins are another group of growth-stimulating phytohormones that are responsible for cell division, plant senescence, seed germination, flower and fruit development, and apical dormancy [113, 114]. Although cytokinins are produced by several growth promoter microbes, few studies have demonstrated their beneficial effects.

Gibberellins are involved in many developmental processes in plants, such as flowering regulation, seed germination, stem and leaf elongation [114], but also the promotion of nodule organogenesis and the negative regulation of the rhizobial infection and root system development [115].

Several bacteria produce and regulate the production of more than one phytohormone, such as the rhizobacteria *Bacillus aryabhattai*, which produces ABA, IAA, cytokinin, and gibberellic acids *in vitro* and promotes soybean growth [116]. Thus, inoculation with endophytic bacteria may benefit legumes via the production or suppression of some phytohormones.

3.2.2 Volatile compounds and other phytostimulators

Some growth promoters' bacteria can regulate plant growth by releasing volatile compounds [86]. For instance, *B. subtilis, Bacillus amyloliquefaciens*, and *E. cloacae* promote plant growth in legumes by releasing volatiles, such as 2,3-butanediol and acetoin [117, 118], while the mutants of *B. amyloliquefaciens* IN937a and *B. subtilis* GB03, blocked in their biosynthesis, did not promote Arabidopsis growth [118]. Studies on growth promotion by *Chryseobacterium* rhizoplane in mung bean indicate that 2,3-butanediol is the molecule causing growth stimulation [119]. Growth promotion mechanisms of volatiles in plants were reviewed by Sharifi and Ryu [120].

Other nonvolatile molecules such as bacterial cell components or secreted compounds have been proposed to be plant growth stimulators. The endophyte *Serratia proteamaculans* was able to promote soybean growth by the production of a lipo-chitooligosaccharide [121]. And the PQQ peptide, previously mentioned to be associated with P solubilization, has also shown growth promotion [99], antifungal activity, and the ability to induce systemic resistance [86]. The role of PQQ in plant-microbe interaction has been reviewed by Carreño-Lopez et al. [122].

Lastly, endophytes can generate allelopathic effects inhibiting the growth of neighboring plants or protecting the host plant from allelopathic effects from adjacent plants [123]. For example, endophytic bacteria of red clover seem to be responsible for the negative allelopathic effects observed over maize, reducing seedling emergence and height [124]. Additionally, some weeds have negative allelopathic effects on legumes, mediated by their endophytic bacteria, which inhibit nodulation [125].

Overall, there is a body of evidence that suggests that enhancing or regulating phytohormone or other phytostimulators via endophytic microorganisms is a viable strategy to increased crop production in agriculture [108], and because of these attributes, endophytes have gained ground in the area of agricultural sustainability.

3.3 Protection against pathogens

Among the major factors restraining agriculture are crop diseases and pests, while one important driver of plant health is the structure and dynamics of the plant-associated microbial communities [126]. In recent years, a deeper understanding of the endophytic microbiome and its potential has been achieved to become a fundamental tool in phytosanitary management and reduce the damage of plant diseases.

Endophytes can decrease the harmful effects of pathogens by different mechanisms, including direct and indirect mechanisms [104]. Direct inhibition of pathogens is mainly mediated by the synthesis of inhibitory allelochemicals such as antibiotics, hydrogen cyanide, iron-chelating siderophores [127], secretion of lytic enzymes, or quorum quenching (QQ) by degrading pathogens autoinducer signals [128]. Indirect biocontrol mainly includes the induction of the plant systemic resistance that inhibits the proliferation of a broad spectrum of phytopathogens [129].

3.3.1 Antibiosis

Most endophytes have been reported to produce secondary metabolites, and some of them exhibit antibacterial and antifungal properties, which help to inhibit

the growth of phytopathogenic microorganisms [44]. Many metabolites with antimicrobial properties synthesized by endophytes have been described so far, such as flavonoids, peptides, quinones, alkaloids, phenols, steroids, terpenoids, and polyketides. Antimicrobial properties of bacterial metabolites were recently reviewed [130]. Hansen et al. [131] studied the microbiome of alfalfa (*Medicago sativa*) nodules and identified two families of molecules produced by *Brevibacillus brevis in planta*, such as antibacterial thyrozidines, and a new set of gramicidin-like molecules, britacidins. They conclude that, in addition to nitrogen fixation, it is likely that legume root nodules are also a source of active antimicrobial production.

3.3.2 Lipopeptides

Lipopeptides are low-molecular-weight cyclic peptides attached to a hydrophobic fatty acid. These molecules are classified into three families: surfactin, iturin, and fengycin. Iturins and fengycins show strong antifungal activities while surfactins exhibit strong antibacterial activity. Antimicrobial lipopeptides can form toroidal-like pores on cell membranes leading to membrane permeation and/or disintegration and protect plants directly suppressing the growth of pathogens or inducing systemic resistance [132]. Recently, 263 different lipopeptides were synthesized by 11 microbial genera, with *Bacillus* being the most abundant [133].

The common bean root microbiome was used to search potential biocontrol agents of *Fusarium* sp., *Macrophomina* sp., and *Alternaria* sp. fungi, causal agents of root rot disease [65]. Biocontrol assays conducted under controlled conditions demonstrated that *B. amyloliquefaciens, B. halotolerans, Bacillus velezensis, Agrobacterium fabrum,* and *Pseudomonas lini* displayed the highest protective effect, and lipopeptide biosynthetic genes encoding surfactin, iturin, bacillomycin, and fengycin were present. These bacteria can produce at least one or more lipopeptides that may be involved in biocontrol activity.

3.3.3 Lytic enzymes

During plant colonization, endophytes produce numerous enzymes, which successively aid the hydrolysis of the plant cell wall. There are numerous types of enzymes such as chitinases, cellulases, hemicellulases, and 1,3-glucanases [70, 134]. These enzymes are also capable of degrading fungal (and oomycetal) cell walls hyphae, spores, and sporangia, thus contributing to the protection of the plant. The isolate *Pseudomonas* spp. EGN 1 was the most promising bioagent for the management of the stem rot (*Sclerotium rolfsii*) in groundnut, mediated by an important protease and cellulase production [57]. While, Brigido et al. [135] evaluated the diversity and functionality of the endophytic bacterial strains in the roots of native legumes from two different sites in Portugal, finding 15 isolates with a high cellulase production.

3.3.4 Hydrogen cyanide

A few bacterial species are known to produce and excrete hydrogen cyanide, a potent inhibitor of cytochrome c oxidase and several other metalloenzymes [136]. The host plant is unaffected by the bacteria or the hydrogen cyanide produced by it. For this reason, hydrogen-cyanide-producing bacteria have an application as biological control agent. Zaghloul et al. [137] isolated a total of 167 endophytic bacterial from roots, nodules, leaves, and stems of faba bean (*Vicia faba*), pea, fenugreek (*Trigonella foenumgracum*), lupine (*Lupinus* spp.), common bean (*Phaseolus vulgaris*), and rice (*Oryza sativa*) at flowering stage. About 82% of the

isolates showed positive results of hydrogen cyanide production. In another recent investigation, ~20 endophytic bacteria isolated from roots and nodules of chickpea (*Cicer arietinum*) and pea showed HCN production [66].

3.3.5 Siderophores

As previously mentioned, siderophores chelate iron in the soil making it more available for plants. Furthermore, by tightly binding the iron, siderophores reduce its bioavailability for plant pathogens and facilitate the death of the phytopathogens [138]. Some of the siderophores are known to be produced by endophytes, such as hydroxymate, phenolate, and/or catecholate types, confer biocontrol activities [139]. Also, the role of siderophores as part of the protective effect of the induced systemic resistance has been described in many studies. The production of siderophores is very common among *Pseudomonas, Frankia, Streptomyces* sp. Several researchers described endophytic bacteria producing siderophores isolated from different legumes as peanut, faba bean, soybeans, chickpea, pea, and bean [65, 66]. Bahroun et al., [140] demonstrate that *Rahnella aquatilis* B16C, *Pseudomonas yamanorum* B12, and *P. fluorescens* B8P isolated from faba bean nodules suppressed *Fusarium solani* root rot in three faba bean cultivars in greenhouse. The three strains were able to produce siderophores and significantly reduced the disease severity. Zhao et al. [54] obtained 276 isolates from root nodules of soybean, six of which showed antagonistic to the pathogenic fungus *Phytophthora sojae* 01. The isolates were identified as *Enterobacter, Acinetobacter, Pseudomonas*, *Ochrobactrum*, and *Bacillus* genera. The high correlation of siderophores production and the fungal inhibition of nodule endophytic bacteria in that study supported the idea that the ferrous absorption by endophytic bacteria may be a viable inhibitory mechanism.

3.3.6 Quorum quenching

The regulation of gene expression in response to fluctuations in cell-population density is known as "quorum sensing." Many important bacterial processes are regulated by it. Quorum sensing regulates gene expression depending on the accumulation of a signal molecule in the environment. The signal, called autoinducer, allows the bacteria to perceive the existing population density and jointly executed responses. Gram-negative bacteria use acyl-homoserine lactone (AHL) as an autoinducer, whereas Gram-positive bacteria utilize modified peptides [141]. The bacterial quorum sensing controls a wide variety of physiological processes such as virulence, extracellular polymeric substances (EPS) production, mobility, and biofilm formation among others, which are essential for the establishment of a pathogen in the host plant [142].

Often endophytic bacteria can disrupt quorum sensing. This ability to interfere with bacterial cell-to-cell communication was collectively called "quorum quenching" and can be crucial to prevent the plant colonization by pathogenic bacteria that use quorum sensing to coordinate virulence [143]. Several chemicals and enzymes have been identified that target the key components of bacterial quorum-sensing systems in the recent years (such as [33]). The mechanisms of quorum quenching may be the inhibition of the signal synthesis or detection, signal enzymatic degradation (by enzymes such as AHL acylase, AHL lactonase, and oxidoreductases), or synthesis of structural analogs of the signal [144]. Lopes et al. [145] reported antimicrobial activity against *Pseudomonas syringae* pv. tabaci or *Hafnia alvei* 071 in endophytic bacteria isolated from common bean. The isolates *Microbacterium testaceum* BAC1065, BAC1100, and BAC2153, *Bacillus thuringiensis* BAC3151, and *Rhodococcus erythropolis* BAC2162 exhibited a greater ability to inhibit the response of AHL reporter.

3.3.7 Insecticides

Some metabolites with insecticidal action have been described. The famous *B. thuringiensis* produces crystalline inclusion bodies consisting of delta-endotoxins (also referred to as Cry proteins) during sporulation. These proteins, which are formed by variable-molecular-weight polypeptides (27–140 kDa), are highly toxic for a broad range of pest insects [146]. *P. fluorescens* strains exhibited a protective effect against aphids and some herbivorous beetles and termites [147]. The bacterium *Lysinibacillus sphaericus* (former *Bacillus sphaericus*) produces sphaericolysin, which is toxic for *Spodoptera litura* [148].

3.3.8 Induction of systemic response

Induced systemic resistance (ISR) is a term used for the resistance stimulated by chemicals agents or signals (elicitors) produced by beneficial microorganisms [149], whereby the plant's innate defenses are potentiated against subsequent biotic challenges. In this way, the endophytes enhance the plant defenses against many pathogens [129]. The plant hormones jasmonic acid (JA) and ET are responsible for the regulation of the group of interrelated signaling pathways required to activate ISR. The main routes by which microbes regulate ISR in plants include: (i) phytohormones, (ii) pathogen-associated molecular patterns (PAMPs)/microbe-associated molecular patterns (MAMPs), and (iii) several elicitors (volatile organic compounds, siderophores, phytases, miRNAs, among others) [150]. Bacterial endophyte-mediated ISR has a broad spectrum of effectiveness. It was demonstrated that *Acinetobacter*, *Azospirillum*, *Rhizobium*, *Pseudomonas*, and *Bacillus* are beneficial inducers of systemic resistance in both leguminous and nonleguminous plants [151]. Dey et al. [91] described an endophytic isolate *Klebsiella pneumoniae* HR1 from the root nodules of black mung bean (*Vigna mungo*) capable of reducing the occurrence of *Macrophomina phaseolina*, which is the causal agent of the root rot disease in *Vigna*. The lowest percentage of disease incidence (18.2%) was observed when *K. pneumoniae* was applied in dual mode (seed bacterization + soil drench application). The increased activities of peroxidase (PR9), chitinase (PR3), and β-1,3-glucanase (PR2) in leaves indicated that *K. pneumoniae* HR1 induces a systemic response.

Endophytic bacteria have diverse mechanisms that could contribute, even simultaneously, to protect the plant against the attack of different pathogens, having the potential to produce a more efficient pathogen control on the fields.

3.4 Abiotic stress tolerance

Under abiotic stress conditions (such as drought, salinity, flooding, heat, chilling, or heavy metals), several metabolic responses are shared among plant species. Most of the stresses cause photosynthesis inhibition, oxidative stress, and hormone imbalances ending in reductions of shoot growth and yield impairments [10, 97, 152–154]. In addition, some of the responses are interconnected, for instance, reactive oxygen species and hormones mutually affect each other at early and late phases of abiotic stress (reviewed by [155]).

Endophytic bacteria can protect the host plant against some of those deleterious effects, by at least two different ways (alone or combined): (i) activation of host stress response systems soon after exposure to stress (named induced systemic tolerance), and (ii) biosynthesis of chemicals, which will contribute to the stress tolerance in the host [9]. Here we focus on three mechanisms by which the bacteria can protect the plant host against abiotic stress: redox status, water balance, and hormone regulation.

3.4.1 Redox status regulation

Oxidative damage (caused by reactive oxygen and nitrogen species) is a common consequence of environmental stress, which may cause damage to lipids, proteins, and overall to any subcellular component [156]. Then, the activation of the enzymatic and nonenzymatic antioxidant system is critical to tolerate adverse conditions. Several endophytic bacteria mediate a higher induction of the antioxidant system under stress. For instance, under salinity, the inoculation of peanut with the halotolerant bacteria *Brachybacterium saurashtrense* JG-06, *Brevibacterium casei* JG-08, or *Haererohalobacter* JG-11 showed lower oxidative damage, ion leakage, and K/Na ratio and higher growth, IAA, and Ca [157], while the inoculation of *B. subtilis* (alone or combined with *Mesorhizobium ciceri*) of chickpea reduced hydrogen peroxide accumulation and improved plant growth [10]. Soybean plant inoculated with *Curtobacterium* sp. SAK1 induced polyphenol oxidase activity, associated with growth protection and hormonal changes [158], while inoculated with *Pseudomonas simiae* increased catalase and peroxidase, but not polyphenol oxidase gene expression under salinity [159]. Also, soybean inoculated with *B. cereus, Pseudomonas otitidis*, and *Pseudomonas* sp. showed a reduction of hydrogen peroxide and membrane oxidative damage caused by PEG-induced drought [160]. However, if these responses are generated by the plant or bacterial enzymes remains unknown.

3.4.2 Water use efficiency regulation

Under stress, plant tissues usually modulate osmotic and water retention, by stomata activity and/or accumulation of osmotically active compounds. The latter compounds, also known as compatible solutes, include sugars (e.g., sucrose, trehalose, etc.), organic acids (e.g., malate), inorganic ions (e.g., calcium), amino acids (e.g., glycine betaine, proline) [161]. An increase in drought tolerance was detected after the inoculation of *Sphingomonas* sp. LK11 (isolated from *Tephrosia apollinea*) in soybean, by the accumulation of sugars and amino acids (glycine, glutamate, and proline) [162], and after the inoculation with *Rhizobium etli* in common bean, by the overexpression of trehalose-6-phosphate synthase [163]. Trehalose is an osmotically active compound that accumulates both in plants and microbes under stress. In particular, the role of trehalose in the tripartite symbiosis between plants, rhizobia, and arbuscular mycorrhiza under abiotic stress has been recently reviewed [164].

The optimal regulation of water use efficiency is critical to improved crop production. On one side is essential to survive dehydration stress (such as drought, salinity, heat, and chilling), but a constitutively highly efficient water use may reduce yields, by reducing CO_2 assimilation. The use of bacteria that contribute to transiently intensify stress-tolerance responses can help to improve productivity in marginal environments. In addition, if the endophytic bacteria enhance the osmocompatible compounds in response to the stress, it is possible to increase not only the tolerance to drought, but also the tolerance to chilling, heat, and salinity stress, which share a "dehydration" component. In the latter case, we expect a partial tolerance due to the ion toxicity, not related to the reduction in water potential.

3.4.3 Hormone regulation

As it was mentioned before, endophytic bacteria can regulate hormone synthesis and degradation and synthesize some of the plant hormone-like compounds by themselves. In addition, specific hormone regulation could also protect against abiotic stress increasing growth, yield, and survival.

Abscisic acid (ABA) is the main plant hormone related to water stress. It stimulates root growth and optimizes water uptake and nutrient acquisition, regulates shoot and root hydraulic conductivity, and upregulates the antioxidant system and compatible osmolytes synthesis [161]. The inoculation of *Sphingomonas* in soybean leaves induced ABA accumulation and reduced chlorophyll degradation and growth inhibition. However, under drought, ABA levels were lower in inoculated plants. So, in this case, the initial increase of ABA might have a role in acclimation to the stress induced by the bacteria inoculation [162]. In addition, ABA may interfere with SA-, JA-, and ET-mediated plant defenses [165], which may have undesired consequences under biotic stress.

Ethylene (ET) is usually considered a plant growth inhibitor, but at low levels, it can promote growth in several plant species. At moderate levels, ET inhibits both root and shoots elongation, while at high levels, enhances senescence and organ abscission [166]. The direct precursor of ET in the plant biosynthetic pathway, 1-aminocyclopropane-1-carboxylate (ACC), is exuded from plant roots together with other amino acids. The enzyme ACC deaminase cleaves ACC into ammonia and alfa-ketobutyrate. Plant growth promoter bacteria that express the enzyme ACC deaminase utilize their products (ammonia and ketobutyrate) as nitrogen and carbon sources, respectively. Bacterial ACC deaminase is not excreted from the bacterial cytoplasm [167]; hence, the decrease of plant ET levels relies on the ability of ACC deaminase expressing bacteria to take up ACC before it is oxidized by the plant's ACC oxidase [167]. When those bacteria are present, ET production could be lowered, relieving stress-induced growth inhibition [168]. For instance, the inoculation of pea (*P. vulgaris*) plants with *Aneurinibacillus aneurinilyticus* and *Paenibacillus* sp., two strains with high ACC activity *in vitro*, increased salt and drought tolerance. The combined inoculation reduced plant ET content and increased root and shoot length and biomass, as well as chlorophyll content [169]. The inoculation of alfalfa plants with *Bacillus megaterium* NMp082, which can produce ACC deaminase activity and IAA *in vitro*, also enhanced their salt tolerance [170]. Lastly, a novel mechanism was proposed in which salt tolerance is mediated by the activation of ET signaling. The inoculation of alfalfa with the bacteria *Enterobacter* sp. SA187 (isolated from a desert plant) increases salt tolerance, and studies in Arabidopsis indicate that the bacteria activate the ET signaling pathway [171]. The different mechanisms by which microorganisms can interfere with ET signaling were reviewed by Ravanbakhsh et al. [167].

Auxins regulate many important physiological processes related to growth and development affecting photosynthesis and responses to stress [161]. Under stress, auxins stimulate root elongation and density, increasing the water and nutrient availability, although they may interfere with SA-dependent plant defenses.

The inoculation of chickpea with *Serratia* sp. in nutrient-deficient soil induced more IAA and higher yields [172], while the same plant inoculated with IAA-producing *B. subtilis* NUU4 in combination with *M. ciceri* IC53 stimulated root and shoot biomass and improved nodule formation under salt stress [173]. Soybean plants inoculated with *B. aryabhattai* strain SRB02, which produces IAA, GA, and ABA, showed higher drought tolerance through stomatal closure, and higher root and shoot rates under high temperatures [116], and the same host treated with *Sphingomonas* sp. LK11 and *Serratia marcescens* TP1 (which produced IAA *in vitro*) stimulated root and shoot growth with increased ABA and GA and reduction of JA [162]. Overall, abiotic stress protection mediated by plant hormones and crop salinity protection mediated by beneficial bacteria have been reviewed [10, 174, 175].

Some primary stresses share the responses among them, such as those that generate dehydration (water or temperature deficit) or oxidative stress (dehydration, hypoxia, ions). For example, the double inoculation of chickpea with *M. ciceri* IC53 and *B. subtilis* NUU4 reduced the infection rate of root rot caused by *Fusarium solani*

in salty soils [173], although the mechanism was not determined. Then, a bacteria strain, inducing a protective mechanism against oxidative stress, can protect the crop against a diversity of stress, which generates redox imbalances. Consequently, knowing the responses that each stress triggers in the plant may allow us to predict which bacteria or group of them could protect the plant against a combination of stresses.

4. Synthetic communities of plant-associated bacteria to a more sustainable agriculture

Natural microbial communities within the plants are complex systems, with unknown functions and interrelationships among the microbial species and with the host plant. Small consortia of bacteria, with a "designed" composition, called "synthetic communities," reduce the complexity of those systems to be studied and used. The goal is to simplify the network while preserving the interactions and most of the functions, which may be lost in single plant-microbe interactions [175]. The use of synthetic communities allow us to ask questions about the performance and stability of the microbial community as well as to study conditions necessary to generate interaction patterns required to provide specific benefits. They are not only valuable as models but also as assays for biotechnological approaches [176].

4.1 How to study synthetic communities?

Manipulative experiments with synthetic bacterial communities can validate the predicted keystone species and, in general, help to find out specific effects of the resulting community under some pathogen infection or environmental condition. Those studies required *in vitro* experiments in gnotobiotic (germ-free) systems [11], where the plant is inoculated with a few or several microbial species, and the diversity is monitored across time. For instance, a gnotobiotic system was used to study the bacteria-colonizing alfalfa nodules [131]. The authors inoculate alfalfa with the four accessory bacterial members *B. brevis* Ag35, *Paenibacillus* sp. Ag47, *Pseudomonas* sp. Ag54, and *Pantoea agglomerans* Ag15, plus the nodulating strain *Sinorhizobium meliloti* RM1021. They observed that the addition of *B. brevis* neutralized the cooperation between *Pseudomonas* sp. Ag54 and *Paenibacillus* sp. Ag47, shifting the community from cooperative to competitive.

Another alternative, it is to use synthetic communities in a non-germ-free environment (more accessible and simpler to set up) to evaluate the protective or antagonist effect of a small group of species under a particular condition. Overall, only a few studies of the kind have been carried out in legumes until now. For instance, Lu et al. [177] described the diversity of nonrhizobial bacteria (32 genera) in legume nodules inoculated with *Bradyrhizobium elkanii* H255, *Rhizobium multihospitium*–like HT221, or *Burkholderia pyrrocinia* with or without the addition of N fertilization. The study suggested a vital role of that group of bacteria in N fixation in legumes.

The synthetic communities are a way to understand how microbial communities are built in the plants but also the base to a more complex (and likely more effective) phytostimulation effects, biological control of diseases, and protection against abiotic stress.

4.2 Can we manipulate the plant microbiome to improve the fitness or yield of legumes?

There are a variety of strategies to manipulate the microbiome of a plant host and could be classified according to the direct target: (i) the microbiome itself,

(ii) the plant genome, or (iii) the holobiome (plant plus microbial community) (reviewed by [39, 178]).

The microbiome (i) can be modified by the exogenous inoculation of the microbe, increasing the abundance of a single strain or a few species together. The first case is the most traditionally used, and there are thousands of examples, such as the inoculation with rhizobia. In those cases, the single strain should be compatible with the host genotype and able to overcome the competence of the native microbiome and the environmental conditions. The second case is open to unexplored scenarios, such as an infinite possibility of a higher number of strains/species combinations. This strategy is just starting to be explored, such as with non-nodulating bacterial species present in the nodules (and sometimes in the rest of the plant) that promote nodulation. For instance, the inoculation of common bean (*P. vulgaris* L.) with *Paenibacillus polymyxa* and *B. megaterium* strains showed a synergistic effect with *Rhizobium* strains on the plant growth [179]. On the contrary, the inoculation of alfalfa with different strains of the mutualistic *P. fluorescens*, showed that the increase in the community richness led to a negative complementary effect causing the loss of the protective effect against pathogens [180]. These results highlight the importance to evaluate the effects of any agricultural treatment or management on the microbial community.

The inoculation with synthetic communities has the advantage (over the use of the native microbiome) to allow the design of a community, which includes distant species (which may provide complementary benefits), or similar species, which increase the efficiency of the community (by using a wider diversity of resources) [19]. However, with the number and diversity of species, it also increases the complexity to handle the system and to commercialize the inoculants.

The plant genome (ii) could be manipulated by traditional breeding, gene editing, or transgenesis, changing the ability of the host to interact with the microbes (such as changing the exudates or volatiles). Instead of only breeding for pathogen resistance or abiotic stress tolerance, this could be a complementary alternative to select crop legumes to be more responsive to the presence of beneficial microbes [181]. For instance, modern accessions of common bean showed a lower abundance of Bacteroidetes and higher of Actinobacteria and Proteobacteria than the wild accession [79], with a gain in the diversity of rhizospheric bacterial and a stronger effect of the bean genotype [182]. In addition, Mendes et al. [183] showed that common bean breeding for *Fusarium oxysporum* resistance altered the functionality of the rhizosphere, unintentionally increasing the host protection against other pathogens. We hypothesize that a similar effect is happening in the endosphere, although it has not been explored yet. Additionally, when using this approach, it is relevant to evaluate that host defenses against pathogens are still functional.

Lastly, the holobiome (iii) could be altered through specific agricultural practices such as crop rotation, mineral, and organic fertilization, tillage practices, etc., favoring a specific community composition or function. Several studies reported the effect of agricultural management on the rhizosphere of legumes and its effect on crop performance. A meta-study showed the effect of crop rotation, intercropping, or companion planting on the rhizospheric microbial richness and diversity [184]. Those agricultural practices did not always have positive effects in richness and diversity, and legume-cereal crop rotation (relevant to reduce N fertilization) showed inconsistent results on the microbiome. A recent study showed that pea-wheat rotations showed no effect in the diversity index, but they affected the specific co-occurrence networks for each crop [185] suggesting a more complex effect of crop rotation that needs to be further studied. Certain chickpea cultivars select a more beneficial microbiome for the subsequent wheat plants, and they were associated with the antagonist species *Penicillium canescens* [186]. Red clover and

potato crops in rotation shared 73% of the bacterial endophytes, and 21% of those species promoted plant growth and yield in potato bioassays [187], while 74% of the shared species showed some degree of *in vitro* antibiosis against *Rhizoctonia solani*, a pathogen of both crops. We hypothesize that changing the rhizosphere will affect the endosphere too, by changing the available microbial pool, but that effect has not been explored at legume endophytic microbiomes.

4.3 Are there collateral impacts of using synthetic communities in agriculture?

Lastly, it is important to consider alive microbes will be released to the environment and into products used or consumed by humans and animals, so the potential risks need to be considered and tested [188]. There is no internationally agreed protocol to be complimented, but recently, Vilchez et al. [189] have proposed an Environmental and Human Safety Index (EHSI) protocol to determine the safety of the bacterial strains. The protocol evaluates microbial and animal sensitivity/pathogenicity and ecotoxicity in different model organisms, and it has been validated for many well-known bacteria. In addition, on the agronomical level, little information is available on the nontarget effects on microbial communities and the resulting impact on the soil function [32].

5. Final remarks and future directions

Agricultural legume crops are usually treated with synthetic chemicals to increase growth, control diseases, and mitigate environmental stress, which has high economic, environmental, and health costs. However, there is a myriad of endophytic bacteria that colonize the plant at least in part of its life cycle that could replace or complement those chemicals with great benefits for the plants. In addition, the huge bacterial diversity could be combined to provide several benefits at the same time. For that purpose, the use of synthetic communities is critical to study how the microbial community evolves within the plant as much as their beneficial effects.

The use of synthetic bacterial communities to improve and make more sustainable legume production is still in early stages of development, but it is a promising field. Using synthetic communities has the theoretical advantage of combining strain benefits and contributing to the survival of the bacteria on the field and inside the plant while producing a package of benefits for the legume. Although it is expected to have more difficulties at the time of commercial production.

On the other hand, changes in the agricultural management with some specific purpose could be a more affordable strategy for most of the small-scale producers in low-income countries, which are the ones in more need of sustainable and accessible technologies. Additionally, the use of soil-native microorganisms could have the advantage to reduce possible adverse consequences on the environment and health.

For the moment, the knowledge about endophytic bacteria in legumes, the possibility to "design" synthetic communities for a specific goal, and to manipulate the holobiome by agricultural practices is still incipient. However, the potential benefits for current agriculture to improve yields and sustainability have a great unexplored potential in the endophytic bacterial microbiome of legume crops.

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Author contributions

LV and MM conceived and planned the overall idea of the review manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 21

Challenges, Progress and Prospects for Sustainable Management of Soilborne Diseases of Cowpea

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Abstract

Cowpea [*Vigna unguiculata* L. (Walp.)], is an important legume crop widely grown in the tropics. Biotic and abiotic stresses cause significant yield reduction in cowpea. In this chapter, we provide a synthesis of information on the damage/economic importance of soilborne diseases of cowpea and present options that can be used to manage these diseases. The aim is to demonstrate that a wide array of control options are available for potential use within an integrated disease management (IDM) framework. Reviewed literature indicated presence of several sources of resistance to *fusarium* wilt (FW) and charcoal rot but few sources for stem rots, collar rot and damping-off. Major resistant genes and quantitative trait loci (QTL) were identified for FW and charcoal rot and these may be exploited in marker assisted selection (MAS). Cultural practices such as crop rotation and compositing were found to be effective against soilborne diseases, however, there is lack of knowledge regarding their adoption. Similarly, several botanicals were found to be effective against several soilborne fungal diseases but these studies were limited to controlled environments necessitating the need for large scale field trials. Several effective microbial control agents (MBCAs) and fungicides exist and can be incorporated in IDM.

Keywords: cowpea, disease management, fungi, host resistance, soilborne

1. Introduction

Cowpea, *Vigna unguiculata* L. (Walp.) is a multipurpose legume providing food for humans and feed/fodder for livestock and also a key source of income for farmers and grain traders especially in the tropical environments [1]. Ecologically, cowpea improves the productivity and sustainability of farming systems especially through its ability to fix substantial amounts of nitrogen from the atmosphere [1, 2]. While the name cowpea is the most popular worldwide especially among the English-speaking regions, it is known by a wide range of names. For instance, in the Francophone countries, the name 'niébé' is often used. In the USA, cowpea is popularly referred to as 'blackeye beans', 'blackeye peas', and 'southern peas' while in India and Brazil, it is referred to as 'lobia' and 'caupi', respectively [1, 2]. Common local names include

'seub' and 'niao' in Senegal, 'wake' in Nigeria, and 'lubahilu' in the Sudan [1, 2]. The species *V. unguiculata* includes cultivated forms (*Vigna unguiculata* ssp. *Unguiculata* var. *unguiculata*), wild annual forms (ssp. *Unguiculata* var. *spontanea*) and wild perennial subspecies [3]. Cultivated cowpea, subspecies *Unguiculata* is divided into five cultivar-groups (Cv-gr.) based on pod and seed characteristics; Cv-gr. *Unguiculata*, Cv-gr. *Biflora*, Cv-gr. *Sesquipedalis*, Cv-gr. *Textilis* and Cv-gr. *Melanophthalmus* [4]. Cv-gr. *Unguiculata* is the largest and comprises of both medium and large seeded grain and forage cowpea types of African origin. Cv-gr. *Melanophthalmus* includes 'blackeye pea'-type cowpeas which is characterised by white flowers/white seeds and thin seed coats [4, 5]. Cv-gr. *Textilis* is a rare form of cowpea mainly grown in West Africa for fibre extracted from its long peduncles [5, 6]. Cv-gr. *Sesquipedalis* (yard long bean, long bean, asparagus bean and snake bean) is commonly grown in Asia for its long (40–100 cm) green, fleshy and wrinkled pods that are often used as 'snap beans' [4, 5]. Cv-gr. *Biflora* is characterised by thick seed testa and erect pods.

Cowpea is consumed in several forms; for instance, in south-eastern USA, Asia and Caribbean, fresh seeds and green pods are mostly consumed while in many parts of Africa and Asia, dry grains are mainly consumed in addition to fresh or dry leaves (as side dish or part of the stew), thus providing significant nutritional value [7–9]. Although leaves are consumed, cowpea is mainly grown for consumption of grains as they are rich in proteins, carbohydrates as well as minerals. The nutrient composition both in grain and leaves is highly variable depending on the environment and genotype under consideration. In an evaluation of 1541 cowpea accessions for grain nutrient composition by [10], protein content ranged from 17.5 to 32.5%, Fe content from 33.6 to 79.5 mg/kg, Zn ranged from 22.1 to 58.0 mg/kg, Ca from 310 to 1395 mg/kg, Mg from 1515 to 2500 mg/kg, K ranged from 11,400 to 18,450 mg/kg and P from 3450 to 6750 mg/kg. Weng et al. also reported a wide range (22.8–28.9%) of seed protein content among the 173 cowpea genotypes [11]. A similar study of 15 genotypes by [12] showed that moisture content ranged from 12.28 to 13.35%, total carbohydrates from 49.37 to 55.74%, crude ash from 2.99 to 3.34%, crude lipids from 0.13 to 0.81%, crude protein from 23.37 to 29.70% and crude fibers from 1.40 to 4.34%. Cowpea samples recorded highest percentage of essential amino acids (60.71%) and non-essential amino acids (39.29%). The mineral content ranged from 1.97 to 2.69 mg/100 g for calcium, 3.23 to 3.90 mg/100 g for magnesium, 205.53 to 223.30 mg/100 g for sodium, 0.80 to 1.23 mg/100 g for zinc, 1071.15 to 1152.62 mg/100 g for potassium and 0.62 to 1.06 mg/100 g for phosphorus. Cowpea has shown great potential for production of fermented yoghurt-like food products with improved bioavailability of nutrients [13, 14]. Cowpea is rich in phenolic acids such as benzoic and cinnamic acid derivatives that are associated with antioxidant properties [15]. In addition, cowpea has a high proportion of polyunsaturated fatty acids (40.1–78.3% of total fats) [16] and these are associated with several healthy benefits.

While cowpea is cultivated globally, most of the production occurs in the developing countries. Recent estimates show that West Africa accounts for over 80% of the total world production [17]. The leading cowpea producing countries in Africa include: Nigeria, Niger, Burkina Faso and Ethiopia with production of 3,576,361, 2,386,735, 652,454 and 374,332 tonnes, respectively. The estimated acreage, production and average yield of cowpea from the selected major producing countries of cowpea are presented in **Table 1**.

Despite the importance of cowpea, abiotic and biotic constraints are major yield limiting factors especially in the developing countries where most of the production takes place. Water availability is the most significant abiotic constraint for yield in cowpea despite the fact that the crop is inherently drought tolerant [9]. Cowpea diseases caused by various pathogens (fungi, bacteria, viruses, nematodes

Rank	Country	Acreage (Ha)	Quantity (t)	Yield (hg/Ha)
1	Nigeria	4,303,005	3,576,361	8311
2	Niger	5,725,433	2,386,735	4169
3	Burkina Faso	1,354,100	652,454	4818
4	Ethiopia	220,037	374,332	17,012
5	Kenya	298,120	246,870	8281
6	Mali	454,274	215,436	4742
7	Cameroon	244,058	215,016	8810
8	Ghana	149,102	202,735	13,597
9	Senegal	290,677	184,137	6335
10	Sudan	339,780	161,000	4738
11	Tanzania	112,657	127,884	11,352
12	Myanmar	122,637	108,021	8308
13	Mozambique	331,424	90,461	2729
14	DRC	175,418	76,292	4349
15	Yemen	26,062	66,190	25,397
16	Malawi	97,825	41,656	4258
17	Madagascar	34,122	31,069	9105
18	Haiti	42,145	30,741	7294
19	Peru	15,794	21,539	13,637
20	China	14,503	14,696	10,133
21	Uganda	33,350	12,697	3807
22	USA	5220	11,750	22,510

Source: FAOSTAT [17].

Table 1.
Top cowpea producing countries in the world.

and parasitic plants) constitute one of the important biotic constraints to cowpea production in all regions where the crop is cultivated [18]. These diseases can infect cowpea at different stages such as during emergence, vegetative and reproductive stages causing substantial plant damage hence leading to yield loss or complete production failure [19]. While there have been some extensive reviews on shoot and pod diseases of cowpea [20], as well as soilborne diseases [21], this manuscript provides an updated synthesis of the economic importance of major soilborne fungal diseases in the world and the available options for their sustainable management. This present review covers past efforts, achievements and gaps in the management of soilborne fungal diseases of cowpea. The management approaches focused on include: resistance breeding/host resistance or pre-breeding, cultural practices, fungicides, microbial biocontrol agents (MBCAs) and use of botanicals.

2. Damage caused by soilborne fungal diseases

Soilborne fungal diseases of cowpea are widespread globally and constitute a major constraint to production especially in the tropical and subtropic environments. Southern blight also referred to as basal stem disease or stem rot, damping-off, collar

rot or seedling blight, *Fusarium* wilt, and charcoal or dry root rot are the prevalent soilborne fungal diseases of cowpea. Notably, Southern blight or stem rot is caused by *Sclerotium rolfsii*, damping off is caused by *Pythium* sp., while collar rot or seedling blight is incited by *Rhizoctonia solani* [22–27]. Among these pathogens, *Sclerotium rolfsii* is identified as the main disease-causing pathogen while the others are referred to as minor pathogens [24–26]. Southern blight is characterised by initial stem decay of plants in the top 2 cm of the soil, general wilting and yellowing of plants followed by drying of foliage and plant death [28]. In advanced stages of infection, the stems exhibit tan to brown sclerotial bodies and white mycelial growth on the epidermis of the stem at the soil surface. Non-germinated diseased seeds have a brown blotchy colour or a soft rot and often disintegrate when touched. Germinated seedlings may fail to emerge above the soil line and are characterized by water-soaked lesions girdling the hypocotyl. Emerged seedlings have necrotic tap roots with few lateral roots while infected hypocotyls above the soil surface have light brown lesions [29]. While the disease is widely recognised as important, there are limited studies aimed at assessing its economic impact. Fery and Dukes reported yield losses of up to 53% in susceptible cultivars mainly due to reduction in the number of pods per plant [28]. Similarly, Thies et al. [30] reported significant seedling losses and reductions in seed weight/seed number as a result of *Rhophitulus solani* infection.

Charcoal rot or dry root rot caused by *Macrophomina phaseolina* [31] is another serious constraint to cowpea production especially in the drier savannas and Sahel [18]. Yield loss of up to 10% due to charcoal rot has been reported in the Sahelian zone of West Africa [32]. For instance, in Niger and Senegal alone, charcoal rot was estimated to cause yield loss of up to 30,000 tons of grain valued at USD146 million [32]. *Fusarium* wilt (FW) caused by *Fusarium oxysporum* f.sp. *tracheiphilum* (*Fot*) is associated with characteristic symptoms such as chlorosis, wilting and stunting at seedling or flowering stage or and/or early pod development resulting in plant mortality with significant yield losses [33–36]. Significant yield losses ranging from 35 to 65% or total loss due to *fusarium* wilt alone or in combination with nematode infestation were reported [33–36]. In Brazil, yield losses of 8.3–86.5% due to wilt were also reported [37].

3. Management approaches for soilborne diseases of cowpea

Effective management of soilborne fungal diseases requires use of a number of approaches which can be grouped into four categories: (1) host resistance or use of tolerant varieties, (2) adoption of best cropping practices, (3) seed treatments and (4) protection of seedlings [38]. However, none of these approaches is effective when used alone thus necessitating the need for their combination within the framework of integrated disease management (IDM) approach if sustainability is to be achieved.

3.1 Utilization of host resistance

Host resistance is the most effective, economical and environmentally friendly approach for managing soilborne fungal diseases of cowpea. This approach mainly involves deployment of resistant and/or tolerant plant varieties, which support lower pathogen populations or better tolerate injury; and the integration of such varieties with other approaches within the IDM framework. In this section we provide a synthesis of available information about genetic resources for resistance, genetics of resistance, identification of markers associated with disease resistance and their potential for use in breeding programs.

3.1.1 Genetic resources for resistance to soilborne diseases

Several screening studies have been conducted both under the field and greenhouse conditions to identify sources of resistance against major soilborne fungal diseases of cowpea. Majority of the studies have targeted resistance to *fusarium* wilt (FW) and charcoal rot while screening trials for southern blight, stem rots, collar rot and damping-off have been limited, hence more studies are needed on these aspects.

Oyekan reported resistance to FW in TVu109-2, 347, 984, 1000 and 1016-1 cowpea varieties under both field and greenhouse conditions [39]. Five cowpea cultivars with resistance to three FW races (1, 2 and 3) were identified in another study [40]. The cultivars were: Magnolia, Iron PI293520, Iron TVu 990, Iron TVu 1072 and Iron TVu 1611. Roberts et al. identified CB3, CB46, 7964 and 8517 as having resistance to FW [36]. Similarly, Hall et al. [2] reported varieties CB3 and 7977 as sources of resistance to FW. Moreover, CB 46 and CB 88 were reported to have resistance only against race 3 of FW while CB27 and CB50 gave resistance against both race 3 and race 4 of FW [41, 42]. Following screenhouse/greenhouse studies, four FW resistant cowpea genotypes namely: Asontem, Danila, IT89KD-88 and NE 70 were identified [43, 44]. Other genotypes that could be used as resistance donors for FW are: TVu 134, TVu 410, TVu 901-1 and MNCO1-649F-2-1 [45, 46]. Genotypes TVu 134, TVu 410 and TVu 901-1 share the same resistance gene [45, 46]. Wu et al. reported 10 highly resistant genotypes to FW. These were: Fei 8, CB46, IT93K_503_1, UCR5040, Zhijiang dwarf No. 1, Jiacaidou, Heiziyacao, Fan, Zhuyan long bean and Qiyezai [47] representing the Chinese asparagus bean, and the African cowpea.

For resistance to southern blight/basal stem disease, cowpea genotypes: CO-4, Brown Crowder, Carolina Cream, L-25, IT89-KD-374, IT86-D-715 and IT99K-1122 were identified [28, 48–50, 57]. According to Adandonon [24] Sèwé, Kpodji, Kumassi and Cameroon cowpea genotypes showed resistance to both stem rots and damping off under field conditions. The potential sources of resistance to charcoal rot include: IT04K-217-5, Komsare, Gaoua local-2, 58-57, Kaya local and SP369A profil-39B [51, 52]. Singh and Lodha found moderate resistance to charcoal rot in 26/4/1, V 16, K 39, 25/8/2 and CO3 genotypes [53]. In field experiments conducted over 3 years, IT98K-499-39, Suvita 2, IT93K-503-1 and Mouride were found to be highly resistant to charcoal rot [54]. Cowpea cultivar Caloona was reported to be resistant to *Phytopthora vignae*, the causal agent for Phytopthora root rot or foot rot [55]. Under field conditions, the genotype IT86D-326-2 was found to be moderately resistant to damping-off and stem rots caused by *S. rolfsii* [26].

3.1.2 Inheritance of resistance to soilborne diseases

Most studies on inheritance of resistance to soilborne fungal pathogens of cowpea have relied on Mendelian genetics. These studies have mainly focused on FW resistance with few studies on charcoal rot and southern blight. Inheritance studies focusing on other pathogens such as *Pythium* sp. and *Rhizoctonia solani* are largely missing in literature. Literature on genetic inheritance of resistance to FW suggests that it is controlled by a single dominant gene [46]. Resistance to race 1, 2 and 3 was reported to be controlled by a single dominant gene [45, 56]. Dominant monogenic inheritance makes it possible to effectively use backcrossing for transfer of resistance to susceptible backgrounds [46]. However, additive gene effects were also reported to control resistance [44]. For southern blight, resistance is conditioned by single dominant genes which are non-allelic in two resistant genotypes namely: Carolina Cream and Brown Crowder [57]. Inheritance to charcoal rot was found

to be controlled by additive gene action and thus quantitative in nature [54, 58]. Resistance to *P. vignae* (race 2) in cultivar Caloona is controlled by a single dominant gene [55, 59] and it is expressed throughout the life of the plant in all tissues [55].

3.1.3 Identification of resistant loci and markers for resistance to soilborne pathogens

Efforts to identify resistant loci and development or deployment of molecular markers in breeding for resistance to soil-borne fungal diseases in cowpea have been restricted mainly to FW and charcoal rot. Little or no progress has been made on markers used or developed for other pathogens. For instance, a single SSR marker (C13-16) that can discriminate between resistant and susceptible genotypes for FW resistance was identified [45]. This marker can easily be used in low resourced laboratories in several developing countries [45]. Two independent loci (QTLs), *Fot4-1* and *Fot4-2*, which confer resistance to FW race 4 were identified in three cowpea RIL populations derived from three crosses: IT93K-503-1 × CB46, CB27 × 24-125B-1 and CB27 × IT82E-18/Big Buff. Locus *Fot4-1* was located on linkage group 5 while *Fot4-2* was located on linkage group 3 [34]. *Fot4-1* was derived from an African breeding line, IT93K-503-1 and *Fot4-2* was derived from a US blackeye dry grain cultivar, CB27 [34]. While the locations of *Fot4-1* and *Fot4-2* were identified, generation of tightly linked markers is yet to be done. For resistance to FW race 3, Pottorff et al. [33] identified a single QTL (*Fot3-1*) from a RIL population derived from CB27 × 24-125B-1 cross. The *Fot3-1* locus is located on linkage group 1. Four SNP markers, 1_1107, 1_0860, 1_1484 and 1_0911 linked to *Fot3-1* locus were identified making transfer of FW resistance into susceptible cultivars through MAS more likely [33]. Using a genome wide association study, 17 SNPs associated with FW resistance were reported [47]. The 17 SNPs were: 1_0075, 1_1111, 1_1147, 1_0251, 1_0895, 1_0691, 1_0897, 1_0298, 1_0410, 1_0857, 1_0981, 1_1369, 1_0330, 1_1062, 1_0629, 1_0318 and 1_1504. SNP 1_0981 was used to design a PCR primer (1_0981CAPS-F: 5′-AAGTTGCAGAGCACCACAGA-3′ and 1_0981CAPS-R: 5′-TAAAAGGACCACTGCACACG-3′) to distinguish between resistant and susceptible lines due to its strong association with FW resistance [47]. This primer set can readily be used in marker assisted selection. QTL analysis of a RIL population derived from a cross between IT93K-503-1 and CB46 revealed nine QTLs: *Mac-1*, *Mac-2*, *Mac-3*, *Mac-4*, *Mac-5*, *Mac-6*, *Mac-7*, *Mac-8* and *Mac-9* against charcoal rot and these QTLs were associated with eight SNP markers: 1_0709, 1_0853, 1_0604, 1_0201, 1_0079, 1_0804, 1_0678 and 1_0030, respectively [54].

3.2 Adoption of good agronomic practices

Agronomic practices that can delay or discourage the survival and development of pathogens can play a role in the management of soilborne fungal diseases. This is because many of the pathogens are relatively weak requiring a favourable environment for infection to occur [38]. Several agronomic practices that modify the growing environment such as seedbed preparation, soil pH management, planting dates, seed rate, plant density, soil fertility and moisture management, cropping systems (crop sequence and intercropping, cover crops), and soil solarisation have been reported as efficient in the control of soilborne pathogens [38]. However, few studies have been carried out on management of cowpea soilborne fungal diseases.

For instance, rotation of cowpea with a gramineous/cereal crop such as fonio (*Digitaria exilis*) and millet (*Pennisetum glaucum*) leads to rapid reduction of microsclerotia of *Modiolula phaseolina* in soils [32, 60]. Fonio and millet planted continuously for 3 years significantly reduced microsclerotia densities in soils at a rate of

81% after the second year; 86% after the third year under fonio and 56 and 66% for the second and third year under millet, respectively [32, 60]. Composting heavily *M. phaseolina* infected cowpea residues raises temperature (52–60°C) leading to complete destruction of *M. phaseolina* microsclerotia [32, 61]. Addition of six tonnes of compost alone or supplemented with 50 kg NPK ha^{-1} gave 28–45% lower area under disease progress curves (AUDPC) with a 43–66% higher cowpea production. Furthermore, addition of compost combined with *C. rosea* in planting holes sharply reduced AUDPC (up to 4-fold) and increased the grain yield 2–5-fold [32, 61].

Combined use of solarization and organic soil amendments is highly effective in controlling soilborne fungal pathogens [32, 61, 62]. For instance, there was a 78 or 96% reduction in charcoal rot disease severity, when millet residues or paunch amendments were applied in combination with solarization, respectively. Soaking of seed in an antioxidant, spermine (SP) at 10 mg L^{-1} before planting followed by foliar application of potassium (K) as potassium chloride (KCl) at 2% and zinc (Zn) as zinc sulphate $ZnSO_4$ at 0.01% gave the highest germination percentage and lowest incidence of damping-off disease at 96.34 and 3.66%, respectively [63]. The same treatments (SP + K + Zn) also significantly reduced the incidence of charcoal rot by up to 83.30% [63].

3.3 Role of microbial biocontrol agents (MBCAs) against soilborne fungal diseases

The pathogens causing soil-borne diseases such as *R. solani*, *Pythium* spp., *Fusarium* spp., *S. rolfsii*, and *M. phaseolina* on cowpea either survive in soil or are introduced from seeds therefore both seed treatment and soil application of MBCAs or chemicals are recommended. In particular, management of soilborne pathogens of cowpea through MBCAs is more effective. Application of beneficial microbes for the control of plant diseases can be successfully used particular within the framework of an IDM system due to their manifold mode of actions (**Figure 1**). The use

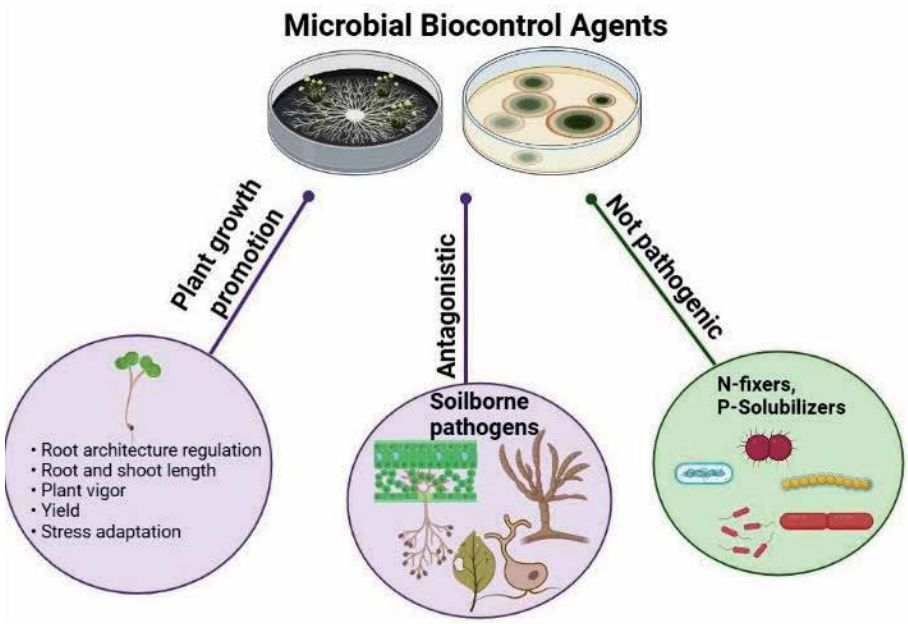

Figure 1.
Showing manifold performance of microbial biocontrol agents (MBCAs).

of MBCAs with other management practices such as cultural practices, cover crops and organic amendments is known to be less harmful than chemical fungicides in the management of soilborne diseases [64].

The beneficial microbes that have been frequently used for the control of soil-borne diseases of cowpea include: *Trichoderma* species, *Pseudomonas* species and *Bacillus* species [65, 66]. *Bacillus* species have been used against root rot and postharvest diseases [67, 68]. In a study by [69], *Bacillus firmus* coated cowpea seeds when sown in soil amended with radish compost had lower mortality at 3–4% induced by *Modiolula phaseolina* compared to non-amended soils (13.8–20.5%). Cowpea seeds treated using *Trichoderma* strain Kd 63, and soil sprinkle with *Trichoderma* IITA 508 (5 g/L, 10^9 colony forming units (CFU)/g) exhibited higher control of stem rot caused by *S. rolfsii* [70]. Besides, Adandonon et al. [70] found that seed treatment with *Moringa* followed by soil sprinkle application of *Trichoderma* resulted in 94 and 70% stem rot control under greenhouse and field conditions, respectively with significant increase in seed yield.

Application of *Trichoderma* species with organic amendments increased the population and efficacy of *Trichoderma* as well as increased defense response in host species and seed yield [71, 72]. In India, Singh et al. [73] used six organic substrates for multiplication and efficacy testing of *T. harzianum* against collar rot disease caused by *Rhophitulus solani*. They found that of the six substrates, *T. harzianum* multiplied in spent mushroom compost contained the highest population density (15×10^7 CFU/g) up to 240 DAI and exhibited potential efficacy against collar rot. The treated plants showed reduced seedling mortality, enhanced shoot and root length, number of leaves as well increased seed yield. Similar results were reported by El-Mohamedy et al. [74] in greenhouse experiments. They reported that soil amendment with *T. harzianum* multiplied on sugar cane bagasse (10% w/w) of soil reduced root rot incidences by 73.9, 73.9 and 78.6% caused by *R. solani*, *F. solani* and *M. phaseolina* at pre-emergence stage, respectively. The management of soil-borne pathogens through soil amended with organic materials including MBCAs may be attributed to: (i) increasing efficacy of native microbes resulting in suppression of pathogens through competition or specific inhibition, (ii) releasing degradation compounds viz., ammonia, carbon dioxides, saponins, nitrites or enzymes which are generally lethal to the pathogens, (iii) inducing defense mechanisms of hosts and (iv) glucanase and cellulose being prevalent in the soil at a high concentration as a result of cellulose and lignin biodegradation [75]. Besides, the efficiency of *Trichoderma* may be also due to the presence of several volatile and non-volatile antifungal metabolites, a combination of competition and mycoparasitism [75, 76]. Both *Trichoderma* species and bacterial agents produce many mycolytic enzymes, thus playing a key role in the degradation of cell wall of target pathogens [77].

In recent times, bio-priming as a seed treatment that integrates the biological aspects of disease management has been used as an alternative method for mitigating many seed and soil-borne pathogens, and it has emerged as another alternative to chemical fungicides. Also, seed coating with MBCAs is the most efficient treatment for mitigating root rot diseases as shown by many researchers [78, 79]. In this regard, bio-coated cowpea seeds with *Bacillus* species demonstrated a significant ($P < 0.05$) increase in shoot and root length, seed germination and leaf area with increased seed yield [80]. In addition, the bacterium was found as potential antagonists against *M. phaseolina*, *R. solani*, *F. oxysporum*, *F. solani* and *S. rolfsii*. It was also reported [81] that priming of seed with *T. harzianum* at a rate of 4 g/kg of seed along with the application of vermi-compost with 20% neem cake (w/w) mixed with antagonists significantly controlled root and collar rot resulting in increased yield of cowpea.

One of the requirements for execution of MBCAs are the development of suitable formulation and delivery systems [82]. Fabrication procedures for these agents are dependent on enough and efficient biomass formation, which must be carried out carefully in order to retain viability at the end of processing and deployment. Seed treatment with different formulations of *T. koningii* and *T. harzianum* containing 6.8×10^7, 2.0×10^{10} and 1.0×10^7 CFUs/ml significantly controlled dry root rot in cowpea as higher plant survival was reported in treatment plots compared to control plots [83]. In another trial conducted by [84], it was observed that some strains of *P. fluorescence*, *B. subtilis* and *Trichoderma* spp. were found to be potential antagonists in control of FW caused by *F. solani* in chickpea which evidenced that these MBCAs have cross bio-efficacy against the same pathogens of different hosts. Besides, during application of MBCAs, ventilation and drainage of the field should be maintained to avoid high relative humidity, which favours germination of pathogen spores [85].

More recently, biofilms based on MBCAs have been used for the control of many soilborne diseases. In particular, these biofilms are microbial communities adhering to the biotic and abiotic surface, and they are fixed in the organic matrix of biological origin that provides structure and stability to the microbial community. Due to multi-layers of microbial cells, these biofilms play a major role in plant-microbe interaction. For example, seed treatment with *T. harzianum* and *Bacillus* biofilm-based formulations have shown potential disease control caused by *R. solani* and *Pythium aphanidermatum* with only 0–14% disease incidence and increased yield 44–48 g/plant compared to controls [86]. Moreover, the rhizosphere soil of cowpea plants applied with biofilms formulations showed higher propagules of *T. harzianum*. These results are in agreement with earlier researchers who also reported an increase in population of beneficial microbes after application in soil [87–89].

In addition to *Trichoderma*, *Pseudomonas* and *Bacillus*, other MBCAs have also been reported as effective agents against soilborne diseases of cowpea. For example, Hamed et al. [90] reported that *T. asperellum*, *T. roseum* and *Chaetomium globosum* also possessed efficient antagonistic activity against FW and stem rot pathogens, but less than *Trichoderma* species. Some other MBCAs have been found effective against soilborne pathogens of other crops. For instance, the arbuscular mycorrhizal fungus (AMF), *Glomus clarum* has been found to be effective against *R. solani* by reducing the mortality in bean plants [91]. Soil drenched with AMF (*Glomus deserticola* and *Gigaspora gigantean*) before planting and inoculation of *M. phaseolina*, after 10 days of germination, the crop showed higher growth parameters. However, simultaneous treatments of *Gnypeta deserticola*, *G. gigantea* and *M. phaseolina* were the most effective for both growth parameters and reduction of charcoal rot disease severity [92]. Amendments such as soil application of biochar have been reported to improve soil carbon sequestration, soil fertility and plant growth, especially when combined with organic compounds such as compost. This in turn improved plant vigor and the ability of plants to resist pathogen attack [93]. For instance, soil amended with 15% compost was 71.4% effective in controlling damping-off while combination of 15% compost + *mycorrhizae* and 3% w/w biochar + *mycorrhizae* showed 61 and 73.3% efficacy against damping-off [93]. *In vitro* studies conducted also showed that PDA amended with 15% compost reduced *R. solani* mycelial growth by 54% while no mycelial growth occurred on PDA amended with 3% w/v biochar [93].

In addition, research has demonstrated that besides diseases control, MBCAs also increased nitrogen fixation ability. For instance, *B. subtilis* and *T. longibrachiatum* had no negative effects on the nitrogen fixing ability of *Bradyrhizobium* [94]. The application of antagonists in soil through seed treatment and soil application decreased sclerotia germination of *S. rolfsii* which resulted in decreased disease incidence and increased nitrogen fixation ability by *Bradyrhizobium*. Likewise, in

beans and soybean, *Bacillus-Rhizobium* inoculants have been used to control root rot caused by *F. solani* [95]. Therefore, more investigation is required to see the effect of *Bacillus-Rhizobium* combination on soilborne diseases of cowpea.

3.4 Role of botanicals against soilborne fungal diseases

The fungicidal properties of aromatic and medicinal plants have been recognized since prehistoric times. Worldwide, plant based natural chemicals and their application for plant protection is one of the focus areas of research. Earlier, plant extracts of many medicinal plants such as neem (*Azadirachta indica*) [96] and garlic (*Allium sativum*) [97] have been used for control of many soilborne fungi. A study by [70] reported that application of *Moringa* extract at a concentration of 15 kg leaves/10 L of water (w/v), exhibited the highest stem rot control in cowpea. In another study, application of *Acacia nilotica* and *Prosopis juliflora* extracts with compost reduced charcoal rot incidence in cowpea by exhibiting <5.8% disease incidence with 28.3% increase in seed yield [98]. Using *P. juliflora* also controlled root infecting fungi (*R. solani*, *Fusarium* spp. and *M. phaseolina*) of cowpea [99]. Through soil amendment method, leaves, stem and flower powder at the rate of 0.1, 1.0 and 5% w/w suppressed the disease incidence and enhanced growth parameters like weight, shoot and root length, leaf area and number of nodules per plant. Soil amended with *Aerva javanica* leaf powder at 1%w/w was effective against several root fungi; *Fusarium* spp., *R. solani* and *M. phaseolina* [100]. In another study by Dawar et al. [101], it was reported that leaves, stem, bark and fruit powder of *Eucalyptus* species have the potential to reduce the infection of root infecting fungi viz., *Fusarium* sp., *R. solani* and *M. phaseolina* in mung bean and chick pea. Therefore, the efficacy of *Eucalyptus* species needs to be tested against soilborne pathogens of cowpea. These results suggest that in resource-deficient farming systems, certain on-farm wastes can be effectively utilized for managing soilborne pathogens, as well as for enhancing crop productivity.

In another study by Dawar et al. [102], charcoal and root rot of cowpea was controlled by seed coating with *Paecilomyces variotii* followed by soil drenching with *Datura alba* Nees extract. Another species of *Datura*, that is, *D. fastulosa* was also reported to be effective against charcoal rot in a pot experiment [103]. The efficacy of *D. alba* reported in this study may be due to presence of some compounds such as 6*B*-tigloxytropane-a-ol, tigloidine (3*B*-tigloyloxytropane), tropine, hyoscyamine, apoatropine and scopolamine present in *Datura* species [104]. Besides, Zainab et al. [105] reported that seed powder of *Adenanthera pavonina*, *A. indica*, *Leucaena leucocephala* and *Eucalyptus* spp. controlled root rot diseases at 0.1 and 1% w/w concentration and extract of *Avicennia marina* (5% w/w) has been found to suppress the growth of charcoal rot fungus in beans [106]. Similar results were reported by [107] who controlled several root rot fungi through seed treatments with *Trichoderma* + leaf extract.

In addition to control of root rot diseases, plant extracts are reported to increase seed germination through decreasing disease incidences [108]. For example, soil application of 1–3% dry leaf biomass of *A. indica* with *T. harzianum* efficiently decreased (20–25%) disease incidence caused by *M phaseolina* in cowpea with improved plant growth attributes [109]. Although extracts of *A. indica* and *Garcinia cola* have shown 77 and 92% inhibition activity against damping-off pathogen, *P. aphanidermatum* [110], they have not been tested under field conditions. Therefore, further experiments are required to validate their efficacy under field conditions.

Besides plant extracts, essential oils extracted from higher plants has also been found effective against some soilborne pathogens. For example, essential oils from wild oregano and black cumin applied at the concentration of 0.16 µl/cm^3 of air

have been found effective against *M. phaseolina* and *S. sclerotiorum* under *in vitro* conditions. Similarly, Alice et al. [111] and Kazmi et al. [112] revealed that neem oil was effective against *M. phaseolina*, cinnamon bark and lemongrass essential oils were effective against *R. solani* at 5 mg/paper disc [113]. In addition to essential oils, their chemical constituents such as *trans*-cinnamaldehyde, neral, geranial, salicylaldehyde and hydrocinnamaldehyde have also shown 100% inhibition of growth of *R. solani* at 2.5 mg/paper disc in a laboratory study [113]. However, literature on field efficacy is lacking and therefore, necessitates further investigation in this domain. Since these are only observations of *in vitro* experiments, these investigations should be continued under field conditions as well in order to get more reliable data on prospects of using essential oils in the management of soilborne diseases of cowpea with the aim of keeping the environment and consumer's health safe. The efficacy of different plants extracts reported may be due to the presence of several constituents, that is, tannins, saponins, alkaloids, glycoalkaloids, alkenyl phenols, flavonoids, terpenoids, sesquiterpenes lactones and phorbol esters [114]. The active ingredients identified in these plants can be used for the development of next-generation fungicides.

3.5 Synthetic fungicides for management of soilborne fungal diseases

Most of the pathogens causing root rot diseases in cowpea are soilborne. Therefore, seed treatment prior to sowing is important followed by soil drenching. In integrated disease management, fungicides are an important component for disease management. The majority of systemic fungicides need to be applied before the occurrence of disease or at the appearance of the first symptoms to be effective. Fungicides have 'curative' properties, that is, they are active against those pathogens that have already infected the plant, tend to have a higher risk of pathogens developing resistance to the fungicide. In Benin, the only registered fungicide used on cowpea is Super-Homai 70% PM (active ingredient: methylthiophanate 35%, thiram 20% and diazinon 15%) (SPV, Benin). Unfortunately, there has been a problem regarding the efficacy of this product against pathogens [79].

Control of fungal soilborne diseases of cowpea is achieved by several fungicides. Combined application of carbendazim and mancozeb at the rate of 2 g/L as soil drenching, controlled 14.28% collar rot disease, while 57.4% disease incidence was reported in control plots [86]. Seed soaking with potassium sorbate (9%) or sodium benzoate (20 mM) followed by their foliar spray efficiently reduced root rot incidence caused by *F. solani* and *R. solani* [115]. It was found that Dithane (M-45) gave best control against *R. solani*, *F. oxysporum* and *F. solani* when compared with Benomyl 85 and Bavistin 87% [100]. These results were confirmed by the observations of [116] who reported that these fungicides were effective against root rot diseases of blackgram. Likewise, mancozeb, copper oxychloride, carbendazim and metalaxyl have been used for control of *F. solani* in other arable crops [117, 118]. Treating seeds with broad-spectrum fungicides also helps in controlling other soilborne/seedborne fungi and the decay of seeds. For example, carbendazim (0.2%) and etaconazole (0.1%) have been used for control of *M. phaseolina* in chickpea via application through seed treatment and soil drenching [119]. Similarly, fosetyl-Al, metalaxyl, propamocarb-hydrochloride, and azoxystrobin were used against *Pythium* spp. [120] and azoxystrobine fungicides have been widely used against *R. solani* in other crops [121]. These fungicides can be evaluated against *Pythium* species, *R. solani* and *M. phaseolina* isolated from cowpea for their further application against the cowpea pathosystem.

Furthermore, there has been investigations on the sensitivity of isolated *M. phaseolina* to fungicides under *in vitro* conditions and the efficacy of fungicide

application to seed and soil to reduce the population of microsclerotia [111]. Relatedly, Adekunle et al. [83] reported that seeds treated with benomyl at 0.5 g a.i/50 g resulted in 95% plant survival against charcoal rot pathogen. However, control of *M. phaseolina* through chemical fungicides is still complex and neither profitable nor advisable [122]. Although, various studies have reported the efficacy of fungicides against soilborne pathogens of cowpea, they are pathogen-specific and their regular use may cause fungicide resistance. Therefore, more systemic fungicides should be screened against soilborne pathogens of cowpea in order to get more potential fungicides. Furthermore, to reduce the fungicide resistance problems, their mixed application in seed treatment or fungicide rotation strategies should be recommended. Nevertheless, it is very essential to highlight that continuous use of fungicides has a harmful impact on beneficial soil microbial communities, leading to poor soil fertility with reduced productivity [123]. The use of MBCAs in conjunction with fungicides may be one of the strategies for the management of soilborne diseases of cowpea.

3.6 Role of micronutrients and herbicides against soilborne pathogens

Improved plant nutrition through well-balanced fertilization particularly for micronutrients is critical in management of soilborne diseases [38]. A study by [124] reported that amending soil with manganese at a rate of 10 μg/g of soil as MnSO4.H2O reduced the severity of root rots caused by *R. solani* and *R. batiticola* by 42.7 and 42%, respectively. Similarly, soil application of herbicide, Basalin 50% E.C (fluchloralin [*N*-(2-chloroethyl]-2,6-dinitro-*N*-propyl-4-trifluoromethylaniline) at a 5 μl a.i/kg soil significantly reduced incidence of seedling mortality (post-emergence damping-off caused by *R. solani*) compared to 63% in untreated controls [125]. *In vitro* studies involving the same herbicides, Fluchloralin and Lasso 50% E.C (alachlor [2-chloro-2′-6′-diethyl-*N*-(methoxymethyl] acetamide) at rates of 10 μl a.i/L at pH 8 inhibited mycelial growth of *R. solani* by 37–38% [125]. Both herbicides reduced damping-off in potted plants kept at 30°C.

4. Challenges and future prospects

Over 95% of the global cowpea production [17] occurs in the least developed countries by resource constrained smallholder farmers with limited knowledge on integrated pest and disease management options. Several cowpea genotypes with resistance or tolerance to several soilborne diseases were identified in many studies conducted in a few locations. This has hindered their widespread use because of adaptability/suitability to a restricted range of geographical conditions. Therefore, variety screening/evaluation should be conducted in diverse geographies across years when developing cowpea lines with disease resistance. Breeding for durable resistance to most soilborne fungal pathogens is still a challenge in many breeding programs due to pathogen diversity and monogenic nature of host resistance [23, 25, 26, 45]. Correct identification of causal pathogens/agents associated with soilborne diseases using rapid and reliable diagnostic assays is therefore needed.

Marker assisted selection (MAS) offers a great opportunity to improve efficiency in selecting progenies with desirable traits. This is because through MAS, selection for resistance can be carried out even in the absence of disease and at early stages of plant development [126]. Use of markers in breeding for resistance to soilborne fungal pathogens in cowpea is however lacking although a few markers were identified.

In many cowpea producing countries, many MBCAs have been experimentally tested and several are commercially available. However, their use or application is still on a very small scale. This is partly because of lack of sensitization of farmers who assume that a crop cannot be grown successfully without application of synthetic fungicides [127]. Creativity and appropriate guidance through proper extension advice is therefore needed to cause mind-set change among farmers who are still inclined to using synthetic pesticides. Many botanicals and bio-based products were evaluated in controlled environments in many studies but their effectiveness under field conditions is not yet fully known. Also, the application rates of some botanicals are unusually high [70] thus additional studies on refining their efficacy are needed.

Globally, resistance to synthetic fungicides is increasingly becoming a big problem. This problem is likely to worsen in many African countries where over 95% of the cowpea cultivation takes place due to laxity in application of fungicide regulations coupled with poor extension services to educate farmers. For instance, there is limited or lack of national, regional or international policies to guide enforcement of sustainable solutions/practices [127]. Unknowingly, majority of farmers think that registered pesticides are safe for the environment and for man, so there is no incentive for them to change. Also, farmers rarely rotate fungicides with different modes of action due to limited knowledge and extension on IDM [128].

Environmental factors such as soil moisture and temperature that greatly contribute to disease development in the field were reported to have an effect on the level of disease development [38]. For instance, initial inoculum load and soil moisture were the main factors responsible for incidence of damping-off and stem rots in cowpea [26]. A good understanding of all key predisposing factors that trigger development of soil-borne diseases is therefore needed.

5. Conclusions

Soilborne fungal diseases poses a major challenge to production of cowpea globally thus necessitating the need for sustainable management approaches that enhance production while also preserving the environment. Stem rot, damping-off, collar rot, *fusarium* wilt and charcoal rot are the main cowpea soilborne diseases. Several management options both chemical (such as synthetic fungicides) and non-chemical (cultural, physical, host-plant resistance and biological) have been researched on by several investigators. Adoption of an integrated disease management framework is the most effective option to sustainably manage these diseases. Described literature revealed that cowpea genotypes with resistance to FW and charcoal rot have been identified and only a few for stem rots, collar rot and damping-off by evaluating cowpea genotypes under natural/artificial conditions. Some of the identified sources of resistance were specific to few strains/races of the pathogen and regions where they were tested. Therefore, evaluation of resistant genotypes for these diseases at multi-locations in a coordinated approach would help in deploying host resistance at a larger scale. Reviewed literature showed that most of the genetic studies focused on *fusarium* wilt resistance and to a small extent charcoal rot and southern blight. Resistance to FW is conditioned by a single dominant gene making it easier to effectively use backcrossing for transfer of resistance to susceptible backgrounds. However, such resistance is most often less durable and thus can easily be broken down. Reviewed literature also showed that molecular markers are available for FW and charcoal rot, however, there is need for their validation before they are widely deployed in breeding programs. More effort is required to develop the molecular markers for other soilborne diseases.

Use of cultural or agronomic practices such as rotation of cowpea with cereal crops (fonio and millet), application of compost and synthetic fertilizers (NPK) was shown to reduce infestation by charcoal rot. However, there is a knowledge gap regarding how much of these practices have been adopted by farmers to manage soilborne fungal diseases in cowpea.

Several studies reported the efficacy of synthetic fungicides against soilborne pathogens of cowpea however, most of these fungicides are pathogen-specific and their regular use may cause fungicide resistance. Therefore, more systemic fungicides should be screened. Furthermore, to reduce the fungicide resistance problems, their mixed application in seed treatment or fungicide rotation strategies should be recommended. However, continuous use of fungicides has a harmful impact on beneficial soil microbial communities, leading to poor soil fertility with reduced productivity.

Concerning the use of MBCAs, several beneficial microbes (*Trichoderma*, Pseudomonas and Bacillus) have been frequently used for the control of soil-borne diseases of cowpea either as seed dresser or soil application. However, their effective use requires the development of suitable formulation and delivery systems. Similarly, several botanicals or plant-based products have been extensively evaluated in the control of soilborne fungal diseases of cowpea but few have been adopted or reached the market due to lack of large-scale field trials. Concerted and well-coordinated efforts among various stakeholders are therefore needed to evaluate prospective MBCAs, and botanical products in fields at multi-locations and commercialization of superior products.

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Conflict of interest

The authors declare that they have no conflict of interest.

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