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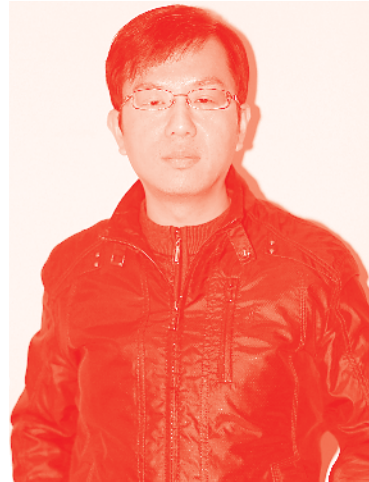
*Edited by Everlon Cid Rigobelo
and Ademar Pereira Serra*



Nitrogen Fixation

*Edited by Everlon Cid Rigobelo
and Ademar Pereira Serra*

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Nitrogen Fixation

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Edited by Everlon Cid Rigobelo and Ademar Pereira Serra

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Biochemistry

Volume 11



Dr. Everlon Cid Rigobelo graduated from Agronomy School Universidade Estadual Paulista Campus of Jaboticabal, Brazil, in 2000. He received a MSc in Animal Science Microbiology and a PhD in Microbiology from the same university where he also works as a researcher. Dr. Rigobelo has experience in genetics and epidemiology, and is active in the fields of microbial biotechnology, molecular genetics and bacterial genomics. He works with plant growth-promoting rhizobacteria and he previously worked with probiotic strains to reduce the spread of *Escherichia coli* in animal production. He has also served as editor for several books, including *Probiotic in Animals*, *Plant Growth*, and *Symbiosis*



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Scope of the Series

Biochemistry, the study of chemical transformations occurring within living organisms, impacts all of life sciences, from molecular crystallography and genetics, to ecology, medicine and population biology. Biochemistry studies macromolecules - proteins, nucleic acids, carbohydrates and lipids –their building blocks, structures, functions and interactions. Much of biochemistry is devoted to enzymes, proteins that catalyze chemical reactions, enzyme structures, mechanisms of action and their roles within cells. Biochemistry also studies small signaling molecules, co-enzymes, inhibitors, vitamins and hormones, which play roles in the life process. Biochemical experimentation, besides coopting the methods of classical chemistry, e.g., chromatography, adopted new techniques, e.g., X-ray diffraction, electron microscopy, NMR, radioisotopes, and developed sophisticated microbial genetic tools, e.g., auxotroph mutants and their revertants, fermentation etc. More recently, biochemistry embraced the ‘big data’ omics systems.

Initial biochemical studies have been exclusively analytic: dissecting, purifying and examining individual components of a biological system; in exemplary words of Efraim Racker, (1913 –1991) “Don’t waste clean thinking on dirty enzymes.” Today however, biochemistry is becoming more agglomerative and comprehensive, setting out to integrate and describe fully a particular biological system. The ‘big data’ metabolomics can define the complement of small molecules, e.g., in a soil or biofilm sample; proteomics can distinguish all the proteins comprising e.g., serum; metagenomics can identify all the genes in a complex environment e.g., bovine rumen. This Biochemistry Series will address both the current research on biomolecules, and the emerging trends with great promise.

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Preface

Nitrogen (N) is the most abundant gas in the Earth's atmosphere, although it is exceptionally unreactive. Biological nitrogen fixation (BNF), the process by which gaseous N_2 is converted into ammonia (NH_3) via the enzyme nitrogenase, is crucial for the availability of nitrogen in the terrestrial ecosystem. Some bacteria have the remarkable capacity to fix atmospheric nitrogen to ammonia under ambient conditions, a reaction only mimicked on an industrial scale by a chemical process. This microbiological process converts atmospheric nitrogen into a plant-usable form, thus decreasing the necessity of the use of chemical fertilizers in crop production. In many systems, N fixers have driven the accumulation of fixed N on long time scales, bringing N supply dose to equilibrium with other potentially limiting resources.

BNF includes symbiotic (nodule formation) and free-living bacteria N fixation, defined as N fixation occurring without formal plant–microbe symbioses. The ability of microorganisms to use nitrogen gas as the sole nitrogen source and engage in symbioses with host plants confers many ecological advantages. However, it also incurs physiological penalties. BNF is highly regulated at the transcriptional level by sophisticated regulatory networks that respond to multiple environmental cues.

The present book chapters in this volume cover approaches different aspects related to of this fantastic phenomenon, such as biofertilizer, organic nitrogen in agricultural systems, nitrogen fertilization for sustainable crop production, and others.

I would like to thank my wife Fernanda, my daughter Maria Eduarda and my son João Henrique for making my life happier. I also thank my deceased father Angelo Sidney Rigobelo, who lives in my mind and in my heart every day of my life.

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Role of Biofertilizers in Plant Growth and Soil Health

Murugaragavan Ramasamy, T. Geetha and M. Yuvaraj

Abstract

Biofertilizers nowadays have been realised for shifting fortunes in agriculture. It has been proven successful technology in many developed countries while in developing countries exploitation of bioinoculants is hampered by several factors. Scientific knowledge on bioinoculants and its usage will pave way for its effective usage. At the same time overlooking the significance of ensuring and maintaining a high quality standard of the product will have negative impact. Hence a proper knowledge of bioinoculants and its functioning will pave way to tap the resources in a better way. Thus the chapter provide overview knowledge about different bacterial, fungal and algal biofertilizers, its associations with plants and transformations of nutrients in soil. Adopting a rational approach to the use and management of microbial fertilizers in sustainable agriculture thrive vast potential for the future.

Keywords: biofertilizers, microorganisms, diazotrophics, bioinoculants, biological nitrogen fixation

1. Introduction

One of the present day challenges in agriculture is eco-friendly practices. Though the benefits of Green revolution has been reaped by us in terms of production, the other side of it, i.e., over usage of chemical fertilizers and its subsequent deterioration of soil health has been realised these days [1]. Hence awareness of practicing organic agriculture has been taken to various spheres and products of organic agriculture are fetching up huge market. One of the organic agriculture practices includes usage of biofertilizers in farming. Biofertilizers are likely called as bioinoculants as they are the preparations containing living or latent cells of microorganisms that facilitate crop plants uptake of nutrients by their interactions within the rhizosphere once applied through seed or soil. It accelerate bound microorganism processes within the soil that augment the extent of convenience of nutrients in a very type simply assimilated by plants [2]. Use of biofertilizers has several other advantages as well like they are cost effective, eco-friendly and renewable source of plant nutrients hence forms one of the important components of integrated nutrient management. As of now we could not claim bio-inoculants as a right alternative to chemical fertilizers but in near future the scientific understanding of the same will pave way for its right use and reap full benefits [3]. In addition to this in global scale, recent published works on biofertilizers states about the varied role of bioinoculants viz., other than nutrient transformations in different crops. To mention few, increase in root growth has been observed in wheat due to inoculation of bioinoculant consortia. Likewise *Rhizobium* inoculation increases deaminase

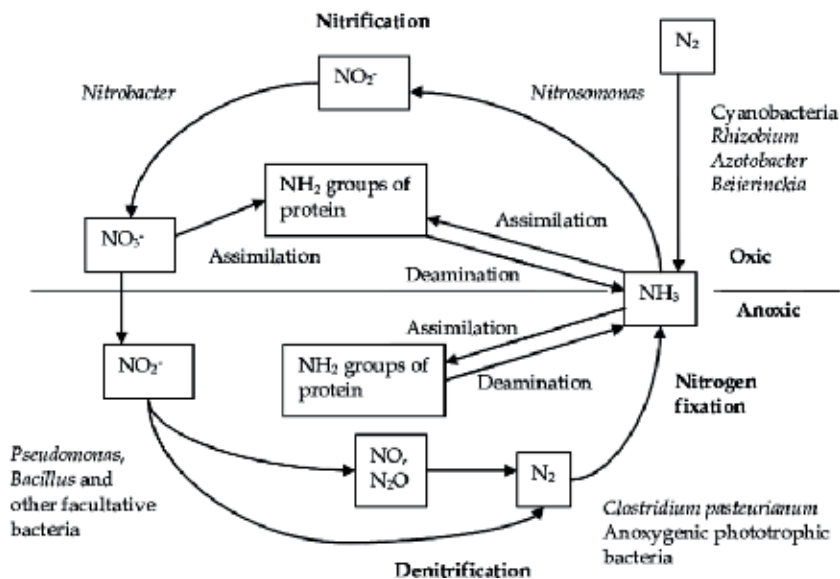
activity in pulses crops. Hence this chapter focuses on different bioinoculants and its uses in farming.

2. Importance of soil microbes in nutrient transformations

It is well established fact that soil microbes have versatile enzyme systems hence perform various nutrient transformations in soil which is very important for maintaining soil equilibrium and its health [4]. Among the nutrient transformations nitrogen and phosphors transformations forms significant importance, since they are the major plant nutrients derived from the soil.

3. Nitrogen transformations

Nitrogen cycle involves of transformations of nitrogen by particular group of soil microbes into organic, inorganic and volatile forms. In addition, a small part of the large reservoir of N_2 in the atmosphere is converted to organic compounds by certain free living microorganism or by plant microbe association that makes the element available to plant growth [5]. The atmospheric nitrogen constitutes about 78% in gaseous form which cannot be utilised by plant and other living organisms which is referred to as biological nitrogen fixation [6]. The details of nitrogen transformations occurring in soil with the role of microbes involved has been depicted below:



4. Biological nitrogen fixation

Biological nitrogen fixation is a component of nitrogen cycle which involves fixing up of atmospheric nitrogen by particular soil microorganisms. Nitrogen fixing ability has been restricted only to certain bacteria and few actinomycetes which belong to various groups and they are referred to as diazotrophs [7]. Diazotrophic microbes are ubiquitous to soil and are classified according to mode of nitrogen fixation to plants **Table 1**.

S. No.	Groups	Examples
1.	Free-living	<i>Azotobacter</i> , <i>Beijerinckia</i> , <i>Clostridium</i> , <i>Klebsiella</i> , <i>Anabaena</i> , <i>Nostoc</i>
2.	Symbiotic	<i>Rhizobium</i> , <i>Frankia</i> , <i>Anabaena azollae</i>
3.	Associative Symbiotic	<i>Azospirillum</i>

Table 1.
 Groups of important diazotrophic organisms according to mode of nitrogen fixation.

The process of biological nitrogen fixation has been first documented in anaerobic bacterium *Clostridium pasteurianum* from which the enzyme nitrogenase has been isolated [8]. However today the organism has not been commercially used for the purpose. The nitrogen fixation is mediated by nitrogenase enzyme which reduces gaseous nitrogen to ammonia. All diazotrophs seemed to possess the enzyme and found to deliver quite similar mechanism of nitrogen fixation.

5. Important diazotrophs in commercial use

Rhizobium is the most studied bioinoculant which forms symbiotic association with legume plants. It was first shown by Boussingault that leguminous plant can fix atmosphere N₂ which Hellriegel and Wilfarth clarified that the process is done by bacteria residing in the roots of leguminous plants [9]. The purified bacterium was put into various examinations and now well-developed nitrogen fixing strains are available in various commercial production units.

This bioinoculant is specific for legume crops and forms nodules in the roots of the plants. It enriches the soil fertility also after harvesting of the crop. Hence it is the most preferred bioinoculant [10]. Other than root nodulating *Rhizobium* some of the strains found to nodulate stem known as *Azorhizobium* present in *Sesbania rostrata*. *Rhizobium* species are specific to legume crops because of nod factors they produce [11]. However some leguminous plants found to develop effective nodules on inoculation with the *Rhizobia* obtained from the nodules from other legume groups which is referred to as cross inoculation grouping

Table 2.

1.	<i>Rhizobium leguminosarum</i>	CIG	Host it can nodulate
	bv. <i>viciae</i>	Pea	Peas, lentils, vicia
	bv. <i>phaseoli</i>	Bean	<i>Phaseolus</i> spp
	bv. <i>trifoli</i>	Clover	<i>Trifolium</i> spp
2.	<i>R. meliloti</i>	Alfalfa	Alfalfa, clover, fenugreek
3.	<i>R. loti</i>	Lotus	Trifoli, lupine
4.	<i>R. fredii</i>	Soybean	Soybean
5.	<i>R. spp</i>	Cowpea group	<i>Vigna</i> , <i>Arachis</i> , <i>Cajanus</i> , <i>Dolichus</i> , <i>Sesbania</i> , <i>Acacia</i> , <i>Prosopis</i> , green gram and blackgram
6.	<i>R. spp</i>	Chickpea group	Chickpea

Table 2.
 Cross inoculation grouping of *Rhizobium*.

5.1 *Azospirillum*

Azospirillum is considered as very important diazotrophs as it form associative symbiotic relationship with the roots of graminaceous plants. It is generally recommended for rice crop [12]. The organism is microaerophilic, some are aerobic motile and gram negative in nature hence suits well for rice field conditions. It was first isolated by Beijerinck and was named as *Sprillum lipoferum* later named as *Azospirillum*. In addition to nitrogen fixing ability, they also produce growth promoting substances such as IAA [13]. Some of the important species of *Azospirillum* has been listed below:

1. *A. brasilense*
2. *A. lipoferum*
3. *A. amazonense*
4. *A. halopraeferens*
5. *A. irkense*
6. *A. dobereineriae*
7. *A. largimobilis*

5.2 *Azotobacter*

Azotobacter are gram negative free living bacterium in the rhizosphere soil of many plant species, discovered by Beijerinck. The bacterium is very well recognised diazotroph and fixes atmospheric nitrogen in its habitat. Owing to its versatile adaptability and nitrogen fixing ability, they are commercially used in agriculture for many crops and are known with a brand name azotobacterin. Some species of *Azotobacter* known to produce alginic acid, a compound used in medical industry and in food industry it is used as additive in ice creams and cakes [14]. Apart from its nitrogen fixing ability, it also synthesise many phytohormones such as auxins and helps in promoting growth of the plants [15]. They are involved in mobilising heavy metals in the soil thus used for bioremediation purposes as well. Many species of *Azotobacter* are pigment producers and found to degrade aromatic compounds in the agriculture lands.

5.3 *Gluconoacetobacter diazotrophics*

They are endotrophic bacterium which resides inside the stem of sugarcane as it prefers high sucrose and acid content for its survival. They have the ability of capturing atmospheric nitrogen and converting into ammoniacal form [16]. Moreover they are known for stimulating plant growth by tolerant to acetic acid. The bacterium was first discovered in Brazil by scientists Vladimir A. Cavalcante and Johanna Dobereiner. They are originally known as *Acetobacter* belong to Acetobacteriaceae family and got the current name due to carbon source requirement. Besides nitrogen fixing ability they are known to synthesis indole-3-acetic acid which promote the growth of the associated plant species [17]. Also reports suggest this bacterium controls pathogen especially *Xanthomonas albilineans* in sugarcane. Thus in recent years it is the most recommended bioinoculant for sugarcane.

Apart from these bacterial bioinoculants, cyanobacteria also fixes nitrogen which are referred as algal bioinoculants.

6. Algal bioinoculants

6.1 Algal biofertilizers

The potentiality of algal biofertilizers are realised long before by 1939, when WHO attributed the tropical rice natural fertility to green blue chlorophytic algae. Among algae, only blue green algae have biological nitrogen fixing ability due to the presence of heterocysts cells in them [18]. This bioinoculant is recommended only for rice crop and was proved to improve soil fertility by nitrogen fixation and organic matter enrichment after harvest. In some places, practice of culturing algae as dual crop along with rice has been done which found to inhibit small weed growth during cropping. Apart from this some of the algal species also promote growth by producing growth promoting substances [19].

The following list is some of the nitrogen fixing algal species:

- a. Examples of unicellular nitrogen fixing algae: *Gloeothece*, *Gloeobacter*, *Synechococcus*, *Cyanothece*, *Gloeocapsa*, *Synechocystis*, *Chamaesiphon*, *Merismopedia*.
- b. Filamentous non heterocystous forms of *Cyanobacteria*, *Oscillatoria*, *Spirulina*, *Arthrospira*, *Lyngbya*, *Microcoleus*, *Pseudanabaena*.
- c. Filamentous heterocystous forms *Anabaena*, *Nostoc*, *Calothrix*, *Nodularia*, *Cylindrospermum*, *Scytonema*.

6.1.1 *Anabaena azollae*

Anabaena is a special type of algae which forms symbiotic association with free floating water fern *Azolla*. Water fern is bilobed in nature and algae resides in the roots of the fern. The common species of algae forming symbiotic association with *Azolla* are *A. microphylla*, *A. filiculoides*, *A. pinnata*, *A. caroliniana*, *A. nilotica*, *A. rubra* and *A. mexicana*. This algae takes shelter and carbon from the water fern and in turn fixes atmospheric nitrogen. They need sunlight and water for its multiplication and hence can be used for rice crop as dual crop. *Azolla* as dual crop in crop estimate to reduce nitrogen requirement by 20–25% [20].

6.2 Phosphate solubilizing and mobilizing bio inoculants

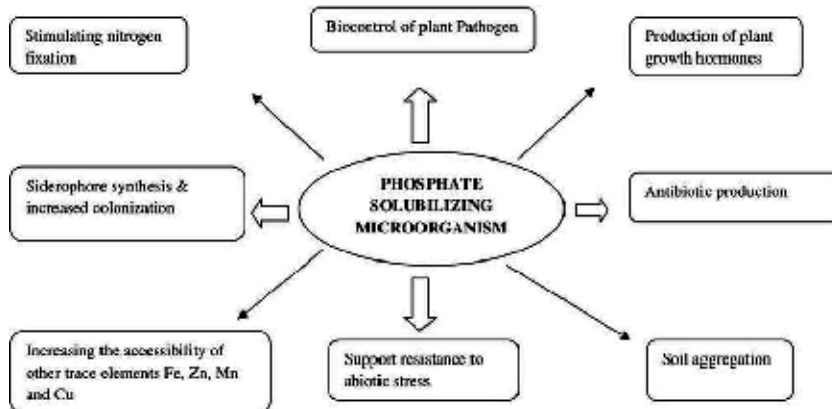
Next to nitrogen phosphorus is the key element for plant growth. Most of Indian soils contain significant amounts of inorganic form of phosphorus, but it is unavailable for plants as it is in the insoluble form. Hence it needs to be solubilised for plant use [21]. Moreover, phosphorus is also available in organic forms in soil which need to be mineralised for plant utilization. Thus mineralization and solubilisation of phosphorus in soil becomes important with respect to plant growth [22].

6.3 Phosphorus solubilising bioinoculants

The fate of phosphorus is that it forms apatite's with the salts present in the soil. In acid soil phosphorus will becomes Aluminium phosphates and Iron phosphates

while in alkaline soils it becomes calcium phosphates or sodium phosphates and becomes unavailable to plants [23]. In order to make these form of phosphorus to available form some of the bioinoculants produces organic acids which convert them to soluble form like hypophosphites which can be taken by plants [24]. Examples of phosphorus solubilising Bacteria: *Bacillus megatherium* var. *phosphaticum*, *Bacillus megatherium* var. *phosphaticum*, *Bacillus subtilis*, *Bacillus circulans*, *Pseudomonas striata* Fungi: *Penicillium* sp., *Aspergillus awamori*.

The phosphorus transformations occurring in soil has been depicted below:



6.4 Phosphorus mobilising bioinoculants

Mycorrhiza is the special type of relationship between fungi and plants Existence of mycorrhizal fungi has been date back 450 million years ago [25]. The relationship is mutualistic in nature and the fungal members who enter into the relationship are members of Zygomycetes, Ascomycetes and Basidiomycetes [26]. Mycorrhizal fungi contribute According to the reports; roots of about 95% of all kinds of vascular plants are normally involved in symbiotic associations with mycorrhizae [27]. For angiosperms, gymnosperms, ferns and some mosses mycorrhizal association appears to be the norm. In the relationship fungus gets a supply of carbon from the associated plant and in turn plants gets lots of benefits from the fungus which is listed below:

- Fungi hyphae increases the root area hence produces more vigorous plants.
- Hyphae surrounding are thinner than roots, but longer than it hence absorb nutrients and water from deeper layers of soil. This helps the plants to tolerate drought.
- Mobilises phosphorus from distant places.
- Gives plants disease tolerance.
- Contribute to nutrient recycling due to production different enzymes.

Mycorrhizas are commonly divided into *ectomycorrhizas* and *endomycorrhizas* based on the mode of hyphal formation. The former do not penetrate the individual cells within the root, while the hyphae of later penetrate the cell wall and forms structures inside the cell membrane [28].

Ectomycorrhizas, are formed between the roots of around 10% of plant families, mostly woody plants including the birch, eucalyptus, oak, pine, and rose families, orchids, and fungi belonging to the *Basidiomycota*, *Ascomycota*, and *Zygomycota*. Thousands of ectomycorrhizal fungal species exist, hosted in over 200 genera [29]. In ectomycorrhizas the hyphae of the fungus do not penetrate the cells of plant roots. In ectomycorrhizae, the mycelium of the fungus forms a dense sheath over the surface of the root. These hyphae form a network in the apoplast, but do not penetrate the root cells. Ectomycorrhizae form a sheath and the fungus grows between the plant cells producing “Hartig net” [30].

One of the more important ectomycorrhizal fungi is *Pisolithus tinctorius*. Symbiosis begins when fungal spores germinate and emerging threadlike structures called hyphae, enter the epidermis of plant roots [31]. After colonization of the root, the fungus sends out a vast network of hyphae throughout the soil to form a greatly enhanced absorptive surface area. This results in improved nutrient acquisition and uptake by plant roots, particularly elemental phosphorus (P), zinc (Zn), manganese (Mn) and copper (Cu) and water. In return, the plant provides carbohydrates for the fungi [32].

6.5 Endomycorrhizae (AM fungi)

Endomycorrhizae form an association in which the hyphae penetrate and colonize epidermal and fleshy cortical cells of plant roots. The most common type of Endomycorrhizae is arbuscular endomycorrhizae (AM). Arbuscular mycorrhizae are characterized by the formation of unique structures such as arbuscles and vesicle by fungi within the plant root cortical cells [33]. Once the roots are colonized, individual hyphae extend from the root surface outward into the surrounding soil forming a vast hyphal network that absorbs nutrients and water that would otherwise be unavailable to the plant’s root system. Endomycorrhizae can occur on most seed bearing plants, rain forest tree species, most agriculture crops and a vast variety of ornamental greenhouse crops.

Fungi forming AM associations include about 150 species belonging to genera *Gigaspora*, *Glomus*, *Sclerocystis*, *Acaulospora* and *Entrophospora*. Colonization of roots begins by the secretion of enzymes by arbuscular endomycorrhizae allowing hyphae to penetrate the epidermal and fleshy cortical cells of plant cells. Two to 3 days after colonizing the cell, the hyphae form structures within plant cells called arbuscules which resemble tiny trees and serve to facilitate the transfer of nutrients within the cortical cells.

Arbuscular endomycorrhizae provide the plant with certain mineral elements and water from the soil, and in turn, the plant provides sugars and other carbohydrates for the fungus. Between the cells, sac like structures, called vesicles may form midway or at the terminal ends of the hyphae. Vesicles contain lipids and serve primarily as storage organ for the fungus [34]. Vesicles can also serve as propagules that can colonize other parts of the plant root. Arbuscular endomycorrhizae hyphae also give rise to spores. Spores have very thick walls, which makes them very resistant to freezing and intense heat so they can survive for long periods of time. For this reason, spores are ideal for incorporating into growing media and for use as inoculants. It takes 2–6 weeks for arbuscular endomycorrhizae fungi to completely colonize plant roots and will remain with the plant throughout its life.

6.6 Plant growth promoting rhizobacteria (PGPR)

Other than these above mentioned bioinoculants some of the bacterium like *Pseudomonas*, *Bacillus thuringiensis* and fungi like *Trichoderma viride* are involved

in the control of pests and diseases of plants by colonising the rhizosphere of many plants. They are referred to as biocontrol agents. Among the biocontrol agents *Pseudomonas* is largely used in seed treatments and soil application in large number of crops. They are known for siderophore production which chelates iron in the rhizosphere region thus creating iron deficiency for the pathogenic microbes. Apart from these report suggests that they are also involved in nitrogen fixation and nutrient transformations in soil. Some bacteria living in rhizosphere affect plant growth positively and some are detrimental. Rhizosphere bacteria that favor plant growth are termed as plant growth promoting rhizobacteria (PGPR). They improve plant growth directly by producing plant growth regulators such as auxins, gibberellins and cytokinins; by eliciting root metabolic activities and/or by supplying biologically fixed nitrogen. Consequently, germination, root development, nutrient and water uptake are improved. Other PGPR affect plant growth by indirect mechanisms such as biocontrol activity by suppression of bacterial, fungal and nematode pathogens. The mechanism of biocontrol include competition for colonization space and for nutrients, antibiosis, excretion of hydrogen cyanide and other volatile compounds, synthesis and absorption of siderophores, excretion of lytic enzymes (chitinases, glucanases) and systemic resistance. The well-known PGPR include *Azotobacter*, *Azospirillum*, *Azoarcus*, *Klebsiella*, *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Enterobacter*, *Burkholderia*, *Serratia*, and *Rhizobium*.

7. Conclusion


In developing countries, the most important challenge is to produce sufficient food for the growing population from inelastic land area. Products of biological origin can be advantageously blended to replace a part of the energy-intensive inputs. It is in this context, biofertilizers can provide to the small and marginal farmers an economically viable lever for realizing the ultimate goal of increasing productivity. These microbes siphon out appreciable amounts of nitrogen from the atmospheric reservoir, solubilise phosphorus and enrich the soil with this important but scarce nutrient. The crop-microbial-soil ecosystem can, therefore, be energized in sustainable agriculture with considerable ecological stability and environmental quality.

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Organic Nitrogen in Agricultural Systems

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Abstract

This work summarizes information about organic nitrogen (N) in the agricultural system. The organic N forms in soils have been studied by identifying and quantifying the released organic compounds when soils are acid treated at high temperature, in which the following organic N fractions are obtained: hydrolyzable total N, subdivided into hydrolyzable NH_4^+ -N, amino sugars-N, amino acids-N, and unidentified-N and acid insoluble N, a fraction that remains associated with soil minerals after acid hydrolysis. Nitrogen mineralization and immobilization are biochemical processes in nature. This chapter summarizes how these processes occur in the agricultural system. Then, soluble organic nitrogen (SON), volatilization and denitrification processes, and biological nitrogen fixation (BNF) as a key component of the nitrogen cycle and how it makes N available to plants are also discussed. Finally, we discuss the use of organic fertilizers as N source to satisfy the worldwide demand for organic foods produced without synthetic inputs.

Keywords: biological N fixation, immobilization, mineralization, organic fertilization

1. Introduction

Nitrogen (N) is the fourth most abundant element in cellular biomass and comprises most of the Earth's atmosphere. In the surface layer of most soils, over 90% of N occurs in organic forms. Soil organic N can be divided into two categories: (1) N from organic residues and (2) N from soil organic matter or humus [1]. All these materials are important in maintaining or improving soil fertility and plant nutrition through direct and indirect effects on microbial activity and nutrient availability [2]. Analysis of organic fractions has been highlighted due to the increasing application of organic fertilizers and their direct and indirect effects on crop growth and yield and soil attributes. Thus, we will discuss about organic N forms, N mineralization and immobilization, volatilization and denitrification, soluble organic N, biological N fixation, and organic fertilization with emphasis on N.

2. Organic nitrogen

Nitrogen is an essential element for plants, being constituent of important biomolecules such as adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), chlorophylls, amino acids and proteins (glyco- and lipoproteins), nitrogenous bases and nucleic acids, and various enzymes [3, 4]. Soil organic N consisting of proteins, chitins, amino acids, and nucleic acids represents about 90–98% of total soil N [1, 5]. Mineralized N forms are transient in the soil so that the existing amount depends on numerous processes such as mineralization, immobilization, nitrification, denitrification, leaching, and plant uptake. Therefore, the study of mineral N may not represent the N availability during the crop growing. On the other hand, the study of organic N fractions and their transformations over time can help in predicting the N availability for crops, in estimating the N supply to the soil, and in evaluating the potential release of mineral N by organic fertilizers.

Many compounds account the soil organic N, being approximately 40% protein material (proteins, peptides, and amino acids), 5–6% amino sugars, 35% heterocyclic nitrogen compounds (including purines and pyrimidines), and 19% NH_3 , with $\frac{1}{4}$ fixed as NH_4^+ . Thus, protein materials and heterocyclic compounds predominate in the total soil N, and organic N fractionation may inform about the mineralization susceptibility of compounds [6]. The organic N forms in soil have been studied by identifying and quantifying the released organic compounds when soils are acid treated at high temperature. The organic N fractions obtained by acid hydrolysis are hydrolyzable total N, subdivided into hydrolyzable NH_4^+ -N, amino sugars-N, amino acids-N, and unidentified-N and acid insoluble N, a fraction that remains associated with soil minerals after acid hydrolysis [7].

The fractionation allows separating the labile N forms from the soil, such as amide-N and amino-N (acid hydrolyzable), which can be rapidly synthesized in the mineralization process, releasing inorganic N (NH_4^+ and NO_3^-) to the soil solution. However, most of the organic N can compose more stable fractions in the soil, such as non-hydrolyzable-N and unidentified-N. Variation in the non-hydrolyzable-N may be related to soil management, because the higher the hydrolysis intensity of organic N fractions in the soil, the higher the presence of finer particles that form clay-metal-humus complexes that constitute the non-hydrolyzed N. In Brazil, studies are reported in soils from Amazônia [8], São Paulo [9–12], and Espírito Santo [13].

In Latosols and Argisols from Amazônia, determination of the organic N forms indicated that the immobilization was mainly from microbial origin and the ^{15}N immobilized in the soil was found as acid-soluble N and undistilled-N [8]. In São Paulo, in sugarcane-cultivated soil, amino acid-N fractions predominated, and, after 12 weeks incubation, the total hydrolyzable-N did not vary, but the hydrolyzable NH_4^+ -N decreased [9]. In soil samples under different cover plants [10], the amino acid-N fraction predominated, with the following distribution: 14–38% hydrolyzable NH_4^+ -N, 36–52% adenosine triphosphate as NH_4^+ -N + amino sugars, 10–32% amino sugar-N, 26–46% amino acid-N, and 3–28% unidentified-N.

Moreover, in São Paulo, in a soil under maize cultivation, it was observed that topdressing N fertilization decreased the N content of the most labile fractions (hydrolyzable NH_4^+ -N and amino sugars-N) in the surface layer of the soil, and the amino acid-N and amino sugar-N fractions were considered the organic N reservoirs that control the soil N availability [11]. In contrast, fertilization with cattle manure [12] increased the most easily mineralized (up to 100 days) organic N fractions and subsequently increased the more stable organic N fractions, mainly in clay soil. In Espírito Santo, in soil under eucalyptus, [13] observed that the amino-N

was predominant (39%), followed by unidentified-N (27%), amide-N (18%), and hexosamine-N (15%).

Several theories have been developed to explain the resistance of some N compounds to microbial attack. It is mentioned that N compounds are probably protein constituents (amino acids, peptides, and proteins) that are stabilized by reactions with lignins, tannins, quinones, and reducing sugars. Moreover, N compounds would adsorb to the clay fraction of soil and thereby would be protected against the action of protease enzymes. Also, the formation of organic N complexes and polyvalent cations (iron and aluminum) is another biologically stable form of protection [14]. Accumulation and/or decrease of organic C and N is more dynamic in sandy soils than in clayey ones, probably due to the highest oxygenation capacity and lower residue input of sandy soils due to its low productive potential, which gives it less resilience.

3. Nitrogen mineralization and immobilization in the agricultural systems

Nitrogen mineralization and immobilization are biochemical processes widely discussed in the literature. We will focus on how these processes occur in the agricultural system. N mineralization occurs through hydrolysis and biodegradation of soil organic matter when N content in the substrate exceeds the metabolic N requirement by microbial cells. The process is mediated by heterotrophic soil microorganisms [15] that use nitrogenous organic substances as a source of C, N, and energy, releasing NH_4^+ ions as a residue (ammonification). In its turn, immobilization is defined as the transformation of inorganic N (NH_4^+ , NH_3 , NO_3^- , NO_2^-) to microbial forms. Microbiota assimilates inorganic forms of N by incorporating them into the amino acids, which will participate in protein synthesis during soil biomass formation [14].

N mineralization and immobilization occur simultaneously and oppositely in the soil. The net balance between these processes is controlled by several factors: (a) environmental, such as soil temperature, aeration, and moisture; (b) soil physical, such as texture, structure, and size of aggregates [16]; (c) soil chemical, such as pH; (d) agricultural management system adopted [17]; and (e) quality parameters of the decomposing waste (such as C/N, C/P, and C/S ratios), content of easily decomposable and recalcitrant fractions, type of associated decomposers, size and activity of microbial biomass, and inorganic N availability [18]. Carbon/nitrogen (C/N) ratio less than 25 in organic waste favors N mineralization and fast decomposition, while greater than 30 strongly favors N immobilization and fast decomposition [19]. The crop developmental stage also influences waste C/N ratio. For instance, wastes from millet plants cut at the flowering or milky grain stages present high C/N ratio which delays mineralization. On the other hand, wastes from millet cut at the flag leaf stage, even though phytomass is lower, present less C/N ratio which favors N mineralization for the next crop [20].

In residue plant, considering ^{13}C -CPMAS NMR spectral regions [21], observed that the carbonyl C and N-alkyl and methoxyl C regions had the most significant positive correlation with N mineralization, while the di-O-alkyl C and O-alkyl C were strongly associated with N immobilization. This study demonstrates that the biochemical quality of organic C defined by ^{13}C -CPMAS NMR is capable of predicting N dynamic pattern better than C/N ratio. Abbasi et al. [22] observed positively correlated with the initial residue N contents and negatively correlated with lignin content C/N ratio, lignin/N ratio, polyphenol/N ratio, and (lignin +

polyphenol)/N ratio indicating a significant role of residue chemical composition and quality in regulating N transformations and cycling in soil.

In the N compartments, N from the most labile fractions is released in the early mineralization process, and its mineralization estimate can be used to adjust the nitrogen fertilization recommendations. In fact, it was observed that the mineralization potential and the respective mineralization rate can be used to predict the N availability for plants in the agricultural system. Camargo et al. [23] found that the potentially mineralizable nitrogen values in 10 soils from Rio Grande do Sul ranged from 108.6 to 210.8 mg kg⁻¹.

In respect to the management system adopted, time is essential for N mineralization, mainly in the no-tillage (NT) system. Siqueira et al. [24] found that in soil under NT system for 12 and 22 years, the averages for N mineralization were 0.19 and 0.26 g m⁻² day⁻¹, respectively. For organic compounds such as sludge, the N mineralization rate is generally below 50%, 5–38% [25], 14–43% [26], 7–16% [27], and 24–31% [28]. Among the species used in straw production, Fabaceae plants stand out for fixing atmospheric N₂ and presenting low C/N ratio tissues, in addition to the high soluble compound content and low lignin and polyphenol contents. This fact favors the fast decomposition and mineralization, with significant N input to the soil–plant system, but with reduced soil cover, which is essential for NT system [29]. On the other hand, Poaceae plants present relatively high dry matter content and high C/N ratio (> 30), which increase the persistence of soil cover although increase N immobilization [30, 31].

4. Nitrogen volatilization and denitrification

Volatilization is the main cause of N loss where ammonia gas (NH₃) is produced according to the simplified equation: NH₄⁺ + OH⁻ ↔ NH_{3(g)} + H₂O. NH₃ loss increases with increasing soil pH. Ammonium ion (NH₄⁺) can be adsorbed by soil colloids (clays in humus); thus the largest losses are found in sandy soils and poor in soil organic matter (SOM). Denitrification is another factor that favors N loss, which is mainly controlled by organic matter content, pH, and soil temperature. This process is performed by anaerobic bacteria such as *Pseudomonas*, *Bacillus*, *Micrococcus*, and *Achromobacter*, which are heterotrophic and get energy from carbon, through oxidation of organic compounds. Some autotrophic species also participate in the process such as *Thiobacillus denitrificans* and *T. thioparus* [32].

NH₃ losses by volatilization in agriculture occur due to many factors: ambient temperature, soil moisture at fertilization time, urease enzyme activity, soil pH, cation exchange capacity, soil cover, rainfall after fertilization, and SOM content [33, 34]. Tasca et al. [34] reported 4.6-fold less NH₃ volatilization when topdressing urea was performed at 18°C temperature, compared to 35° C, which demonstrates that N losses increase with increasing temperature. Low volatilization rates are also reported under higher soil moisture values, around 20%, because fertilizer hydrolysis facilitates the NH₄⁺ diffusion, making it less susceptible to volatilization, even considering the increased soil biological activity in that moisture. In contrast, higher N losses occur under around 10% humidity values, because the NH₄⁺ incorporation is inefficient, resulting in higher N-NH₃ emissions [34]. Moreover, NH₃ losses by volatilization are higher during the driest periods of the year. Soil moisture at fertilization time directly interferes with urea hydrolysis and consequently with NH₃ volatilization losses. Thus, soil wetting soon after urea application is more important than the soil moisture at the application time [35]. According to Ros et al. [36], water applied after urea fertilization or the occurrence of rainfall may decrease NH₃ volatilization if it is sufficient to dilute the hydroxyl (OH⁻)

concentration around the urea granules produced during the hydrolysis, besides providing the incorporation of urea in the soil.

Plant cover also influences N-NH₃ volatilization. Pinheiro [37] found the removal of sugarcane straw from the soil decreased NH₃ volatilization rates. The analysis of topsoil and straw indicated higher urea and NH₄⁺ retention in the largest amounts of straw on the soil, besides effective urea hydrolysis occurring directly in the straw. These results demonstrated a direct contribution of the straw mulches on NH₃ volatilization. However, despite NH₃ volatilization decreases with straw removal, the choice of straw amount to be removed cannot be based only on NH₃ volatilization of N fertilizer. Analyzing fertilizer mixtures in laboratory, Vitti et al. [38] found that mixing urea (330 mg) with ammonium sulfate (300 mg) significantly reduced N-NH₃ losses (97.47 mg) relative to urea (121.52 mg), without affecting the physicochemical quality attributes of the mixture for technical and agronomic efficiency purpose. In Brazil, urea is the most used mineral N fertilizer, but it has volatilization losses due to the enzymatic hydrolysis that consumes H⁺ and increases soil pH. For that reason, even in acidic soils, urea is subject to N losses by volatilization [39]. In agricultural systems, the largest N losses by volatilization occur 3–5 days after fertilizer application [40]. Santos [41] observed that from total N-NH₃ loss by volatilization, 92.5% occurred until the fifth day after fertilization, negatively affecting the corn grain yield.

Fertilizer type may also influence N-NH₃ volatilization. The application of polymer and organic compound-coated urea promoted the lowest ammonia losses by volatilization [42, 43]. In soil under pasture (*Brachiaria decumbens*), Lana et al. [44] observed NH₃ losses 2 days after urea application (2765 mg) and that the use of an inhibitor (NBPT) reduced the volatilization peak by up 4 days. The use of urea plus Uremax NBPT 500® decreased volatilization by approximately 75% after 11 days. Also, adding acid fertilizers may reduce NH₃ losses by 29% [45]. According to Gurgel et al. [46], mineral fertilizers mixed with urea and humic acid (5 and 10%) and urea and zeolite (10%) reduced N-NH₃ losses up to 38%. Results were even more effective in sandy soils.

The use of liquid and solid organic biofertilizers such as poultry and swine residues are also alternative means to reduce N losses, since N is present in biofertilizers as organic form, thereby requiring more time to be mineralized by microorganisms for plant uptake. Niraula et al. [47] reported that cattle manure applied in corn had 11% lower cumulative NH₃ emission than urea, without affecting grain yield, despite having higher CO₂ and CH₄ emissions. Thus, after comparing the ammonia volatilization levels reported in 92 studies, Bouwman et al. [48] concluded that the average NH₃ emissions from the synthetic urea fertilizer and manure slurry were 21.0 and 21.2% from applied N fertilizer, respectively. Moreover, acidification has been a resource used to minimize urea volatilization with liquid waste. Park et al. [49] observed the application of acidified slurry reduced NH₃ emissions by 78.1%, N₂O emissions by 78.9%, and NO₃⁻ leaching by 17.81% compared to control (non-pH-controlled pig slurry), over the course of the experiment.

Quantifying ammonia volatilization from various organic N sources (castor bean cake, bokashi, legume fertilizers, cattle manure), Rocha et al. [50] observed (i) the N loss rate by NH₃ volatilization varies from 3 to 25% in winter/spring and 2 to 38% in summer/autumn among the studied organic fertilizers; (ii) when incorporating organic fertilizers into the soil, volatilization was significantly lower than when they are maintained on the soil surface, with a volatilization reduction by 80% for castor cake, 78% for bokashi, and 67% for legume fertilizer, while for cattle manure there was no difference; and (iii) when on surface, potential NH₃ volatilization from the total N applied in winter/spring and summer/autumn seasons, respectively, was 25.5 and 38.1% for castor cake, 16.6 and 13.7% for bokashi, 8.2 and 8.8% for legume fertilizer, and 3.4 and 2.4% for cattle manure.

In Planosol under irrigated rice, the addition of cover plants on the soil and water management by intermittent irrigation were practices that mitigated N₂O emissions. Zschornack et al. [51] observed an increase in N₂O emissions by more than 200% in a drained area than continuous water blade area. Thus, soil drainage during rice cultivation increases N₂O emissions by stimulating nitrification and denitrification processes. In addition, N₂O emissions depend on the input waste quality and increase significantly when legumes are inserted into cover plants. Moreover, analyzing biochar in rice, He et al. [52] suggested that the combination of biochar and HQ (urease inhibitor-hydroquinone) or the combined application of urease and nitrification inhibitors to soil enriched with biochar at least 1 year previously could be an effective practice for reducing NH₃ emissions and increasing rice yields.

Finally, microorganism respiration may also contribute to retaining N into the soil. By dissimilatory nitrate reduction to ammonium (DNRA), a respiratory process antagonistic to denitrification, nitrate is used by microorganisms, mainly *Bradyrhizobium* and *Mesorhizobium* bacteria, as electron acceptors. This process results in N retention and production of the less mobile ammonium cation (NH₄⁺), thereby reducing the contribution to the total N₂O pool [53]. In addition to N fixation, the potential N retention by microorganisms through DNRA becomes a relevant feature in the reduction of N losses by denitrification [54]. This suggests DNRA may act as a mechanism for conserving N in agricultural systems.

5. Soluble nitrogen

Soluble organic nitrogen is a labile source of N for microorganisms and is an important soluble N reservoir in agricultural soils. Plant species (associated or not with mycorrhizae) can directly uptake simple organic N present in the SON pool [55]. The SON pool is composed of high (protein oligomers), medium (small peptides) [56], and low molecular weight compounds (monomers such as amino acids) [57]. As plants uptake organic and inorganic N, the relative proportion of these different N sources in soils is a determinant of N management.

SON is suggested as a transitional phase during N transformation between soil organic matter and inorganic N (NH₄⁺-N) and considered an intermediate step in microbial mineralization of organic N [58]. The SON pool can regulate the N transformation rate in the soil, i.e., the ammonification and nitrification rates, affecting the substrate associated with different plant species. Thus, soil organic N fractions and SON pools are important indicators of soil fertility and plant nutrition requirements [59], inferring the potential supply of N mainly in low N mineralization soils [60].

Besides an important component of soil total soluble N, SON plays a key role in N cycling and therefore in determining soil N availability in agricultural systems [61]. The amount of SON represents a relatively high proportion of the total soluble nitrogen (TSN) pool. It has been reported that SON constitutes 17–90% and 32–50% of TSN in pasture and agricultural soils, respectively [46, 47, 62, 63]. Like in mineral N, SON dynamics are affected by mineralization, immobilization, leaching, and plant uptake, but its pool size is more constant than mineral N [64]. Although remains unclearly understood, SON is an important pool in N transformations and plant uptake.

Biotic and abiotic processes are involved in the SON generation in soil [58]. By biotic processes, SON can be produced directly from microbial turnover and indirectly through the microbial excretion of extracellular enzymes [61]. However, as plants and microorganisms can compete for soil organic N, it is also possible that SON reservoirs vary spatially due to the variation in activity and density of

microbial population between different types of agricultural management. Zhang et al. [65] reported that SON fractions were significantly and positively correlated with the no-tillage system practices and that this agricultural system is beneficial and effective for increasing soil N turnover.

Proteins are the most abundant nitrogen compounds in SON. Depolymerization of these organic macromolecules in monomeric SON (amino acids) can be considered rate-limiting for the total N cycling in soils [66]. Soil amino acids can contribute, in relative and absolute terms, to the SON pool in agricultural soils, which was observed in soil under fertilized sugarcane [55]. Also, plants can use proteins as N source without the help of other organisms [67]. Although the relative contribution of amino acids to N supply for crops remains unclear, all studied plants have shown the ability to uptake and metabolize amino acids as well as soils containing amino acids [68].

Organic agriculture practices can increase the content of SON, protein, and free amino acids in the soil as a result of frequent and long-term inputs of organic matter. In addition, agricultural production quantity may also influence the SON pool abundance. However, the effect of organic cultivation on specific free amino acids and protein pools remains unclear [66].

Soil organic matter, pH, total C, total N, and C/N ratio are the main factors affecting soil SON abundance. SON dynamics can be significantly affected by mineralization and immobilization during microbial growth and decomposition of organic matter. Besides that, agricultural practices such as irrigation management, fertilization, plowing, harrowing, harvesting, and the plant growth stage can also play an important role in SON dynamics [59, 63]. Furthermore, high temperatures may increase the SON content by stimulating decomposition of organic matter [69]. Knowing the temporal dynamics of organic N pools in the soil may help to understand how these pools are affected by soil properties, climate and crop management, and whether SON can contribute to N supply of crops.

6. Biological nitrogen fixation

Nitrogen in the gaseous form (N_2) represents 78% of the atmospheric gases but is inert and unavailable to plants. Only nitrogen-fixing microorganisms, including bacteria, cyanobacteria, and fungi, are able to break the triple bond between the atoms ($N \equiv N$) of the atmospheric nitrogen, thus transforming it into ammonia (NH_3) through the nitrogenase enzyme ($N_2 + 8H^+ + 6e^- \rightarrow 2NH_3 + H_2$) [70]. Biological nitrogen fixation is a key component of the nitrogen cycle and responsible for most of the nitrogen available to plants.

BNF is performed by symbiotic, endophytic, or free-living microorganisms [71, 72]. Symbiotic bacteria associate with plants forming root nodules (rhizobia), where they fix nitrogen while benefiting from plant photoassimilates. It has been observed that this symbiosis occurs not only in plants from the Leguminosae family [71] but also in cereals such as rice, maize, and wheat from the Poaceae family [73]. BNF also occurs in nonsymbiotic associations. Endophytic bacteria colonize plant tissues and fix N while benefiting from plant photoassimilates, although the amount of N fixed is lower than in symbiosis [73, 74]. Also, free-living microorganisms inhabiting rhizosphere, soil region around plant roots, fix nitrogen while feeding on root exudates (amino acids, peptides, proteins, enzymes, vitamins, and hormones), which stimulate growth of diazotrophic bacteria from genera *Acetobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Paenibacillus*, and *Pseudomonas* [71].

Nitrogen-fixing microorganisms occur naturally in soil [71] and in water [72] or colonize seeds [74]. However, in the agricultural environment, conventional practices such as plowing, harrowing, chemical fertilization, and pesticide application reduce the soil microorganism populations, which make these areas depending on the application of nitrogen fertilizers [75, 76]. Chemical fertilizers require a great amount of energy to be produced, energy that is derived from fossil fuels. Moreover, they are potential soil and water contaminants and expensive and scarce for many developing country farmers [77]. Therefore, strategies have been studied to increase BNF by plants and thus reduce dependence on chemical fertilization.

Conservation practices such as minimum tillage, no tillage, and cover crops stimulate BNF as they increase the population and activity of soil microorganisms (bacteria, actinomycetes, and mycorrhizae) [78, 79]. In addition to capturing soil N, reducing N loss by leaching, and becoming an N source for succeeding crops, mixing cover crops (legumes and grasses) provide additional N through BNF [79, 80].

Another alternative for increasing BNF is to inoculate nitrogen-fixing microorganisms in crops. Inoculated into the seeds, roots, or leaves, these microorganisms may increase the formation of root nodules, stimulate root growth, improve nutrient uptake, stimulate antioxidant defense system, increase tolerance to biotic (pest and pathogen) and abiotic (drought and salinity) stresses, and thereby increase crop productivity. Inoculation of nodulating as well as endophytic fungi or bacteria stimulates growth in both legumes and grasses and represents a viable and sustainable alternative (**Table 1**). Among the most used microorganisms are *Rhizobium* and *Bradyrhizobium* genera bacteria inoculated in legumes and *Azospirillum* and *Enterobacter* genera in grasses (**Table 1**).

Studies also focus on the application of nitrogen-fixing microorganisms through irrigation water, on the genetic improvement for BNF by legume crops [96], on becoming plants able to self-fertilize by stimulating root fungal associations in grasses, and on providing cereals with the nitrogen-fixing enzyme (nitrogenase) [77]. Estimations indicate these practices can reduce fertilizer application costs by billions of dollars annually.

Crop	Scientific name	Inoculated microorganism	Reference
Rice	<i>Oryza sativa</i>	<i>Bacillus amyloliquefaciens</i> , <i>Enterobacter cloacae</i> , <i>Klebsiella variicola</i>	[81, 82]
Sugarcane	<i>Saccharum officinarum</i>	<i>Gluconacetobacter diazotrophicus</i> , <i>Herbaspirillum seropedicae</i> , <i>H. rubrisubalbicans</i> , <i>Burkholderia tropica</i> e <i>Azospirillum amazonense</i>	[83]
Cowpea	<i>Vigna unguiculata</i>	<i>Actinomadura</i> , <i>Bradyrhizobium elkanii</i> , <i>B. pachyrhizi</i> , <i>B. yuanmingense</i> , <i>Paenibacillus graminis</i> , <i>Rhizophagus irregularis</i>	[84–88]
Common bean	<i>Phaseolus vulgaris</i>	<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i> , <i>R. tropici</i>	[89]
Maize	<i>Zea mays</i>	<i>Azospirillum brasilense</i> , <i>Herbaspirillum seropedicae</i>	[90, 91]
Soybean	<i>Glycine max</i>	<i>Bradyrhizobium japonicum</i> , <i>Bacillus megaterium</i> , <i>Methylobacterium oryzae</i> ,	[92, 93]
Wheat	<i>Triticum aestivum</i>	<i>Azospirillum brasilense</i> , <i>A. insolitus</i> , <i>Enterobacter</i> sp., <i>Microbacterium arborescens</i> , <i>Serratia marcescens</i> , <i>Zoogloea ramigera</i>	[94, 95]

Table 1. Legume and cereal crops and nitrogen-fixing microorganisms used for inoculation.

7. Nitrogen and organic fertilization

The worldwide demand for organic foods, produced without the use of synthetic inputs, has driven the use of conservation practices, especially fertilization using organic wastes. The application of organic wastes to the soil improves soil fertility by increasing the organic matter (OM) and nutrient contents, such as N and phosphorus (P), and soil microbiota population, as well as improving the cation exchange capacity (CEC) [97].

Organic fertilization improves yield and quality of vegetables such as lettuce (*Lactuca sativa* L.) [98], tomato (*Solanum lycopersicum* Mill.) [99], and carrot (*Daucus carota* L.) [100]; fruits such as papaya (*Carica papaya* L.) [101], citrus (*Citrus* spp.) [102], and raspberry (*Rubus idaeus* L.) [103]; and annual crops such as maize (*Zea mays* L.) [104] and cowpea (*Vigna unguiculata* (L.) Walp) [105]. Most organic fertilizers used as N source are derived from (a) agricultural wastes (cattle, swine and poultry manure), slaughterhouses (bone and blood meal), composting, and vermicomposting; (b) agro-industrial wastes (oilseed pies, sugarcane bagasse, and vinasse) and biochar; and (c) household wastes and sewage sludge composting (Table 2).

N input by organic fertilizers occurs predominantly through mineralization of organic N, although some mineral N fractions may be released [107, 119]. The organic N mineralization rate is regulated by N fractions and C/N ratio of the decomposing waste, as well as by environmental temperature and humidity [120, 121]. Under favorable conditions, high N content organic fertilizers mineralize quickly similarly to synthetic fertilizers, while those with low N content and high C/N ratio mineralize slowly [122]. Thus, knowing the mineralization rate allows choosing the best organic fertilizer to be used in agriculture (Table 2).

Manures are the main used organic fertilizers worldwide, especially as N source, though the amount and quality of N in manure may vary according to animal species, age, and feed. Forage-based diets increase the residue production, although reduce the quality that is provided by a concentrate-based diet [97, 119]. Cattle, equine, sheep, goat, and swine manures present similar N content, ranging from 0.77 to 3.90%. In its turn, poultry litter may have 2.80–4.60% N content, due to concentrate-based feed supplied to poultries, being a fast mineralizing fertilizer [106, 107]. Thus, manure fertilization has been efficient for many crops, such as sweet pepper (*Capsicum annuum* L.) [123] and radish (*Raphanus sativus* L.) [124].

Residues from the castor bean (*Ricinus communis* L.; *Euphorbiaceae*) chain stand out due to the high N content which is found in the pie (7.54% N), in the oil extraction residue (12.82% N), and in the pulp from direct oil transesterification for biodiesel production [106, 125–127]. Castor pie mineralization rate is more intense than in other composts and thus quickly releases N and other readily available nutrients to plants. As reported by [126], evaluating microbial respiration, who obtained mineralization rates 6 times faster than those obtained in cattle manure and 14 times faster than in sugarcane bagasse, other pies, such as peanut (*Arachis* spp.) and cotton (*Gossypium* spp.), may also have high N (4.0–7.0%) content and similar mineralization characteristics [128, 129].

The product obtained from the composting of organic wastes is rich in stable organic matter. Wastes are transformed through biological decomposition, and the process is affected by environmental conditions and N content. As nitrogen compounds are food for microbiota, N deficiency in waste may retard the maturation process, and the excess may increase the N volatilization as ammonia (NH₃), consequently affecting N stabilization processes in composting [130]. Also, humus from vermicomposting (usually by using *Eisenia foetida* species) is highly stable and presents high contents of N and humic acids, which indicate a better relationship between the mineralization and humification processes of OM, with decreasing C/N ratio [115, 131].

Source	N content (%)	C/N ratio	Reference
Cattle manure	0.8–3.2	16.0–21.0	[97, 106]
Equine manure	1.4–3.9	21.9–25.0	[97, 107]
Sheep manure	1.2–1.8	9.0–29.0	[108, 109]
Swine manure	1.9–2.8	10.0–12.0	[97, 107]
Poultry litter	2.8–4.6	4.2–22.0	[97, 106, 107]
Blood meal ¹	11.8–12.9	—	[110, 111]
Bone meal	4.1–4.2	4.0–7.0	[97, 112]
Meat and bone meal	5.5–6.6	6.0	[106, 112, 113]
Castor pulp ¹	12.8	—	
Castor pie	5.2–7.5	6.0–9.0	[97, 106, 112]
Cotton pie ¹	4.5	—	[106]
Filter pie	1.5–1.8	21.0–24.0	[97, 112]
Sugarcane bagasse	0.9–1.5	85.0	[106, 111, 114]
Vinasse	0.3–1.2	4.0–17.0	[97, 112]
Compost	0.7–2.6	11.3–64.0	[107, 115]
Humus	1.3–2.6	11.0–34.0	[115, 116]
Millicompost	2.0–2.2	15.0–19.0	[98, 117]
Biochar	0.1–5.0	7.0–400.0	[118]
Sewage sludge	0.8–3.5	9.0–50.0	[97, 112]
Household waste	0.9–2.6	7.0–27.0	[97, 107, 112]

¹C/N ratio not found.

Table 2.
Nitrogen content and carbon/nitrogen ratio (C/N) in organic fertilizers.

In addition to the earthworms, arthropods that constitute the edaphic macrofauna [87, 132, 133] are also of great interest. Millipedes (Myriapoda: Diplopoda) fragment and feed on organic wastes and excrete low C/N ratio feces (2.2% N) producing the millicompost [134–136]. Studies suggest that millicompost is similar to vermicompost and commercial substrates in relation to N supply and other macro- and micronutrients for seedling production, such as in lettuce (*Lactuca sativa*) [98] and pitaya (*Hylocereus* spp.) (Cactaceae) [137].

In relation to slow-release organic fertilizers, biochar is an alternative. A by-product from carbonization (pyrolysis) of biomass under low-oxygen atmosphere, biochar is fine-grained carbonaceous material with decomposition resistance [118]. N content in biochar depends on the source material (biomass) as well as on the pyrolysis temperature. Biochars from wood have high C/N ratio and low N content (0.1%), while those from manures have low C/N ratio and high N content (5.0%). For instance, biochar from eucalyptus wood (*Eucalyptus urophylla* S. T. Blake and *Corymbia citriodora* (Hook.) K.D. Hill and L.A.S. Johnson) contains 0.66 and 0.48% N, respectively, while from coffee husks (*Coffea* spp.) contains 2.74% N [138]. Besides slowly releasing nutrients, the use of biochars increases N uptake via ion exchange and NH₃ removal by adsorption, stimulates immobilization (reducing NO₃⁻ losses), and reduces N₂O emissions [139–142]. Moreover, biochar improves mycorrhizal associations and nitrogen biological fixation [118].

Urban wastes have also been used in agriculture. Sewage sludge showed to be an excellent N source (0.80 and 3.47% N) besides slowly mineralizing. N mineralization rates from 20 to 38% were found after 105 days [143], which depends on source material characteristics and treatment processes as well as on heavy metal content that accelerate or limit mineralization [107, 144]. Slaughterhouse residues, such as bone and blood meal, present high N rates, but they are not yet used in agriculture because studies on its adoption and behavior as organic fertilizer are scarce [97, 110, 112].

8. Concluding remarks

In the surface layer of most soils, the soil organic N can be divided into two categories: N from organic residues and N from soil organic matter or humus. N mineralization and immobilization processes occur simultaneously and oppositely in the soil. The net balance between these processes is controlled by several factors such as environmental conditions, soil physicochemical factors, agricultural management adopted, quality of the decomposing residues, and content of easily decomposable and recalcitrant fractions. As organic agriculture increases soluble organic nitrogen content, this fraction has been extensively studied. Also, being biological nitrogen fixation a key component of the nitrogen cycle and responsible for most of the nitrogen available to plants, it was also discussed in this chapter.

Finally, we discussed nitrogen and organic fertilization, since the worldwide demand for organic foods produced without the use of synthetic inputs has driven the use of conservation practices, especially fertilization using organic wastes. Most organic fertilizers used as N source is derived from agricultural and agro-industrial wastes, slaughterhouse wastes, composting and vermicomposting, biochars, household wastes, and sewage sludge composting.

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Nitrogen Fertilization II: Management Practices to Sustain Crop Production and Soil and Environmental Quality

Upendra M. Sainju, Rajan Ghimire and Gautam P. Pradhan

Abstract

Improved management practices can be used to sustain crop yields, improve soil quality, and reduce N contaminations in groundwater and the atmosphere due to N fertilization. These practices include crop rotation, cover cropping, application of manures and compost, liming, and integrated crop-livestock system. The objectives of these practices are to reduce the rate of N fertilization, enhance N-use efficiency, increase crop N uptake, promote N cycling and soil N storage, and decrease soil residual N. This chapter discusses improved management practices to reduce N fertilization rate, sustain crop yields, and improve soil and environmental quality. The adaptation of these practices by farmers, producers, and ranchers, however, depends on social, economic, soil, and environmental conditions.

Keywords: crop yields, environmental quality, management practices, nitrogen fertilizer, nitrogen-use efficiency, soil quality

1. Introduction

Legume-integrated crop rotations provide opportunity to reduce N fertilizer rates due to increased N supply by legume residues to succeeding crops compared with nonlegume monocropping [1, 2]. As little or no N fertilizer is applied to legumes during their growth, inclusion of legumes in rotation with nonlegumes helps to reduce the overall N rate for a crop rotation, which increase farm income by reducing C footprints and lowering the cost of N fertilization [1, 3]. Legumes also fix atmospheric N and release it for as long as 3 years, increasing yields of succeeding crops compared with nonlegume crops in crop rotations [4]. Crop rotations also reduce disease, pest, and weed infestations [5], improve soil structure and organic matter storage [6], increase water-use efficiency [7], and enhance soil health through microbial proliferation [8]. Crop rotation can also increase N uptake efficiency of diverse crops and reduce soil residual N compared with monocropping [2].

Cover cropping has many beneficial effects on sustaining crop yields and improving soil and environmental quality. Cover crops planted after the harvest of cash crops use soil residual N, reducing N leaching. The additional residues supplied by cover crops increase soil organic matter and fertility [9, 10]. Legume cover crops reduce N fertilization rates and enhance crop yields, but nonlegume cover crops are

more effective on enhancing C sequestration [11, 12]. Similarly, integrate crop-livestock system, while reducing feed cost and supplying meat, milk, and wood, enhances N cycling and soil fertility, and control weeds [13, 14].

Continuous application of NH_4 -based N fertilizers to nonlegume crops can reduce soil pH compared with legume-nonlegume crop rotations where N fertilizer is not applied to legumes [15]. After 16–28 years of management implications, soil pH was reduced by 0.22–0.42 from the original level in continuous nonlegumes compared with crop rotations containing legumes and nonlegumes [15]. Soil acidification from N fertilization to crops primarily results from (1) increased removal of basic cations, such as calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na) in crop grains and stover due to increased yield; (2) leaching of soil residual $\text{NO}_3\text{-N}$, Ca, and Mg; and (3) microbial oxidation (or nitrification) of NH_4 -based N fertilizers that release H^+ ions [16]. Alkalinity produced during plant uptake of N or conversion of inorganic N to organic form, however, can partly or wholly counter the acidity from nitrification [17]. Increased toxicity of aluminum (Al), iron (Fe), and manganese (Mn) and reduced availability of most nutrients, such as P, Ca, Mg, K, and Na, during acidification can reduce crop growth and yield [18].

Here we discuss various management strategies to reduce N fertilization rates, increase N-use efficiency, and decrease N leaching and N_2O emissions due to N fertilization. These practices will reduce the cost of N fertilization while sustaining crop production and reducing soil and environmental degradation.

2. Management practices

Management practices that reduce N fertilization rates without affecting crop yields and quality are needed to reduce soil and environmental degradation, as soil degradation is directly related to increased N rates. Some of these practices include crop rotation, cover cropping, application of manure and compost, and integrated crop-livestock system. These practices can increase N inputs, reduce N fertilization rates, conserve soil organic matter, and enhance soil health and environmental quality without affecting crop yields compared with traditional management practices. We discuss these practices as follows.

2.1 Crop rotation

Crop rotations that include legumes and nonlegumes in the rotation can substantially reduce N fertilization rates compared with nonlegume monocropping because legumes supply N to the soil due to their greater N concentration from atmospheric N fixation than nonlegumes. As no N fertilizer is applied to legumes, overall N fertilization rate is lower for the legume-nonlegume rotation than continuous nonlegumes while still maintaining crop yields. Sainju et al. [19] observed that annualized crop biomass and grain yields under rainfed condition were similar or greater with legume-based rotations that included pea, durum (*Triticum turgidum* L.), canola (*Brassica napus* L.), and flax (*Linum usitatissimum* L.) than with continuous durum (**Table 1**). Crop rotation is an effective management practice to control weeds, diseases, and pests [7]; reduce the risk of crop failure, farm inputs, and duration of fallow; and improve the economic and environmental sustainability of dryland cropping systems [20]. Diversified crop rotations can efficiently use water and N compared with monocropping [7, 21]. For instance, wheat and barley can efficiently utilize soil water in wheat-pea and barley-pea rotations than continuous wheat and barley. This is because pea uses less water than wheat and barley, resulting in more water available for succeeding crops in the rotation [7, 21].

Crop rotation†	Annualized biomass yield (Mg ha ⁻¹)	Annualized grain yield (Mg ha ⁻¹)
CD	3.32b‡	1.77a
D-C-D-P	4.02a	1.76a
D-D-C-P	3.90a	1.70a
D-F-D-P	3.39b	1.63ab
D-D-F-P	3.56b	1.54b

†Crop rotations are CD, continuous durum; D-C-D-P, durum-canola-durum-pea; D-D-C-P, durum-durum-canola-pea; D-F-D-P, durum-flax-durum-pea; and D-D-F-P, durum-durum-flax-pea.
 ‡Numbers followed by different letters within a column are significantly different at $P \leq 0.05$ by the least square means test.

Table 1. Effect of crop rotation on average annualized crop biomass (stems and leaves) and grain yields of durum, canola, flax, and pea from 2006 to 2011 in eastern Montana, USA (Sainju et al., 2017d).

Crop rotation can enhance or maintain soil organic C and N levels compared to monocropping. Both soil C and N stocks can be influenced by the quality and quantity of residue returned to the soil from crops involved in the rotation [12, 22]. Crop rotation can sequester C at $200 \pm 120 \text{ kg C ha}^{-1} \text{ year}^{-1}$, reaching equilibrium in 40–60 years compared with monocropping [23]. Sainju [24] found that soil organic C at 0–5 and 5–10 cm was similar in no-till malt barley-pea rotation (NTB-P) and no-till continuous malt barley (NTCB), both of which had greater soil organic C than no-till malt barley-fallow (NTB-F) and conventional till malt barley-fallow (CTB-F) due to greater amount of crop residue returned to the soil and reduced mineralization of soil organic matter (**Figure 1**). Similarly, Sainju et al. (2017d) found that soil total C at 0–125 cm was similar to continuous durum and rotations that included durum, canola, pea, and flax, except D-D-F-P (**Table 2**). Soil total N at 0–120 cm was greater with spring wheat-pea rotation than continuous spring wheat (**Table 3**) [25].

In an experiment evaluating the effects of crop rotation and cultural practice (traditional and ecological) on N balance in dryland agroecosystems, Sainju et al. [26, 27] observed that N fertilization rates were lower with legume-based crop rotations (D-C-D-P, D-D-C-P, D-F-D-P, and D-D-F-P) than nonlegume monocropping (CD) (**Table 4**). Traditional cultural practices included conventional till, recommended seed rate, broadcast N fertilization, and reduced stubble height and ecological practices included no-till, increased seed rate, banded N fertilization, and increased stubble height. They found that both total N input and output were greater with legume-based rotations than nonlegume monocropping due to pea N fixation and increased grain N removal. As a result, N balance was positive, indicating N surplus in legume-based rotations, and negative, indicating N deficit in nonlegume monocropping. This suggests that external N input is lower to sustain crop yields in legume-based crop rotations than nonlegume monocropping.

Legume-nonlegume rotation can also resist soil acidification compared with continuous nonlegumes. Sainju et al. [18] reported that soil pH at 0–7.5 cm after 30 years of experiment initiation was 0.13–0.44 greater and at 7.5–15.0 cm was 0.11–0.29 greater with spring wheat-barley/pea rotation (FSTW-B/P) than continuous spring wheat (NTCW, STCW, and FSTCW) (**Table 5**). They explained this as a result of lack of N fertilization to pea and reduced N fertilization rate to spring wheat following pea whose residue supplied N to spring wheat because of higher M concentration than spring wheat and barley residues. Soil residual NO₃-N, which can pollute groundwater through leaching, was lower with legume-based crop rotations containing durum, canola, pea, and flax than continuous durum (**Table 6**), suggesting that legume-based crop rotations can reduce N fertilization rate and the potential for N leaching compared with nonlegume monocropping.

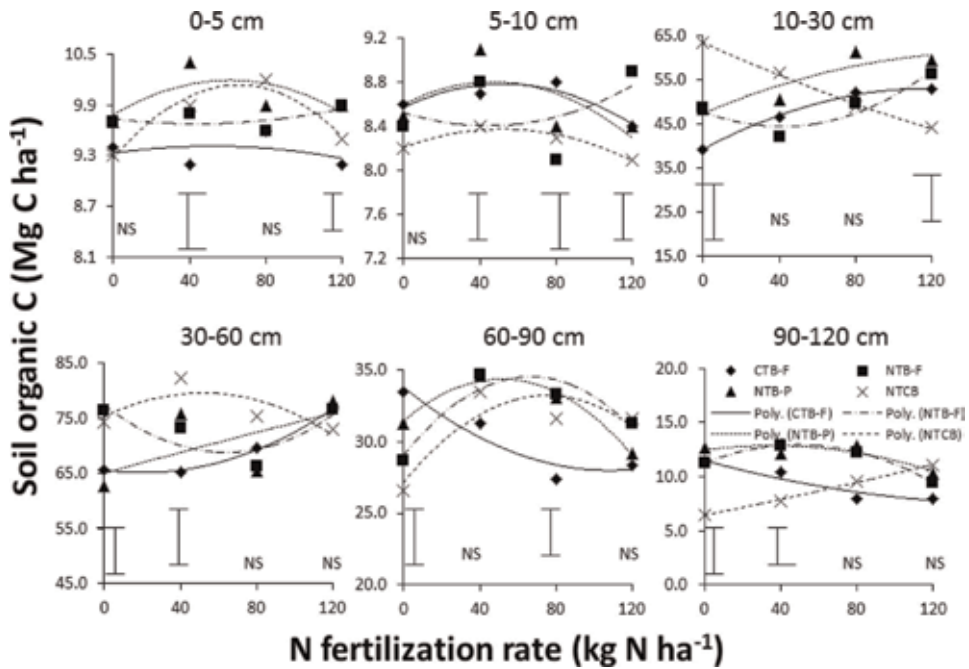


Figure 1. Soil organic C at the 0–120 cm depth as affected by 6 years of N fertilization rates to malt barley in various cropping systems in eastern Montana, USA. CTB-F denotes conventional till malt barley-fallow; NTB-F, no-till malt barley-fallow; NTB-P, no-till malt barley-pea; and NTCB, no-till continuous malt barley. Vertical bars denote least significant difference between tillage and cropping sequence treatments within a N rate at $P = 0.05$ [24].

Crop rotation†	STC at 0–125 cm (Mg C ha ⁻¹)
CD	394.6a‡
D-C-D-P	395.4a
D-D-C-P	387.1a
D-F-D-P	395.4a
D-D-F-P	370.2b

†Crop rotations are CD, continuous durum; D-C-D-P, durum-canola-durum-pea; D-D-C-P, durum-durum-canola-pea; D-F-D-P, durum-flax-durum-pea; and D-D-F-P, durum-durum-flax-pea.

‡Numbers followed by different letters within a column are significantly different at $P \leq 0.05$ by the least square means test.

Table 2. Soil total C (STC) at the 0–125 cm depth after 6 years as affected by crop rotation in eastern Montana, USA [19].

2.2 Cover cropping

Cover crops have been grown successfully in regions with mild winter to provide vegetative cover for reducing soil erosion. Cover crops are usually grown in the fall after the harvest of summer cash crops and have many benefits for sustaining crop yields and improving soil and water quality. Winter cover crops use soil residual N that may otherwise leach into groundwater after crop harvest in the fall, thereby reducing soil profile $\text{NO}_3\text{-N}$ content and N leaching [29, 30]. Summer cover crops are grown in the summer to replace fallow when no other crops are grown. Depending on the species, cover crops can maintain or increase soil organic C and N

Crop rotation ^a	STN (Mg N ha ⁻¹)							
	0–5 cm	5–10 cm	10–20 cm	20–40 cm	40–60 cm	60–90 cm	90–120 cm	0–120 cm
CW	0.82	0.91	1.46	2.34b ^b	2.11	2.29b	2.11	12.03b
W-P	0.85	0.90	1.53	2.66a	2.24	2.55a	2.23	12.96a
W-B-P	0.79	0.86	1.44	2.43ab	2.17	2.35b	2.22	12.17b
W-B-C-P	0.81	0.88	1.47	2.54a	2.26	2.51a	2.10	12.62ab

^aCrop rotations are CW, continuous spring wheat; W-P, spring wheat-pea; W-B-P, spring wheat-barley hay-pea; and W-B-C-P, spring wheat-barley hay-corn-pea.

^bNumbers followed by different letters within a column are significantly different at $P \leq 0.05$ by the least square means test.

Table 3.

Soil total N (STN) at the 0–120 cm depth after 6 years as affected by crop rotation in eastern Montana, USA [25].

by providing additional crop residue which increases biomass C and N inputs to the soil [9, 10, 12] and sequester atmospheric C and/or N, thereby reducing the rate of N fertilization to summer crops [9, 10]. Other benefits of cover crops include increased soil aggregation and water infiltration capacity [31], improved water holding capacity [32], and reduced soil erosion [33] compared with no cover crop.

Integrating legumes in crop rotations can supply N to succeeding crops and increase crop yields compared to nonlegumes or no cover crop rotations [10]. In contrast, nonlegume cover crops are effective in increasing soil organic C through increased biomass production compared with legumes or no cover crop [9, 10, 12]. Nonlegumes also reduce NO₃-N leaching from the soil profile better than legumes, or no cover crop do [29]. As none of the cover crops are effective enough to provide most of these benefits, i.e., to supply N, sustain crop yields, increase soil organic matter, and reduce N leaching, a mixture of legume and nonlegume cover crops is ideal to supply both C and N inputs in adequate amounts that help to improve soil and water quality by increasing organic matter content and the potential for reducing N leaching compared with legumes and increase crop yields compared with nonlegumes [12, 34, 35].

Sainju et al. [36] found higher biomass yield with hairy vetch/rye (*Secale cereale* L.) mixture than rye, hairy vetch, or winter weeds, and N concentration in the mixture similar to hairy vetch, except in 2001 (Table 7). As a result, they observed greater biomass C and N contents with hairy vetch/rye mixture than rye and winter weeds and similar to or greater than hairy vetch. The C/N ratio of cover crop biomass, which measures the decomposition rate of the residue, was similar between hairy vetch/rye mixture and hairy vetch.

Because of increased C supply, soil organic C at 0–10 and 10–30 cm was also greater with hairy vetch/rye than other cover crops (Figure 2). At 30–60 cm, soil organic C was greater with hairy vetch/rye than other cover crops, except hairy vetch. Soil total N at 0–15, 15–30, and 0–120 cm was also greater with hairy vetch and hairy vetch/rye mixture than other cover crops (Figure 3). Similarly, soil residual NO₃-N content at 0–120 cm was greater with hairy vetch than other cover crops and is slightly greater than that with 120–130 kg N ha⁻¹ (Figure 4). Nitrogen loss at 0–120 cm during the winter fallow period from November to April was lower with hairy vetch/rye than other cover crops (Table 8). Nitrogen fertilizer equivalence of rye and winter weeds for cotton and sorghum ranged from –129 to 69 kg N ha⁻¹, but those of hairy vetch and hairy vetch/rye ranged from 92 to 220 kg N ha⁻¹ (Table 9), suggesting that hairy vetch and hairy vetch/rye can increase cotton and sorghum yields similar to those by 92–220 kg N ha⁻¹ [11]. These results suggest that hairy vetch/rye mixture can produce crop yields similar to hairy vetch.

Parameter	Traditional (kg N ha ⁻¹ year ⁻¹)					Ecological (kg N ha ⁻¹ year ⁻¹)				
	CD ^a	D-C-D-P ^a	D-D-C-P ^a	D-F-D-P ^a	D-D-F-P ^a	CD	D-C-D-P	D-D-C-P	D-F-D-P	D-D-F-P
N inputs										
N fertilization rate	83A ^b	62B	59B	52B	54B	87A	60B	63B	55B	56B
Pea N fixation	0C	84AB	76B	80AB	75B	0C	84AB	78B	87A	82AB
Atmospheric N deposition	14	14	14	14	14	14	14	14	14	14
N added by crop seed	3	3	3	3	3	3	3	3	3	3
Nonsymbiotic N fixation	5	5	5	5	5	5	5	5	5	5
Total N input	105B	167A	156A	154A	150A	109B	166A	162A	164A	159A
N outputs										
Grain N removal	49B	62A	57AB	54AB	55AB	52AB	65A	64A	63A	54AB
Denitrification	12	10	9	8	9	13	9	10	9	9
Ammonia volatilization	12	9	9	8	8	13	9	9	8	8
Plant senescence	5	7	6	6	6	6	7	7	7	6
N leaching	9	12	12	12	12	9	12	12	12	12
Gaseous N (NO _x) emissions	2	3	3	3	3	2	3	3	3	3
Surface runoff	1	2	1	1	1	1	2	2	2	2
Total N output	91B	105A	98AB	92B	94AB	96AB	107A	107A	103A	94AB
Changes in N level ^c	14B	62A	58A	62A	56A	13B	59A	55A	61A	65A
N sequestration rate (0–125 cm) ^d	50	45	42	46	43	52	48	46	44	40
N balance ^e	-36 (±11)B	17 (±5)A	16 (±4)A	16 (±4)A	13 (±3)A	-39 (±12)B	11 (±3)A	9 (±2)A	17 (±4)A	25 (±5)A

^aCrop rotation are CD, continuous durum; D-C-D-P, durum-canola-durum-pea; D-D-C-P, durum-durum-canola-pea; D-F-D-P, durum-flax-durum-pea; and D-D-F-P, durum-durum-flax-pea.
^bNumbers followed by the same letter within a row are not significantly different at P ≤ 0.05.
^cChanges in N level = total N input – total N output.
^dDetermined from the linear regression analysis of soil total N (STN) at 0–125 cm from the year 2005 to 2011.
^eN balance = changes in N levels – N sequestration rate (0–125 cm).

Table 4. Annual N balance due to the difference between total N inputs and outputs and N sequestration rate under dryland agroecosystems from 2005 to 2011 in eastern Montana, USA [26, 27].

Tillage and cropping sequence ^a	Soil depth					
	0–7.5 cm	7.5–15 cm	15–30 cm	30–60 cm	60–90 cm	90–120 cm
pH						
NTCW	5.33ab ^b E ^c	6.50abD	7.60C	8.35B	8.58A	8.75A
STCW	5.05bE	6.15bD	7.58C	8.25B	8.63A	8.70A
FSTCW	5.02bE	6.33bD	7.80C	8.30B	8.68AB	8.73A
FSTW-B/P	5.46aE	6.44bD	7.60C	8.15B	8.51A	8.59A
STW-F	5.73aE	7.03aD	7.65C	8.25B	8.50AB	8.66A
<i>Contrast</i>						
NT vs. T	0.29	0.26	–0.09	0.08	–0.08	0.04
CW vs. W-F	–0.68***	–0.88**	–0.08	0.01	0.13	0.04
CW vs. W-B/P	–0.43*	–0.11	0.20	0.15	0.16	0.14
Buffer pH						
NTCW	6.45bE	7.10abD	7.43C	7.60B	7.70AB	7.73A
STCW	6.38bE	7.00bD	7.43C	7.58B	7.68A	7.70A
FSTCW	6.43bE	7.05bD	7.45C	7.60B	7.70AB	7.73A
FSTW-B/P	6.66aD	7.13abC	7.44B	7.58B	7.69AB	7.70A
STW-F	6.80aE	7.24aD	7.44C	7.59B	7.66AB	7.72A
<i>Contrast</i>						
NT vs. T	0.05	0.08	–0.01	0.01	0.01	0.01
CW vs. W-F	–0.43***	–0.24**	–0.01	–0.01	0.01	–0.01
CW vs. W-B/P	–0.24*	–0.08	–0.01	0.03	0.01	0.03

^aSignificant at $P = 0.05$.
^{**}Significant at $P = 0.01$.
^{***}Significant at $P = 0.001$.
^aFSTCW, fall and spring till continuous spring wheat; FSTW-B/P, fall and spring till spring wheat-barley (1994–1999) followed by spring wheat-pea (2000–2013); NTCW, no-till continuous spring wheat; STCW, spring till continuous spring wheat; and STW-F, spring till spring wheat-fallow. CW represents continuous wheat; NT, no-till; T, till; W-B/P, spring wheat-barley/pea; and W-F, spring wheat-fallow.
^bNumbers followed by the same lowercase letter within a column among treatments in a set are not significantly different at $P \leq 0.05$.
^cNumbers followed by the same uppercase letter within a row among soil depths in a set are not significantly different at $P \leq 0.05$.

Table 5. Effect of tillage and crop rotation combination on soil pH and buffer pH at the 0–120 cm depth after 30 years of experiment initiation in eastern Montana, USA [18].

The mixture can also increase soil organic matter and reduce N fertilization rate and the potential for N leaching compared with rye and winter weeds. Therefore, legume-nonlegume cover crop mixture can provide several benefits, such as reducing the cost of N fertilization, maintaining crop yields, enhancing soil organic matter, and reducing N leaching compared with either cover crop alone or no cover crop.

2.3 Application of manure and compost

Manure and compost are rich sources of nutrients, and their application can increase soil organic C and total N, improving soil quality and crop production compared to no fertilizer application [37, 38]. Sainju et al. [39, 40] compared soil organic C and total N after 10 years of poultry litter with inorganic N

Crop rotation ^a	NO ₃ -N content at various depths (kg N ha ⁻¹)						
	0–5 cm	5–10 cm	10–20 cm	20–50 cm	50–88 cm	88–125 cm	0–125 cm
CD	2.47a ^b	1.81a	2.43a	8.49a	9.37a	9.17a	33.87a
DCDP	1.82a	1.22b	1.94b	6.47a	7.77a	6.71b	26.32b
DDCP	1.86a	1.19b	1.93b	5.97a	8.07a	6.38b	25.59b
DFDP	1.90a	1.37b	2.20a	6.59a	9.62a	8.64ab	30.60a
DDFP	1.74a	1.28b	2.29a	6.27a	8.63a	6.65b	27.02b

^aCrop rotations are CD, continuous durum; DCDP, durum-canola-durum-pea; DDCP, durum-durum-canola-pea; DDFP, durum-durum-flax-pea; and DFDP, durum-flax-durum-pea.
^bNumbers followed by different letters within a column are significantly different at $P \leq 0.05$ by the least square means test.

Table 6.

Soil NO₃-N content at the 0–125 cm depth as affected by crop rotation and cultural practice averaged across years from 2006 to 2011 in eastern Montana, USA [28].

Cover crop†	Biomass yield (Mg ha ⁻¹)	Concentration		Content		C/N ratio
		C (g kg ⁻¹)	N (g kg ⁻¹)	C (kg ha ⁻¹)	N (kg ha ⁻¹)	
2000						
Weeds	1.65d‡	370b	15b	587d	25d	24b
Rye	6.07b	430a	15b	2670b	68c	29a
Vetch	5.10c	394ab	33a	2006c	135b	12c
Vetch/rye	8.18a	366b	38a	3512a	310a	10c
2001						
Weeds	0.75d	391b	20b	277d	15b	20c
Rye	3.81b	448a	8d	1729b	32b	57a
Vetch	2.44c	398b	32a	964c	76a	12c
Vetch/rye	5.98a	434a	14c	2693a	84a	32b
2002						
Weeds	1.25c	375b	18b	476c	23b	21b
Rye	2.28b	434a	11b	986b	25b	40a
Vetch	5.16a	361b	36a	2094a	167a	10c
Vetch/rye	5.72a	381b	33a	2260a	186a	11c

†Cover crops are rye, cereal rye; vetch, hairy vetch; vetch/rye, hairy vetch and rye biculture; and weeds, winter weeds.
‡Numbers followed by the same letter within a column of a year are not significantly different at $P \leq 0.05$.

Table 7.

Effect of cover crop species on aboveground biomass yield and C and N contents in cover crops from 2000 to 2002 in central Georgia, USA [36].

fertilizer applications, both applied at 100 kg N ha⁻¹ to corn and cotton (Tables 10 and 11). They found that soil organic C and total N at 0–20 cm were greater with poultry litter application than inorganic N fertilization, regardless of tillage practices. As a result, poultry litter application sequestered C at 461 kg C ha⁻¹ year⁻¹ and N at 38 kg N ha⁻¹ year⁻¹ compared to 38 kg C ha⁻¹ year⁻¹ and 4 kg N ha⁻¹ year⁻¹, respectively, with N fertilization. As poultry litter also supplied C at 1.7 Mg C ha⁻¹ year⁻¹ [40] and only 60% of N from poultry litter was available

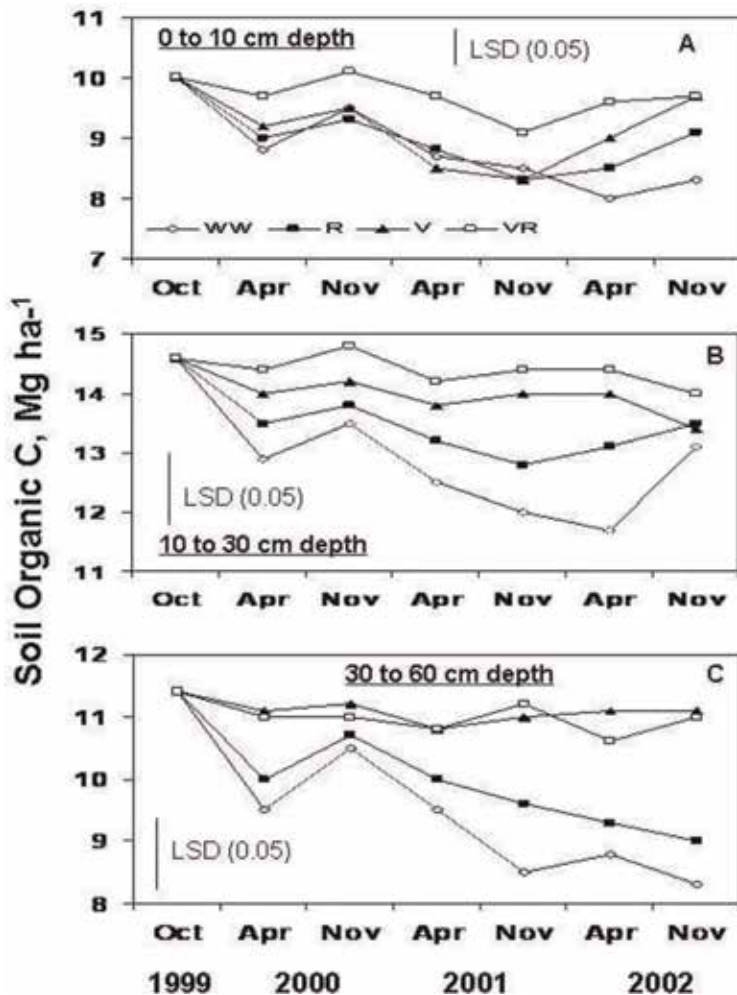


Figure 2. Effect of cover crop on soil organic C at the (A) 0-10 cm, (B) 10-30 cm, and (C) 30-50 cm depths in a chisel-tilled system (October 1999–November 2002, central Georgia, USA). R denotes cereal rye; V, hairy vetch; VR, hairy vetch and rye biculture; and WW, winter weeds. Vertical line with LSD (0.05) is the least significant difference between cover crops within a sampling date at $P = 0.05$ [12].

to crops in the first year [37], Sainju et al. [39, 40] reported that part of non-mineralized C and N from the litter converted to soil organic C and N, thereby increasing their levels with poultry litter application. In contrast, little or no C was supplied by inorganic N fertilizer, and most of N supplied by the fertilizer can either be taken up by the crop or lost to the environment through leaching, denitrification, and volatilization.

Because of lower N availability from poultry litter as a result of reduced N mineralization, total aboveground biomass and N uptake of corn, cotton, and rye cover crop were lower with poultry litter application than inorganic N fertilization (Table 12). Although soil health and quality can be improved with poultry litter application through organic matter enrichment, crop yields can be lower compared with N fertilization. For enhancing soil and environmental quality and sustaining crop yields, both inorganic N fertilizer and manure/compost should be applied as a mixture in balanced proportion as per crop demand after analyzing soil NO₃-N test to a depth of 60 cm. This could reduce N fertilization rate and undesirable consequences of N fertilization on soil and environmental quality.

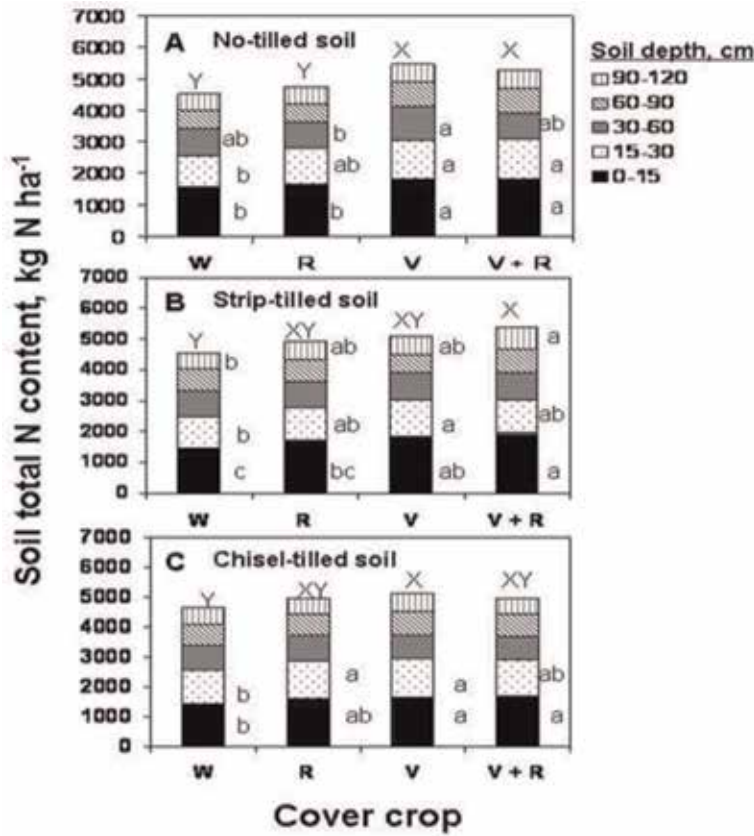


Figure 3.

Effect of cover crop on soil total N at the 0–120 cm depth in (A) no-tilled, (B) strip-tilled, and (C) chisel-tilled soils after 3 years in Central Georgia, USA. R denotes cereal rye; V, hairy vetch; V + R, hairy vetch and rye biculture; and WW, winter weeds. Bars followed by the same lowercase letter within a soil depth are not significantly different between cover crops at $P = 0.05$. Bars followed by the same uppercase letter at the top are not significantly different between cover crops at the 0–120 cm depth at $P \leq 0.05$ [34].

2.4 Integrated crop-livestock system

Integrated crop-livestock systems were commonly used to sustain crop and livestock products throughout the world before commercial fertilizers were introduced in 1950 [41]. The system is still common among producers in developing countries, especially in Africa and Asia where fertilizers are scarce and expensive [42, 43]. The integrated crop-livestock system has the potential to improve soil quality and sustain crop yields [41, 44]. The major benefits of the system are (1) production of crops, meat, and milk, (2) production of crop residue for animal feed, (3) production of manure to apply as fertilizer, (4) use of animals as draft power for tillage, and (5) control of weeds and pests [41, 42].

Animal grazing during fallow periods in wheat-fallow systems can be used to effectively control weeds [14] and insects, such as wheat stem saw fly [*Cephus cinctus* Norton (Hymenoptera: Cephidae)] [13]. The animal usually grazes on crop residues and weeds during the fallow period. Although grazing can reduce the quantity of crop residue returned to the soil, the number of animals grazed per unit area can be adjusted in such a way that crop residue cover in the grazing treatment will be similar to that in the conservation tillage system where soil erosion is minimal [14]. Animal feces and urine returned to the soil during grazing can enrich

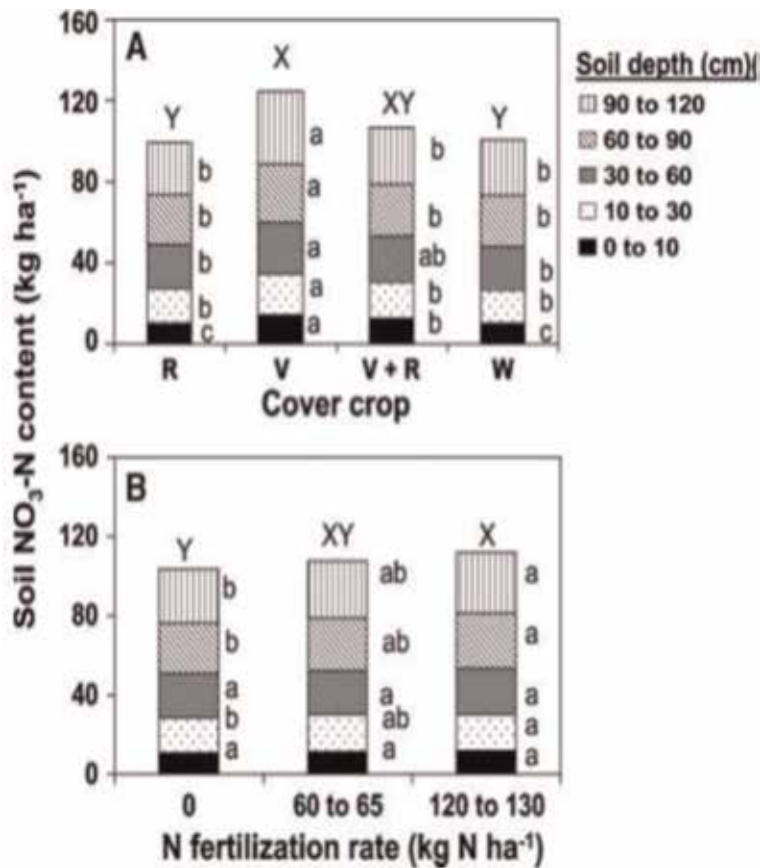


Figure 4. Effect of (A) cover crop and (B) N fertilization rate on soil $\text{NO}_3\text{-N}$ content at the 0–120 cm depth in Central Georgia, USA. R, denotes cereal rye; V, hairy vetch; V + R, hairy vetch and rye biculture; and W, winter weeds. Bars followed by the same lowercase letter within a soil depth are not significantly different between cover crops at $P = 0.05$. Bars followed by the same uppercase letter at the top are not significantly different between cover crops at the 0–120 cm depth at $P \leq 0.05$ [35].

Cover crop†	Total crop residue and soil N‡ (kg N ha ⁻¹)			Total crop residue and soil N§ (kg N ha ⁻¹)		
	November 2000	April 2001	Loss	November 2001	April 2002	Loss
Rye	5057bc¶	4888b	169b	4820b	4764b	56a
Vetch	5455a	5235a	220a	5323a	5244a	79a
Vetch/rye	5249ab	5141a	108c	5222a	5182a	40a
Weeds	4869c	4709b	160b	4725b	4649b	76a

†Cover crops are rye, cereal rye; vetch, hairy vetch; vetch/rye, hairy vetch and rye biculture; and weeds, winter weeds or no cover crop.

‡Include soil $\text{NH}_4\text{-N}$ + $\text{NO}_3\text{-N}$ + organic N contents at 0–120 cm, and N returned to the soil from cotton biomass (stems + leaves) in November 2000 and cover crop biomass in April 2001.

§Include soil $\text{NH}_4\text{-N}$ + $\text{NO}_3\text{-N}$ + organic N contents at 0–120 cm, and N returned to the soil from sorghum biomass (stems + leaves) in November 2001 and cover crop biomass in April 2002.

¶Numbers followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Table 8. Effect of cover crop on N loss from crop residue and soil N ($\text{NH}_4\text{-N}$ + $\text{NO}_3\text{-N}$ + organic N contents) at the 0–120 cm depth during the two winter seasons (from November 2000 to April 2001 and from November 2001 to April 2002) in central Georgia, USA [35].

Parameter	Cover crop				Regression analysis ^a	
	Winter weeds	Rye	Hairy vetch	Hairy vetch/rye	R ²	P
2000 cotton						
Lint yield	—	—	—	—	0.25	0.67
Lint N uptake	—	—	—	—	0.25	0.67
Biomass yield	-13	30	149	93	0.96	0.13
Biomass N uptake	-21	2	165	92	0.99	0.06
Soil inorganic N	-60	-190	220	140	0.64	0.40
2001 sorghum						
Lint yield	7	-64	107	179	0.96	0.12
Lint N uptake	25	-67	167	150	0.96	0.14
Biomass yield	32	-168	194	194	0.99	0.02
Biomass N uptake	69	-84	192	83	0.98	0.08
Soil inorganic N	59	12	116	71	0.86	0.25
2002 cotton						
Lint yield	—	—	—	—	0.28	0.82
Lint N uptake	—	—	—	—	0.24	0.87
Biomass yield	-21	-61	139	205	0.96	0.12
Biomass N uptake	-35	-13	134	160	0.97	0.11
Soil inorganic N	-74	5	176	160	0.70	0.37

^aRegression analysis of N fertilization rates versus cotton and sorghum yields and N uptake and soil inorganic N.

Table 9.

Nitrogen fertilizer equivalence (kg N ha^{-1}) of cover crops and soil inorganic N ($\text{NH}_4\text{-N} + \text{NO}_3\text{-N}$) content at the 0–30 cm depth for cotton and sorghum yields and N uptake from 2000 to 2002 in central Georgia, USA [11].

soil nutrients, improve soil quality, and increase crop yields [44]. The distribution of feces and urine by animals during grazing at the soil surface can be uneven; however, distribution can be more uniform with sheep than with cattle grazing [45].

Hatfield et al. [14] reported that sheep grazing during fallow did not affect soil organic matter and nutrient levels compared to the non-grazed treatment in the North Central Montana. Sheep grazing can increase soil bulk density and extractable P and grass yields compared to cattle grazing [45]. Snyder et al. [46] found similar or greater wheat grain yields with and without animal grazing. Similarly, Quiroga et al. [47] observed that 10 years of cattle grazing did not alter soil P concentration in Argentina. In contrast, Niu et al. [48] in Australia observed greater soil P and K concentrations in sheep camping than in non-camping sites due to increased animal excreta. Cattle and sheep grazing in the pasture can increase soil P and K concentrations compared to non-grazing [45].

Sainju et al. [49] reported that annualized wheat grain and biomass yields were lower with spring wheat-fallow and winter wheat-fallow rotations than continuous spring wheat due to the absence of crops during the fallow period (Table 13). In

Tillage [†]	N source [‡]	SOC concentration (g C kg ⁻¹)		SOC content (Mg C ha ⁻¹)	Changes in SOC from 1996 to 2006 (Mg C ha ⁻¹)	C sequestration rate (kg C ha ⁻¹ year ⁻¹)
		100 kg N ha ⁻¹	0–10 cm	10–20 cm	0–20 cm	0–20 cm
NT	AN	13.5	11.0	40.1	1.47	147
	PL	15.9	10.5	43.7	5.10	510
MT	AN	15.9	11.0	42.6	3.97	397
	PL	15.4	10.6	42.2	3.63	363
CT	AN	14.3	10.7	37.4	-1.20	-120
	PL	15.3	11.8	43.7	5.10	510
LSD (0.05)		—	—	3.1	3.1	310
Means	AN	14.6a [§]	10.9a	40.0b	1.41b	141b
	PL	15.6a	11.0a	43.2a	4.61a	461a

[†]Tillage is CT, conventional till; MT, mulch till; and NT, no-till.

[‡]N source is AN, NH₄NO₃; and PL, poultry litter.

[§]Numbers followed by different letters within a column in a set are significantly different at $P \leq 0.05$ by the least square means test.

Table 10.
 Effect of tillage and N source on soil organic C (SOC) at the 0–20 cm depth after 10 years in Alabama, USA [40].

Tillage ^a	N source ^b	STN concentration (g N kg ⁻¹)		STN content (Mg N ha ⁻¹)	Change in STN from 1996 to 2006 (Mg N ha ⁻¹)	N sequestration rate (kg N ha ⁻¹ year ⁻¹)
		(100 kg N ha ⁻¹)	0–10 cm	10–20 cm	0–20 cm	0–20 cm
NT	AN	1.23	1.03	3.44	-0.23	-23
	PL	1.52	1.02	4.19	0.49	49
MT	AN	1.42	1.01	3.84	0.15	15
	PL	1.49	0.92	3.91	0.21	21
CT	AN	1.31	0.98	3.67	-0.03	-3
	PL	1.51	1.04	4.11	0.41	41
LSD (0.05) ^c		—	—	0.24	0.24	24
Means	AN	1.55b ^d	1.59a	3.65b	-0.04b	-4b
	PL	1.65a	1.59a	4.07a	0.38a	38a

^aTillage is CT, conventional till; MT, mulch till; and NT, no-till.

^bN source is AN, ammonium nitrate; and PL, poultry litter.

^cLeast significant differences between treatments at $P = 0.05$.

^dNumbers followed by the same letter within a column in a set are not significantly different at $P \leq 0.05$.

Table 11.
 Effects of tillage and N source on soil total N and N sequestration rate at the 0–20 cm depth after 10 years in Alabama, USA [39].

Cropping system	N source	Total crop biomass	Total N uptake
	100 kg N ha ⁻¹	(Mg ha ⁻¹)	(kg N ha ⁻¹)
Rye/cotton-rye/cotton-corn		137.0a†	1544a†
Cotton-cotton-corn		110.2b	1247b
	NH ₄ NO ₃	133.3a	1502a
	Poultry litter	111.8b	1289b

†Numbers followed by the same letter within a column in a set are not significantly different at $P \leq 0.05$.

Table 12.

Effects of cropping system and N source on total biomass (stems + leaves) residues of rye, cotton, and corn and N uptake from 1997 to 2005 in Alabama, USA [39, 40].

Year	Cropping sequence† (Mg ha ⁻¹)			Weed management‡ (Mg ha ⁻¹)			Mean
	CSW	SW-F	WW-F	Chem.	Mech.	Graz.	
Annualized grain yield							
2004	5.55a§A¶	2.90aC	3.53aB	3.92aA	4.01aA	4.05aA	3.99a
2005	2.68bA	1.83bB	1.15cC	1.84cA	1.92bA	1.90bA	1.89b
2006	2.57bA	1.45cB	1.70 dB	1.89cA	1.90bA	1.92bA	1.90b
2007	1.86cB	1.18cC	2.95bA	1.89cA	2.03bA	2.00bA	2.00b
2008	2.61bA	1.56bcC	2.22cB	2.09bA	2.17bA	2.14bA	2.13b
Mean	3.05A	1.78C	2.31B	2.32A	2.42A	2.40A	
Annualized biomass yield							
2004	6.60aA	3.10aC	3.57aB	3.61aAB	3.41aB	3.89aA	4.42a
2005	3.28bA	1.65bB	1.94bcB	2.52bA	2.17bcA	2.19bA	2.29b
2006	2.96cA	1.57bcB	1.64cB	1.79bB	2.51bA	1.87bcB	2.06bc
2007	2.18dA	1.55bcB	2.25bA	1.78bA	2.21bcA	2.00bA	2.00c
2008	1.92dA	1.17cB	1.49cAB	1.08cB	1.91cA	1.58cA	1.53d
Mean	2.58A	1.49C	1.83B	1.79B	2.20A	1.91B	

†Cropping sequences are CSW, continuous spring wheat; SW-F, spring wheat-fallow; and WW-F, winter wheat-fallow.

‡Weed management practices are Chem., chemical where weeds were controlled with herbicide applications; Graz., grazing where weeds were controlled with sheep grazing; and Mech., mechanical where weeds were controlled with tillage.

§Numbers followed by the same lowercase letters within a column in a set are not significantly different at $P \leq 0.05$.

¶Numbers followed by the same uppercase letters within a row in a set are not significantly different at $P \leq 0.05$.

Table 13.

Effects of cropping sequence and weed management practice on annualized wheat grain and biomass (stems + leaves) yield from 2004 to 2008 in western Montana, USA [49].

contrast, wheat grain yield was not different among weed management practices where sheep grazing was used among one of the treatments to control weeds along with herbicide application and tillage, although wheat biomass yield was lower with sheep grazing and herbicide application than tillage. Soil organic C, total N, and NO₃-N contents varied among weed management practices and soil depths, but the contents at 0–120 cm were not affected by weed management practices (Table 14).

Weed management†	SOC content (Mg C ha ⁻¹)						
	0–5 cm	5–10 cm	10–30 cm	30–60 cm	60–90 cm	90–120 cm	0–120 cm
Chem.	18.3a‡	19.2a	61.7a	38.0a	32.2a	29.1b	198.4a
Mech.	17.3a	17.4a	58.2ab	38.0a	35.8a	37.0a	203.5a
Graz.	16.9a	17.7a	54.2b	36.1a	31.2a	31.4ab	187.5a
STN content (Mg N ha⁻¹)							
Chem.	1.69a	1.89a	6.48a	4.96a	3.58a	2.79a	21.40a
Mech.	1.61a	1.74b	5.91a	5.00a	3.43a	2.99a	20.55a
Graz.	1.53a	1.79ab	6.33a	5.60a	3.86a	2.87a	22.09a
NO₃-N content (kg N ha⁻¹)							
Chem.	12.6a	12.4a	20.6a	16.0a	18.9b	38.0a	118.6a
Mech.	10.3a	12.0a	21.1a	14.5a	28.8a	37.6a	124.4a
Graz.	9.9a	10.9a	18.7a	17.5a	23.2ab	35.0a	115.2a

†Weed management practices are Chem., chemical where weeds were controlled with herbicide applications; Graz., grazing where weeds were controlled with sheep grazing; and Mech., mechanical where weeds were controlled with tillage.
 ‡Numbers followed by different letters within a column are significantly different at $P \leq 0.05$ by the least square means test.

Table 14. Soil organic C (SOC), total N (STN), and NO₃-N contents at the 0–120 cm depth after 5 years of weed management experiment initiation in western Montana, USA [50].

Soil P, K, and SO₄-S contents at 0–30 cm were lower with sheep grazing than other weed management practices, but pH, electrical conductivity, and Ca, Mg, and Na contents were similar or greater with sheep grazing (Table 15). Consumption of crop residue by sheep during grazing, but little P and K inputs to the soil through urine and feces, reduced soil P and K concentrations with sheep grazing compared with other weed management practices [49]. These results suggest that sheep grazing can reduce the cost of animal feed without seriously affecting crop yields and sustain soil organic matter and nutrients compared with other weed management practices, except P and K which need to be added with inorganic fertilizers to eliminate their deficiency. As soil residual NO₃-N content was not different among weed management practices, long-term study may be needed to evaluate if animal grazing can reduce N fertilization rate for crop production. However, animal grazing can recycle nutrients and control weeds effectively compared with herbicide application and tillage, thereby saving the cost of fertilization and weed control.

Legumes in the crop rotation can supply N from its residue to succeeding crops, thereby reducing N fertilization rates to succeeding nonlegumes. Also diversified crop rotations can use N and water more efficiently and reduce weed, pest, and disease infestations, thereby enhancing crop yields compared with continuous nonlegume monocropping. Cover crops grown to replace the fallow period can reduce soil erosion, enhance soil organic matter, and help to enrich soil health and fertility. Legume covers crop supply N and reduce N fertilization rate. Application of manure and compost can also enhance soil health and quality; however, additional inorganic N fertilization at lower rate is required to sustain crop yield and quality. Similarly, integrated crop-livestock system can help to reduce N fertilization rate by returning N and other nutrients through urine and feces to the soil during animal grazing without affecting crop yields. Some additional N fertilizer, however, may be required for sustainable crop production, because animals

Chemical properties	Soil depth	Weed management (WM)†		
		Chem.	Mech.	Graz.
P content (kg ha ⁻¹)	0–5 cm	34.5a‡	35.7a	30.8a
	5–10 cm	30.4a	29.3a	17.8b
	10–30 cm	81.2a	80.7a	40.1b
K content (kg ha ⁻¹)	0–5 cm	263a	271a	222b
	5–10 cm	176a	191a	139b
	10–30 cm	792a	859a	577b
pH	0–5 cm	6.45a	6.94a	6.72a
	5–10 cm	6.31a	6.64a	6.51a
	10–30 cm	7.06a	7.34a	7.31a
EC (S m ⁻¹)	0–5 cm	0.035a	0.037a	0.035a
	5–10 cm	0.024a	0.024a	0.024a
	10–30 cm	0.025a	0.026a	0.27a
Ca content (Mg ha ⁻¹)	0–5 cm	2.05a	2.06a	2.08a
	5–10 cm	2.14b	2.31a	2.25ab
	10–30 cm	10.70b	11.70ab	12.90a
Mg content (kg ha ⁻¹)	0–5 cm	278a	288a	304a
	5–10 cm	362b	382ab	417a
	10–30 cm	2619a	2593a	2640a
Na content (kg ha ⁻¹)	0–5 cm	11.7a	12.5a	12.8a
	5–10 cm	15.2b	15.2b	18.4a
	10–30 cm	84.8ab	76.6b	95.0a
SO ₄ -S content (kg ha ⁻¹)	0–5 cm	8.5ab	10.0a	7.4b
	5–10 cm	9.0ab	10.6a	7.1b
	10–30 cm	34.0ab	40.8a	28.8b

†Weed management practices are Chem., chemical where weeds were controlled with herbicide applications; Graz., grazing where weeds were controlled with sheep grazing; and Mech., mechanical where weeds were controlled with tillage.

‡Numbers followed by the same letter within a row in a set are not significantly different at $P \leq 0.05$.

Table 15. Effect of weed management practice on soil nutrients, pH, and electrical conductivity (EC) at the 0–30 cm depth after 5 years of experiment initiation in western Montana, USA [49].

return only a part of nutrients through urine and feces to the soil, while most of the crop residue grazed is used to increase the live weight of the animal. The choice of the management practice to reduce N fertilization rate to crops depends on soil and climatic conditions and social, cultural, and economic perspectives of the producers.

2.5 Liming

Soil acidification can be reduced by applying lime. However, lime is bulky and requires in large amount to neutralize soil acidity. The transportation cost to carry lime from manufactures to farms is also high and especially so in hilly regions

where roads are few or lacking. As a result, it is expensive to apply lime and most producers in developing countries cannot afford to apply it. Furthermore, neutralization of soil acidity with lime application is only temporary in nature. This suggests that lime should be applied frequently to neutralize acidity, which increases the cost of production. The best practice to reduce soil acidity is to reduce the rate of N fertilization. Several management practices, such as legume-nonlegume crop rotation, cover cropping, application of manures and compost, and integrated crop-livestock system, can reduce N fertilization rate without affecting crop yields.

3. Conclusions

Degradation in soil and environmental quality can be mitigated, and crop yields can be sustained by reducing N fertilization rates and using novel management techniques that increase N cycling and N-use efficiency. These techniques include legume-nonlegume crop rotation, cover cropping, application of manures and compost, and integrated crop-livestock system. Soil acidity can be neutralized by lime application, but the effect is temporary. It is expensive to apply lime, and many producers in developing countries cannot afford to do so. Adaptation of these techniques to specific places depends on soil and climatic conditions and social, cultural, and economic perspectives of the producers.

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Nitrogen Fertilization in Blackberry

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Abstract

Nutrition studies for blackberry crop are scarce worldwide. This chapter presents several aspects of nitrogen (N) in blackberry (*Rubus* spp.) nutrition. Soil characteristics that can influence nitrogen fertilization are the large discrepancies in the rates recommended in the literature, forms and times of application, sources of nitrogen, differences between cultivars and the main symptoms of N deficiency. The impact of moderate and severe nitrogen deficiency on vegetative growth and yield of ‘Tupy’ blackberry is also presented. In addition, a nitrogen fertilization recommendation system is proposed, based on the organic matter content of the soil, the age of the plants, and the expected productivity of the cultivars.

Keywords: *Rubus* spp., nutritional requirements, cultivar differences, nutritional deficiency, soil organic matter, fertilization recommendation

1. Introduction

Nitrogen (N) is the mineral element that plants generally need in greater quantity, since they serve to form components of plant cells, such as amino acids and nucleic acids, besides participating in the chlorophyll molecule [1]. N deficiency rapidly reduces plant growth, as it causes reduction of cell division and expansion, leaf area, and photosynthesis [2].

In blackberry (*Rubus* spp.), N is the most abundant element and plays a major role in its growth, development, and productivity [3–6]. The optimum leaf content required for a satisfactory performance of blackberry varies from 2.2 to 3.0% of the dry matter of the leaves [7, 8].

The need for N supply may vary according to soil organic matter (SOM) content, yield, growth habit, age, and cultivar [8, 9]. The N rates recommended in the literature vary widely, mainly due to differences between cultivars and soil characteristics, but another important factor is the age of the plants. In the first years, the productive capacity of the plants is smaller, and therefore the demand for nitrogen is also lower. High N rates in the first 2 years can reduce fruit quality and increase disease incidence. On the other hand, low rates from the third year make it difficult to obtain high yield.

Nitrogen fertilization provides immediate effect (same season) and residual (next season). The immediate effect is mainly on the productive capacity of the

floricans and the size of the fruits. Already in the following season, the greatest influence is on the growth and formation of primocanes and floral buds [10].

Considering the N importance to blackberry, this chapter aims to review the dynamics of nitrogen fertilization and present a recommendation proposal.

2. Soil characteristics

The recommended soil pH for blackberries is 5.5 to 6.5 [7, 8, 11]. The availability of N from fertilizers mainly depends on the fertilizer source [12] and the method of application [13]. Soil pH affects nitrification, with ammonium-N sources converted more rapidly to nitrate-N at a pH of 6.0 than at 5.5 [8].

Another issue that must be observed in relation to pH is the presence of aluminum (Al^{3+}) in the soil. In these cases it is essential that the pH is raised to values close to 6.0 through liming, avoiding problems with phytotoxicity by aluminum.

The ideal soil organic matter content is around 2.0 to 4.5% [3, 14, 6]. However, what is observed is that the blackberry is a rustic species, which can be cultivated in soils with a wide range of SOM levels. The difference is that in soils with low levels, less than 2%, there is a need for special care in relation to nitrogen fertilization. On the other hand, when the content of SOM is high, more than 4.5%, the vigor of the plants is driven in a way that increases the frequency and intensity of pruning, as well as decreases the need for N fertilization.

Some studies indicate a lack of effect of fertilization on yield in soils with a SOM content of 3.9%, while in soil with a SOM content of 1.1%, there was a linear increase of the yield in response to increasing rates of N [4, 15]. These results suggest an important influence of SOM on the response of blackberry to fertilization with N.

3. Nitrogen fertilizer rate

There are significant variations in the nitrogen rates recommended by the literature, with values ranging between 0.0 and 200 kg ha⁻¹. Such differences may be related to factors such as soil characteristics, climate, and genotypes [3, 5].

Some authors recommend the application of 34–56 kg ha⁻¹ in the first year, regardless of growth habit, and in the following years, the dose would be from 56 to 78 kg ha⁻¹ for training and semi-erect cultivars and from 56 to 90 kg ha⁻¹ for cultivars of erect habit [8].

Other indications for semi-erect and erect cultivars are 25–45 kg ha⁻¹ at the establishment and 45–70 kg ha⁻¹ in subsequent years [16, 17]. The recommendations for training cultivars are between 25 and 45 kg ha⁻¹ in the year of establishment and between 45 and 60 kg ha⁻¹ in the following seasons [17].

Considering that Tupy and Xavante has semi-erect and erect growth habit, respectively, there was an inversion of the logic indicated in the recommendations of the literature. In this case, probably the greatest nutritional need of the cultivar Tupy may come as a result of its greater yield and export of nutrients, about 40% higher than that obtained with Xavante.

Another feature that diverges between these two genotypes is the presence of thorns. There is a need for new studies in order to verify if there is a possible relationship of this characteristic with the nutritional need.

The amount of nutrients applied in blackberry cultivation can also vary with the age of the plants. The application of N in the year of establishment of the crop is controversial; there are authors who do not recommend the application, due to the risk of damages to the vegetative buds and others that, although emphasizing

that the application is less necessary than in the subsequent years, suggest the application of up to 56 kg ha^{-1} [8, 14]. On the other hand, in a study carried out in the south of Brazil, in an area with a 1.1% SOM content, the maximum productive efficiency rate was 109 kg ha^{-1} in the first harvest after planting and increased up to 155 kg ha^{-1} at the third crop [4]. Thus, these results indicate that there is a need for differential fertilization strategy, depending on the age of the plants.

4. Nitrogen fertilizer application

The N application is usually done in granular form while planting directly in the row but can be carried out during crop development, via fertigation or foliar fertilization [14, 17]. In a study comparing the application methods, it was concluded that the granular fertilization in the spring had a better result than the drip fertigation, mainly due to the higher leaching observed in the drip method [13]. Although it can be used, foliar fertilization has not presented satisfactory results [8], which is justified by the high demand that the plant has in relation to this nutrient.

About the N sources, blackberry responds satisfactorily to several, but ammonium nitrate, urea, and ammonium sulfate are the most commonly used sources [3, 17]. In Brazil, the ammonium sulfate is currently recommended, as blackberries also demand important sulfur amount (about 3 kg per ton of fruit and pruning material removed) [7, 14].

However, research results indicate that after 3 years of using ammonium sulfate as N source, there is a significant reduction of pH (**Figure 1a**) and increase of Al^{3+} content (**Figure 1b**) in the soil, which may have a negative impact on the productivity (**Figure 1c**). In this specific case, when the soil pH was lower than 4.7 and the Al^{3+} content was higher than $0.66 \text{ cmol}_c \text{ dm}^{-3}$, blackberry production decreased. However, further studies on different soil types should be carried out in order to confirm this relationship. Therefore, when using ammonium sulfate as N source, the pH and Al^{3+} content of the soil should be monitored.

As for the time of application, N should be applied in the spring and after harvest [7, 14, 18]. Fertilization carried out at the end of winter or early spring aims to provide fruit production and the growth of primocanes, new stems that will be responsible for fruit production in the following year.

N has an important role in the formation of “primocane” numbers since it stimulates budding of crown buds, thus impacting the number of stems and the yield of the next season [3]. Maintaining the proper number of stems over the years is important. There is a positive correlation between the number of stems and the

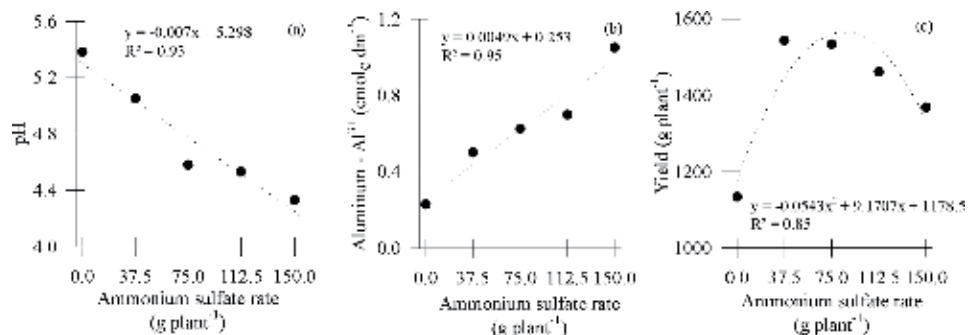


Figure 1. Influence of ammonium sulfate rates on pH (a), aluminum (Al^{3+}) concentration (b), and blackberry “Tupy” productivity (c) [9].

yield of the blackberry, that is, the larger the number of stems (up to 12 stems m^{-1}), the greater the productivity [19].

On the other hand, postharvest fertilization, usually performed after post-harvest pruning, has the function of stimulating the development of primocanes, inducing the formation of vigorous stems and thus capable of supporting high yields and larger fruits in the next season. In blackberry cultivation, there is a significant correlation between stem diameter and fruit size, and more vigorous stems have potential for larger fruit production [19, 20].

5. Nutritional differences between cultivars

It has been observed in the literature that blackberry cultivars present important differences in relation to their nutritional requirements [4, 5, 8, 17]. Some authors recommend fertilization for groups of cultivars with the same growth habit [8, 17]. In addition, there are research results that also indicate differences between cultivars with and without thorns [5, 21]. But, in general, the greatest difference between demands of each cultivar is actually related to their productive capacity, that is, more productive cultivars export larger quantities of N and therefore also demand higher rates of fertilizer. For this reason, the tendency is that fertilization recommendations incorporate the expected productivity as a criterion to define the most adequate N rate.

6. Nitrogen deficiency

Compared to blackberry plants with no N deficiency (**Figure 2a**), N deficiency in leaves is characterized by foliar chlorosis (**Figure 2b**), and in severe deficiency situations, reddish patches may appear distributed throughout the leaf blade (**Figure 2c**). In addition to the leaves, the stems may also exhibit reddish pigmentation, and the greater the deficiency, the greater the intensity of the red (**Figure 2e** and **f**), being this type of pigmentation originated from the anthocyanin accumulation [21]. N deficiency appears on old leaves and progresses to the younger ones. This is due to the fact that N is easily translocated and redistributed within the plant [21]. Therefore, when the nutrient supply in the roots is insufficient, the nutrient of the older leaves is mobilized for the younger ones.

The vegetative growth is the major aspect affected by the deficiency of N, being the foliar chlorosis the first visible symptom. This happens because N-deficient plants present a lower chlorophyll leaf concentration, which can be verified by the lower SPAD (soil plant analysis development) index in leaves of nitrogen-deficient plants (**Figure 3a**), which is an indirect measure of the foliar chlorophyll content. It is noted that the SPAD index was not able to identify differences between moderate and severe deficiency. However, the method clearly identified N deficiency even in a moderate situation. In this way, it is possible that through calibration studies, the SPAD index can be used as a rapid method to evaluate the N leaf content in blackberry plants.

In general, when foliar chlorosis is identified, vegetative growth has generally been compromised. Among the main growth parameters that may indicate N deficiency problems in the blackberry are the length of internodes and the length of the stems. In **Figure 3b**, it is possible to observe the average reduction of 15% in the length of the internodes in plants with moderate N deficiency. However, the length of the stems is further reduced. In plants with moderate deficiency, the reduction in stem length was 35%, whereas in plants with severe deficiency, the reduction was 52% (**Figure 3c**).

N deficiency also causes a reduction in the number and in the mass of blackberry fruits. **Figure 3c** shows a reduction of 63% in the number of fruits produced

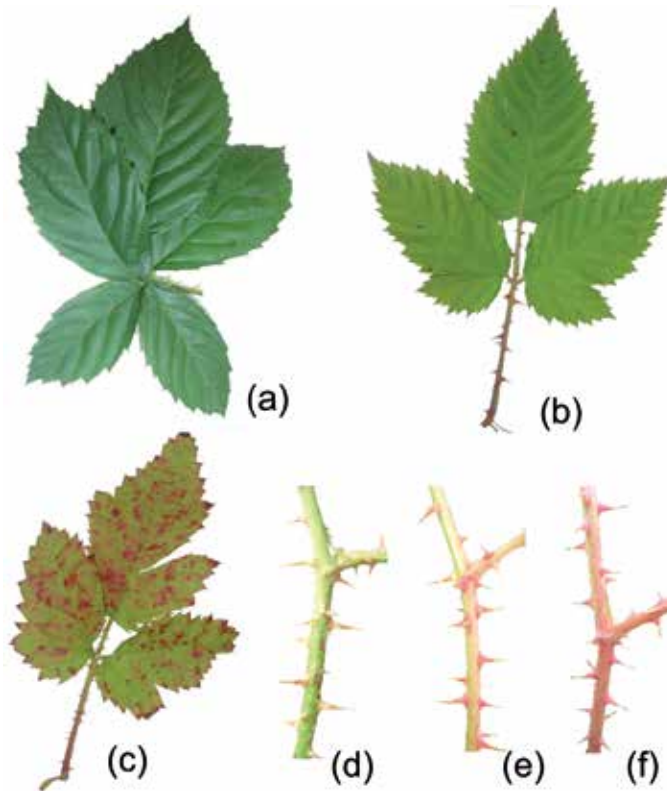


Figure 2. Leaf superior surface with normal aspect, no deficiency symptoms (a), with moderate (b) and severe nitrogen deficiency (c) in Tupy cultivar. Besides stems without deficiency symptoms (d) and with moderate (e) and severe nitrogen deficiency (f) [9].

in blackberry plants with moderate N deficiency and 65% in plants with severe deficiency. Regarding the fruit mass, the reduction was 52% in plants with moderate deficiency and 60% when the deficiency was severe (**Figure 3d**).

The increase in fruit size is the main effect of nitrogen fertilization on the fruit quality of blackberries [22]. In general, nitrogen fertilization induces the formation of vigorous stems which, in turn, provide the formation of larger fruits [20]. In terms of sensory quality, the effects are varied. Nitrogen fertilization performed at the recommended amount increases soluble solids concentration but has little effect on attributes such as pH, acidity, sugar/acid ratio, and firmness [23, 24]. On the other hand, the excess of N can cause reduction of soluble solids, increase acidity and pH, and also decrease fruit firmness.

The accumulation of negative effects caused by N deficiency on vegetative growth and fruit formation in blackberry plants had a catastrophic impact on plant productivity. It was observed a reduction of 71% in the productivity of plants with moderate deficiency and 75% in plants with severe deficiency (**Figure 3f**).

The results presented in **Figure 3** demonstrate that in many aspects there are no significant differences in vegetative growth or fruit yield between plants with moderate and severe N deficiency. This shows that even when N deficiency is moderate, the crop yield is extremely impacted.

Excess of N is characterized by excessive plant vigor, long internodes, thinner stems, dark green leaves, low yield, low quality of fruits, less conservation potential, and greater risk of diseases [8, 14, 21]. In addition, very high rates of N may result in a decrease in the foliar content of manganese (Mn), potassium (K),

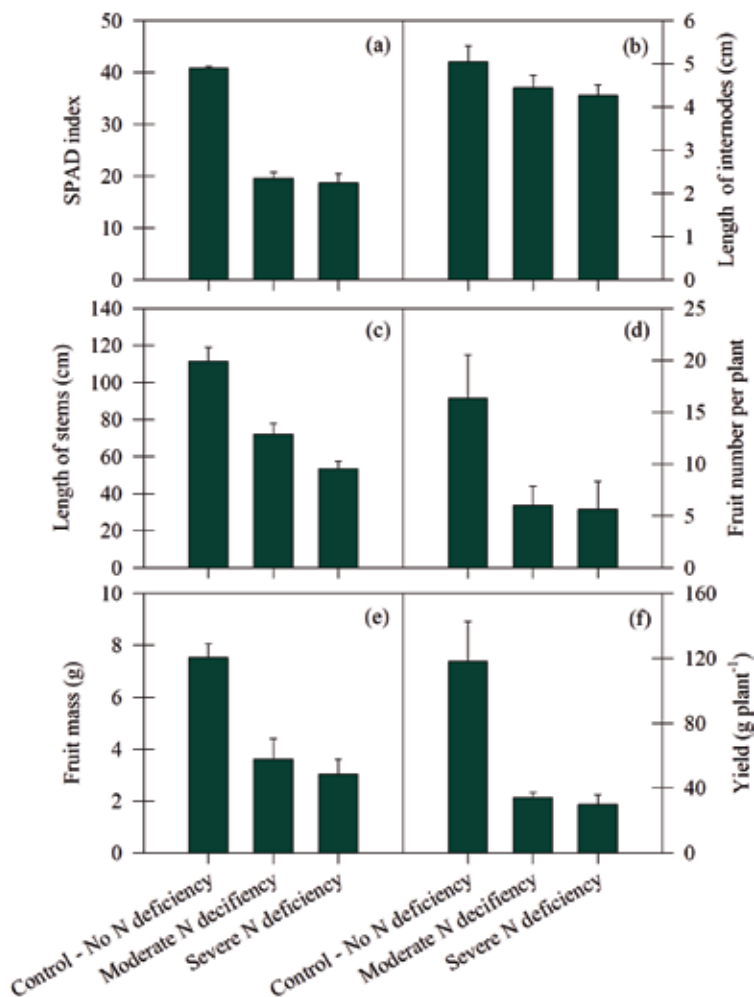


Figure 3. Effects of different levels of nitrogen (N) deficiency on the SPAD index (a), length of internodes (b), length of stems (c), fruit number per plant (d), fruit mass (e) and yield (f) of 'Tupy' blackberry. Deficiency levels: control or no N deficiency; moderate deficiency, and severe deficiency.

calcium (Ca), and magnesium (Mg) and increase of copper (Cu). On the other hand, the reduction of fertilization with N reduces the iron content (Fe) and may reduce production due to nutritional imbalance [4, 25]. The reduction of other nutrients can be attributed to two main factors, competition for the same binding sites and the dilution effect, since N stimulates vegetative growth [4].

7. Nitrogen recommendation fertilization

The fertilization recommendation is divided into **fertilization of preplanting** and **production fertilization** [7]. The preplanting fertilization aims to correct soil problems, as in the case of N, low levels of organic material, and must be carried out during soil preparation, before planting of seedlings, while the production fertilization has the objective to restore to the soil the quantities of N exported by the production of fruits (considers the expectation of production) and natural losses, as, for example, by leaching.

7.1 Preplanting fertilization

Nitrogen fertilization of preplanting is recommended in soils with organic matter content of less than 2.5%. In these cases, it is recommended to apply 40 kg ha⁻¹ of N, and it is preferable to use organic sources, such as compost from manure.

7.2 Production fertilization

Production fertilization begins in the year following to planting of the seedlings and takes into account soil organic matter content, plant age, and production expectation (Figure 4).

The N rate applied in the spring should be divided into three times: the first application should be made at the beginning of the budding; the second, 30 days

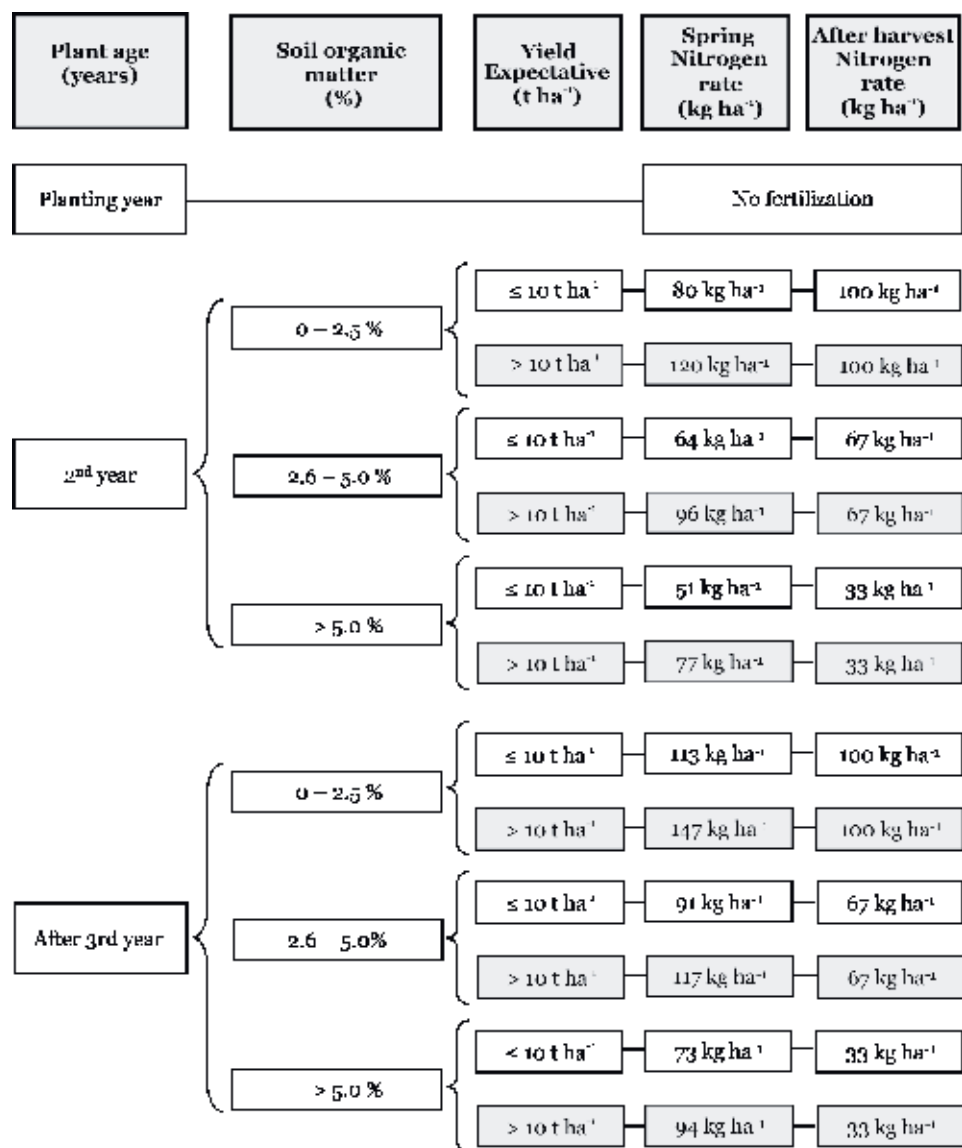


Figure 4.
 Recommendation of nitrogen production fertilization.

Class	Nitrogen content (%)
Insufficient	<2.20
Normal	2.30–3.00
Excessive	>3.00

Table 1.

Tissue N concentration from sampling after harvesting the primocane's leaves [8].

after; and the third, at 60 days after the first application. The application postharvest can be applied in a single rate.

The N may be applied in the form of urea (45% of N), calcium nitrate (14% of N), ammonium nitrate (32% of N), or ammonium sulfate (20% of N). Ammonium sulfate is preferably recommended because the blackberries require sulfur. However, the consecutive use of ammonium sulfate can significantly reduce soil pH, being necessary to monitor this parameter. An alternative to supply the sulfur demand with little impact on soil pH may be the use of agricultural gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$), which also provides calcium and can be applied at a rate of 34–45 kg ha⁻¹ to supply possible S deficiencies of blackberries [8].

The N source application should be performed at the soil surface, along the planting row, approximately 15 cm away from crown of plants.

7.3 Leaf N concentrations

In contrast to other species, in blackberry, leaf analysis is applied to evaluate the nutritional status after harvest, aiming the elaboration of a fertilization program for the next productive cycle (Table 1). For this reason, sampling is performed after harvesting the primocane's leaves. Sampling is performed on the 6th completely expanded leaf from the stem apex.

8. Conclusion

Nutrition studies for blackberry crop are scarce worldwide. However, as presented in this chapter, nitrogen fertilization is one aspect of crop management that has the greatest impact on vegetative growth, fruit yield, and quality. It has been demonstrated in this chapter that even under conditions of moderate N deficiency, there can be significant impacts on blackberry yield. Nitrogen-deficient plants tend to form weak, low-yielding stems. In this way, the application of adequate rates for each type of soil and cultivar is fundamental for satisfactory productivities.

In addition, there are important differences in the demand of N between cultivars, probably due to productive capacity, that is, cultivars that provide higher yields also export larger amounts of N and therefore need larger amount of nitrogen fertilizers.

In this way, a nitrogen fertilization program was proposed, based on SOM (indicator of N availability), plant age, and production expectation.

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Nitrogen Fertilization I: Impact on Crop, Soil, and Environment

Upendra M. Sainju, Rajan Ghimire and Gautam P. Pradhan

Abstract

Nitrogen (N) is a major limiting nutrient to sustain crop yields and quality. As a result, N fertilizer is usually applied in large quantity to increase crop production throughout the world. Application of N fertilizers has increased crop yields and resulted in achievement of self-sufficiency in food production in many developing countries. Excessive application of N fertilizers beyond crops' demand, however, has resulted in undesirable consequences of degradation in soil, water, and air quality. These include soil acidification, N leaching in groundwater, and emissions of nitrous oxide (N_2O), a potent greenhouse gas that contributes to global warming. Long-term application of ammonia-based N fertilizers, such as urea, has increased soil acidity which rendered to soil infertility where crops fail to respond with further application of N fertilizers. Another problem is the groundwater contamination of nitrate-N ($\text{NO}_3\text{-N}$) which can be a health hazard to human and livestock if its concentration goes above 10 mg L^{-1} in drinking water. The third problem is emissions of N_2O gas which is 300 times more powerful than carbon dioxide in terms of global warming potential. This chapter examines the effect of N fertilization on soil and environmental quality and crop yields.

Keywords: crop yields, environmental quality, management practices, nitrogen fertilizer, nitrogen-use efficiency, soil quality

1. Introduction

Nitrogen (N) is a major limiting factor for sustainable and profitable crop production. However, excessive N application through fertilizers and manures can degrade soil and environmental quality by increasing soil acidification, N leaching, and emissions of ammonia (NH_3) and nitrogen oxide (NO , N_2O , and NO_2) gases, out of which N_2O is considered a highly potent greenhouse gas that contributes to global warming [1, 2]. Nitrogen application more than crop's need can also result in reduced yield [3]. Additional N inputs include dry and wet (snow and rain) depositions from the atmosphere, biological N fixation, and irrigation water. Because crops can remove about 40–60% of applied N, the soil residual N (nitrate-N [$\text{NO}_3\text{-N}$] + ammonium-N [$\text{NH}_4\text{-N}$]) after crop harvest can be lost to the environment through leaching, denitrification, volatilization, surface runoff, soil erosion, and N_2O emissions [3, 4]. One option to reduce soil residual N is to increase N-use efficiency. Nitrogen-use efficiency for crops, however, can be lower at high N fertilization rates [5]. Improved management practices can increase N-use efficiency, enhance soil N storage, and reduce N fertilizer application which

reduce N losses to the environment [4]. An account of N inputs, outputs, and retention in the soil provides N balance and helps to identify dominant processes of N flow in the agroecosystem [4].

Economically profitable crop yields could be achieved by recommended N fertilization rates [6]. However, such a yield potential for a crop varies with soil and climatic conditions, crop species, variety, nutrient cycling, and competitions with weeds and pests [6]. Crop production can be optimized and potential for N losses minimized by adjusting N fertilization rates using soil residual and potentially mineralizable N values. Studies show that ~1–2% of soil organic N in the 0–30 cm depth is mineralized every year [6]. Measuring the actual amount of N mineralized is a time taking process. A commonly used method for measuring soil available N and determining nitrogen rates for crops in semiarid regions of northern Great Plains, USA is based on testing $\text{NO}_3\text{-N}$ content in soils to a depth of 60 cm after crop harvest in the fall season of the previous year and deduct the value from recommended N rates for the current crop year [7, 8]. In semiarid regions such as Great Plains of USA, N losses to the environment due to N leaching, volatilization, and denitrification during the winter are considered minimal due to cold weather and limited precipitation in the region.

Nitrogen fertilizers are being increasingly applied to crops to enhance their yield and quality in South Asia, where land available for crop production is limited, the proportion of cultivated land to population is low, and the pressure to increase crop yields to meet the demand for growing population is high. Continuous application of N fertilizers to nonlegume crops and excessive application rates in some places have led to undesirable consequences, such as reduced crop yields and degraded soil and environmental quality from soil acidification, N leaching, and greenhouse gas (N_2O) emissions. In this chapter, we discuss the consequences of N fertilization to crop yields and soil and environmental quality.

2. Crop yields, nitrogen uptake, and nitrogen-use efficiency

Nitrogen fertilization can increase crop yields and N uptake compared with no N fertilization. This has been documented for malt barley (*Hordeum vulgare* L.), cotton (*Gossypium hirsutum* L.), and sorghum (*Sorghum bicolor* [L.] Moench) (Figures 1 and 2, Table 1) by various researchers in Georgia and Montana, USA [9, 10, 14]. It is not unusual to achieve higher crop yield with increased N fertilization rate due to increased soil N availability [11]. Crop yields, however, can remain at similar level or decline with further increase in N rates after reaching the maximum yield. Sainju [9] observed that annualized grain and biomass yields of barley and pea (*Pisum sativum* L.) and their C content maximized at 80 kg N ha^{-1} and then declined, as N rate increased to 120 kg N ha^{-1} (Figure 1). Similarly, Sainju et al. [10] reported that malt barley yield and N uptake increased from 0 to 40 kg N ha^{-1} and then declined with further increase in N rates in no-till and conventional till malt barley-fallow rotation (Figure 2). In no-till continuous malt barley and malt barley-pea rotation, they found that increased N rate from 0 to 120 kg N ha^{-1} continued to increase malt barley yield and N uptake. Increased soil residual N due to fallow as a result of enhanced soil N mineralization from increased soil temperature and water content resulted in a reduced response of malt barley yield and N uptake with N fertilization in no-till and conventional till malt barley-fallow rotation. A study reported a need of 27 kg of total soil and fertilizer N to produce 1 Mg of malt barley grain in irrigated no-till field in Colorado, USA [11].

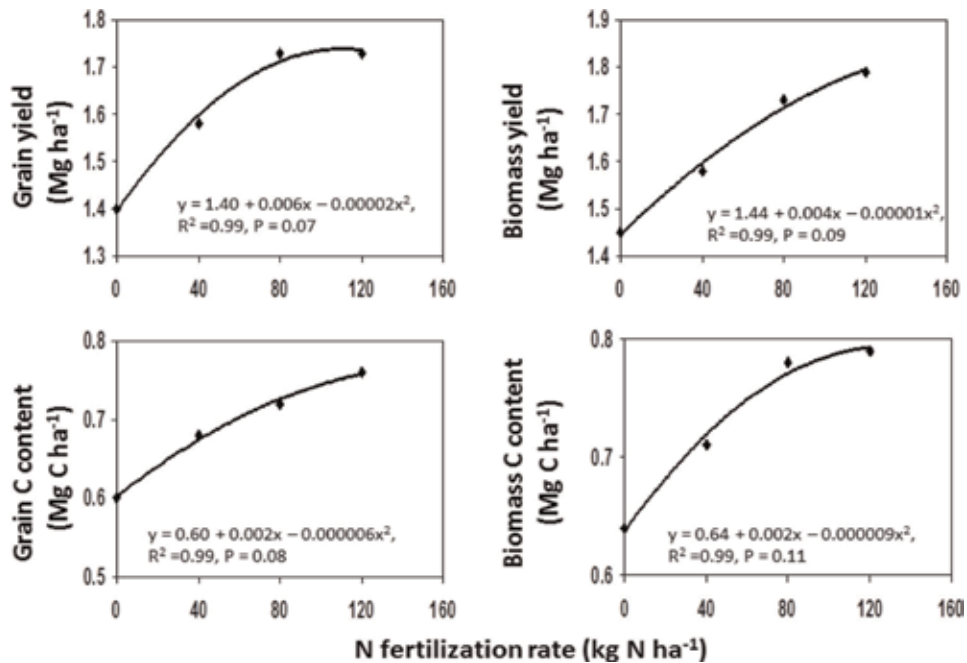


Figure 1. Annualized grain and biomass yields of barley and pea and C content as affected by N fertilization rate in eastern Montana, USA [9].

Increased N fertilization rate can also increase grain quality, such as protein concentration [10, 11]. Increased N fertilization rates increased malt barley grain yield and protein concentration, but reduced kernel plumpness in Canada [12]. While some studies reported malt barley grain protein concentration of $<130 \text{ g kg}^{-1}$ with N rate of $168\text{--}200 \text{ kg ha}^{-1}$ (e.g., [13]) others, observed an increase in protein concentration even with N rates $<150 \text{ kg N ha}^{-1}$ (e.g., [14]). Grain protein and kernel plumpness are important characteristics of malt barley that need to be maintained at critical levels (grain protein $\leq 129 \text{ g kg}^{-1}$, kernel plumpness $\geq 850 \text{ g kg}^{-1}$) for beer production [12]. Therefore, appropriate N fertilization rates are required to malt barley to achieve a balance between optimum grain yield, kernel plumpness, and protein concentration [15].

Sainju et al. [16] evaluated the effect of N fertilization on cotton and sorghum yields and N uptake from 2000 to 2002 in central Georgia, USA (**Table 1**). They found that cotton lint, sorghum grain, and cotton and sorghum biomass yields and N uptake increased from 0 to $60\text{--}65 \text{ kg N ha}^{-1}$ and then remained either at a similar level or slightly increased at $120\text{--}130 \text{ kg N ha}^{-1}$. The response of cotton yield to N fertilization, however, depended on climatic condition, as cotton lint and biomass yields were greater in 2000 than 2002 when the growing season precipitation was below the average. The N fertilizer required for optimizing cotton and sorghum yields varied with the type of tillage and cover crop [16]. Boquet et al. [17] reported that cotton lint yield was lower with no-tillage than surface tillage without applied N, but at optimum N rate, yields were higher with no-tillage. They also found that additional N was required to optimize cotton yield following wheat (*Triticum aestivum* L.) in no-tillage and surface tillage systems without cover cropping, but no N rate was required following hairy vetch cover crop in either tillage practices. Similarly, N fertilization rates to cotton and sorghum can be reduced or eliminated

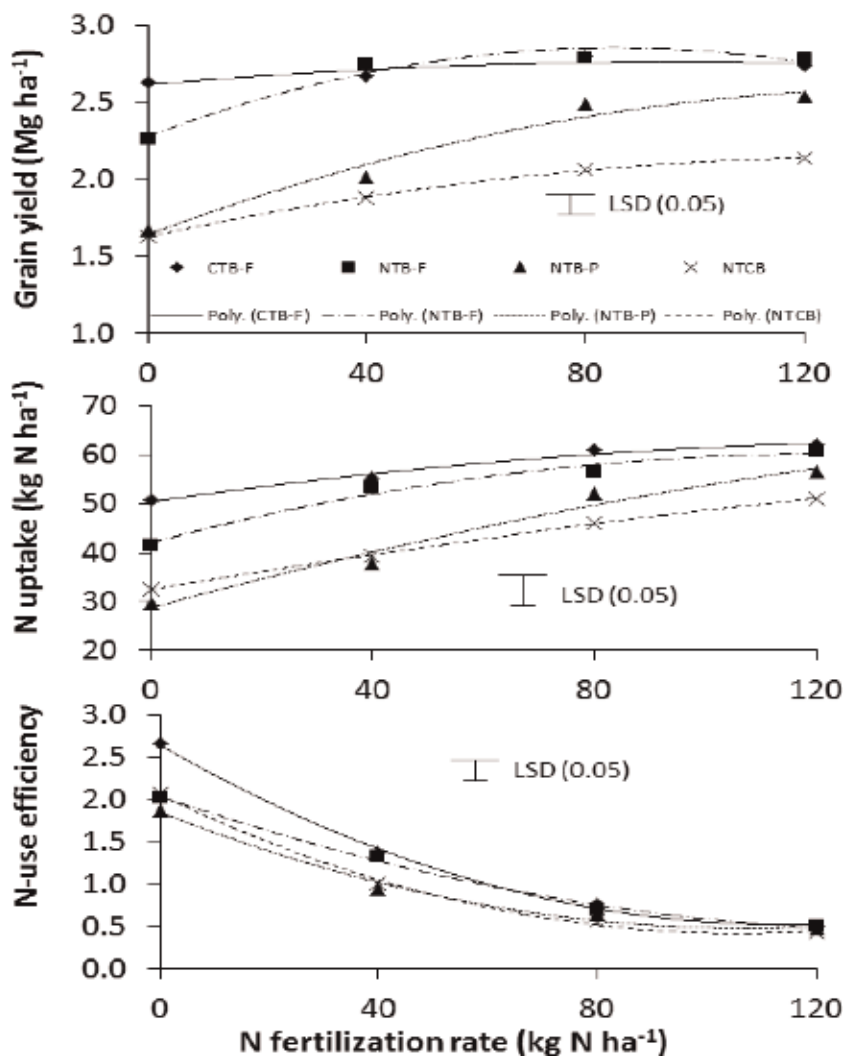


Figure 2. Effects of cropping sequence and N fertilization rate on malt barley grain yield, N uptake, and N-use efficiency in eastern Montana, USA. CTB-F denotes conventional-till malt barley-fallow; NTB-F, no-till malt barley-fallow; NTB-P, no-till malt barley-pea; and NTCB, no-till continuous malt barley. Vertical bar with LSD (0.05) is the least significant difference between treatments at $P = 0.05$ [10].

by using legume cover crops, such as red clover (*Trifolium incarnatum* L.) and hairy vetch (*Vicia villosa* Roth), regardless of tillage practices [18]. The high rate of N fertilization can produce excessive vegetative growth that delays maturity and harvest and reduces cotton lint yield and N uptake [19].

Nitrogen-use efficiency, defined as crop yield or N uptake per unit applied N fertilizer, is a useful measurement of the efficiency of N fertilization to crop yields [5]. Enhancing N-use efficiency can maximize crop yield and N uptake with limited use of fertilizer N while reducing N rate and sustaining the environment [3]. Nitrogen-use efficiency, however, can decrease with increased N fertilization rate due to the inability of crops to utilize N efficiently [5]. Sainju et al. [10] found that N-use efficiency by malt barley decreased curvilinearly with increased N fertilization rate (Figure 2). Varvel and Peterson [5] reported that N removed by corn and sorghum grain was 50% of the applied N at low N rates and at least 20–30% at high N rates.

2000 cotton lint (kg ha ⁻¹)		2000 cotton biomass (kg ha ⁻¹)		2001 sorghum grain (kg ha ⁻¹)		2001 sorghum biomass (kg ha ⁻¹)		2002 cotton lint (kg ha ⁻¹)		2002 cotton biomass (kg ha ⁻¹)		
Treatment	Yield	N uptake	Yield	N uptake	Yield	N uptake	Yield	N uptake	Yield	N uptake	Yield	N uptake
Cover crop ^a												
WW	699b ^b	11b	5200c	124b	2800bc	43ab	12,000ab	133ab	1091a	16a	3667a	74a
R	879a	15a	6300bc	138b	2300c	32b	9400b	81b	940ab	15a	3567a	77a
HV	660b	11b	8200a	239a	3500ab	60a	14,100a	175a	708b	13a	4067a	98a
HV/R	706b	12b	7300ab	194a	4000a	58a	14,100a	138ab	711b	14a	4233a	102a
N fertilization rate (kg N ha ⁻¹)												
0	736a	12a	5700b	135c	2800b	41b	11,600b	108b	1021a	17a	3700a	80b
60-65	783a	13a	7000a	178b	3100b	46b	12,400ab	135a	980a	16a	3900a	86b
120-130	689a	11a	7600a	209a	3700a	57a	13,300a	152a	587b	11b	4000a	97a

^aCover crops are HV, hairy vetch; HV/R, hairy vetch/rye; R, rye; and WW, winter weeds.

^bNumbers followed by the same letters within a column in a set are not significantly different at $P \leq 0.05$.

Table 1.

Effect of cover crop and N fertilization rate on yield and N uptake by cotton lint, sorghum grain, and their biomass (stems + leaves) from 2000 to 2002 in central Georgia, USA [16].

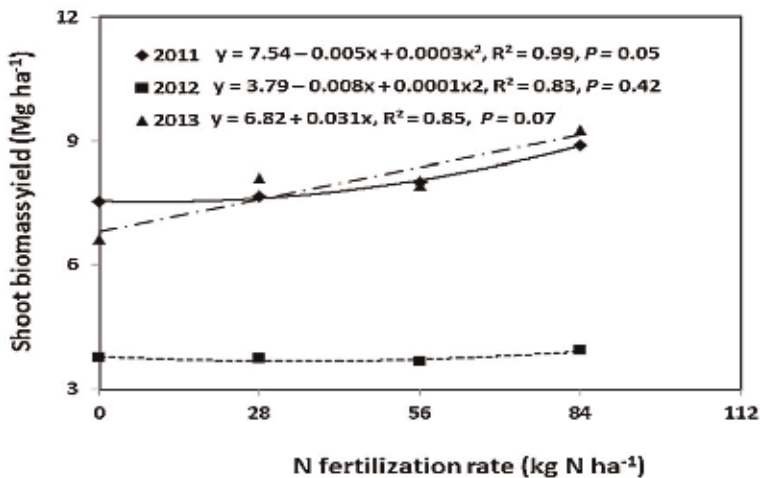


Figure 3.

Linear and quadratic responses of shoot biomass in perennial grasses with N fertilization rates from 2011 to 2013 averaged across grass species in eastern Montana, USA [20].

Nitrogen fertilization can also increase aboveground biomass yield of perennial grasses used for feedstock or bioenergy production. Sainju et al. [20] observed that yields of intermediate wheatgrass (*Thinopyrum intermedium* [Host] Barkworth and Dewey), switchgrass (*Panicum virgatum* L.), and smooth brome grass (*Bromus inermis* L.) increased linearly or curvilinearly with increased N fertilization rate in 2011 and 2013 (**Figure 3**) when the annual precipitation was near or above the average. Biomass yield, however, did not respond to N fertilization in 2012 when the annual precipitation was below the average. Several researchers [21, 22] reported that maximum switchgrass shoot biomass yield reached at 120–140 kg N ha⁻¹ in Iowa and Nebraska, USA, which had 2.5 and 2.2 times, respectively, more annual precipitation than in eastern Montana, USA. Power [23] also observed increased shoot biomass yield with increased N rate for smooth brome grass in North Dakota, USA.

3. Soil acidification

Application of NH₄-based N fertilizers can increase soil acidity due to the release of H ions during hydrolysis [24]. Increased soil acidity following the application of N fertilizers leads to the development of infertile soils that do not respond well to crop yields with further application of N fertilizers [2, 25], thereby resulting in inefficient use of fertilizers [26]. Sainju et al. [27] reported that, after 30 years of tillage and cropping sequence, continuous application of N fertilizers reduced soil pH at the 0–7.5 cm depth from 6.30 at the initiation of the experiment to 5.73 in spring till spring wheat-fallow (STW-F) and to 5.02 in fall and spring till continuous spring wheat (FSTCW) under rainfed condition in eastern Montana, USA (**Table 2**). A similar decline in soil pH at 7.5–15.0 cm was observed from 6.75 at the initiation of the experiment to 6.15 in spring till continuous spring wheat (STCW). Buffer pH, the buffering capacity of the soil to resist changes in pH and is used to measure lime requirement, also similarly decreased with continuous N fertilization in all treatments. Both pH and buffer pH, however, did not change below 15 cm with N fertilization. Because spring wheat was grown once in 2 years in spring wheat-fallow rotation where N fertilizer was applied only to spring wheat, soil pH

Tillage and cropping sequence ^a	Soil depth					
	0–7.5 cm	7.5–15 cm	15–30 cm	30–60 cm	60–90 cm	90–120 cm
pH						
NTCW	5.33ab ^b E ^c	6.50abD	7.60C	8.35B	8.58A	8.75A
STCW	5.05bE	6.15bD	7.58C	8.25B	8.63A	8.70A
FSTCW	5.02bE	6.33bD	7.80C	8.30B	8.68AB	8.73A
FSTW-B/P	5.46aE	6.44bD	7.60C	8.15B	8.51A	8.59A
STW-F	5.73aE	7.03aD	7.65C	8.25B	8.50AB	8.66A
Contrast						
NT vs. T	0.29	0.26	–0.09	0.08	–0.08	0.04
CW vs. W-F	–0.68 ^{***}	–0.88 ^{**}	–0.08	0.01	0.13	0.04
CW vs. W-B/P	–0.43 [*]	–0.11	0.20	0.15	0.16	0.14
Buffer pH						
NTCW	6.45bE	7.10abD	7.43C	7.60B	7.70AB	7.73A
STCW	6.38bE	7.00bD	7.43C	7.58B	7.68A	7.70A
FSTCW	6.43bE	7.05bD	7.45C	7.60B	7.70AB	7.73A
FSTW-B/P	6.66aD	7.13abC	7.44B	7.58B	7.69AB	7.70A
STW-F	6.80aE	7.24aD	7.44C	7.59B	7.66AB	7.72A
Contrast						
NT vs. T	0.05	0.08	–0.01	0.01	0.01	0.01
CW vs. W-F	–0.43 ^{***}	–0.24 ^{**}	–0.01	–0.01	0.01	–0.01
CW vs. W-B/P	–0.24 [*]	–0.08	–0.01	0.03	0.01	0.03

^{*}Significant at $P = 0.05$, 0.01 , and 0.001 , respectively.
^{**}Significant at $P = 0.05$, 0.01 , and 0.001 , respectively.
^{***}Significant at $P = 0.05$, 0.01 , and 0.001 , respectively.
^aFSTCW, fall and spring till continuous spring wheat; FSTW-B/P, fall and spring till spring wheat-barley (1994–1999) followed by spring wheat-pea (2000–2013); NTCW, no-till continuous spring wheat; STCW, spring till continuous spring wheat; and STW-F, spring till spring wheat-fallow. CW represents continuous wheat; NT, no-till; T, till; W-B/P, spring wheat-barley/pea; and W-F, spring wheat-fallow.
^bNumbers followed by the same lowercase letter within a column among treatments in a set are not significantly different at $P \leq 0.05$.
^cNumbers followed by the same uppercase letter within a row among soil depths in a set are no significantly different at $P \leq 0.05$.

Table 2. Effect of tillage and crop rotation combination on soil pH and buffer pH at the 0–120 cm depth after 30 years of experiment initiation in eastern Montana, USA [27].

was less declined in this treatment than continuous spring wheat where N fertilizer was applied every year. From the same experiment, Aase et al. [28] reported an average decline of pH at 0–7.5 cm from 6.3 to 5.7 after 10 years due to continuous N fertilization.

Ghimire et al. [29] found that soil pH at 0–10 cm after 70 years of N fertilization was 5.70 with 0 kg N ha⁻¹ and 5.0 with 135–180 kg N ha⁻¹ under winter wheat-fallow in eastern Oregon, USA (**Figure 4**). Reduction in pH with N fertilization decreased with depth, with no significant effect below 30 cm. A study in China, where intensive farming and high rate of N fertilizer was applied for 20 years, showed that soil pH was dropped by 0.30–0.80 units from the original level [30]. In eastern Oregon, USA, application of total N fertilizer at 2.25 Mg N ha⁻¹ over the 43-year period lowered soil pH by 0.60 units [31]. Liebig et al. [26] reported that, in

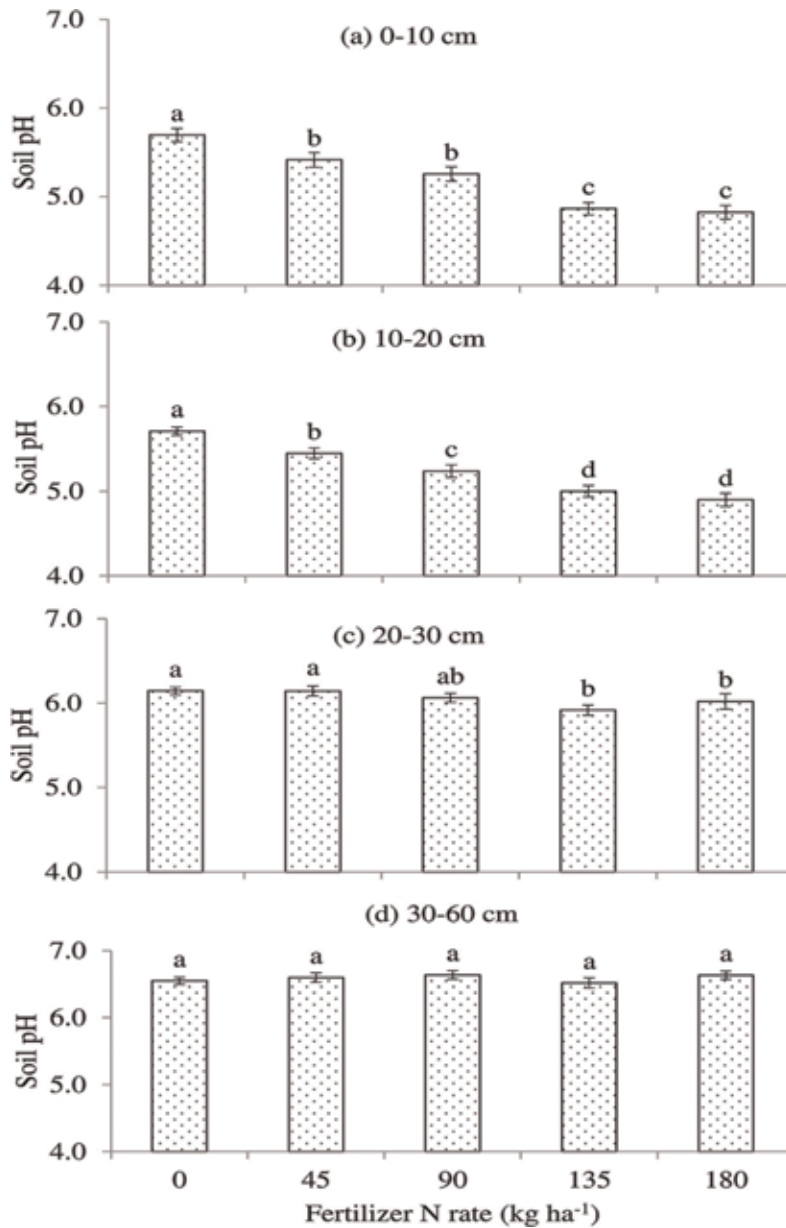


Figure 4. Soil pH at the 0–60 cm depth from N fertilization rates to winter wheat in the winter wheat-fallow rotation after 70 years in eastern Oregon, USA. Bars with different letters at the top are significantly different at $P \leq 0.05$ [29].

North Dakota, USA, soil pH at 0–7.6 cm was lower under continuous corn than corn rotated with legume and other nonlegume crops because of the increased amount of N fertilizer applied. They recommended that soil samples be collected to a depth of 15 cm for measuring changes in soil pH due to N fertilization.

No-till (NT) system can increase soil acidity more than the conventional till (CT) system [32]. This is due to differences in the amount and placement of N fertilizers in the soil and removal of basic cations through grain and biomass removal between the two tillage systems [32]. Nitrogen fertilizers are usually placed at the soil surface, and N rates are usually higher in NT due to the accumulation of

surface residue that partly immobilizes N than CT where fertilizers are incorporated into the soil due to tillage [33]. Because of enhanced soil water conservation, crop yields are higher in NT than CT, especially in dryland cropping systems [34]. As a result, crops remove more basic cations, resulting in increased acidity with NT compared with CT [34]. In contrast, Ghimire et al. [29] reported that soil pH decreased with increased N rate, as tillage intensity increased.

Source of N fertilizer can also have a varying effect on soil acidity. Chen et al. found that soil acidity from N fertilizer sources was in the order $(\text{NH}_4)_2\text{SO}_4 > \text{NH}_4\text{Cl} > \text{NH}_4\text{NO}_3 > \text{anhydrous NH}_3 > \text{urea}$. Similarly, Schroder et al. [25] reported that anhydrous NH_3 produce more acidity than urea. Others [35], however, observed no significant differences in acidity among $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , anhydrous NH_3 , urea, and urea- NH_4NO_3 .

4. Soil organic matter

Soil organic matter refers to soil organic C and N and is a crucial component of soil health and quality [36, 37]. Nitrogen fertilization can increase soil organic C and N by increasing crop biomass yield, and the amount of residue returned to the soil [38]. Russell et al. [37], however, reported no difference in soil organic C with N fertilization rate. Sainju et al. [39] reported that 3 years of N fertilization to cotton and sorghum produced various results on soil organic C at the 0–30 cm depth in strip-tilled and chisel-tilled soils in central Georgia, USA (**Table 3**). Soil organic C at 0–10 and 10–30 cm varied with N fertilization rates in strip-tilled soil, but increased in chisel-tilled soil due to differences in tillage intensity. In strip tillage, only crop rows are tilled, leaving the area between rows undisturbed, and N fertilizer is applied in crop rows. In contrast, the land is tilled using discs in chisel tillage after N fertilizer is broadcast. Differences in N fertilization methods between tillage practices probably affected soil organic C due to N fertilization rates.

Sainju [9] observed different trends of soil organic C at the 0–120 cm depth with 6 years of N fertilization rates in various cropping systems in eastern Montana, USA (**Figure 5**). Soil organic C at 0–5 and 5–10 cm peaked at 40 kg N ha^{-1} and then declined with further increase in N rates in no-till malt barley-pea (NTB-P) and continuous no-till barley (NTCB). In no-till malt barley-fallow (NTB-F) and

N rate (kg N ha^{-1})	Soil organic C (Mg C ha^{-1})				
	0–10 cm	10–30 cm	30–60 cm	60–90 cm	90–120 cm
Strip-tilled soil					
0	10.1a ^a	16.0a	10.9	7.2	5.5
60–65	9.3b	14.4b	10.2	4.5	5.3
120–130	10.3a	14.7ab	9.8	7.3	5.8
Chisel-tilled soil					
0	8.9b	12.5b	10.1	7.4	5.9
60–65	9.6a	13.4b	10.1	7.3	5.3
120–130	9.3ab	14.8a	10.6	7.9	6.1

^aNumbers followed by the same letter within a column in a set are not significantly different at $P \leq 0.05$.

Table 3. Effect of 3 years of N fertilization rate on soil organic C at the 0–120-cm depth in strip-tilled and chisel-tilled soils under cotton and sorghum in central Georgia, USA [39].

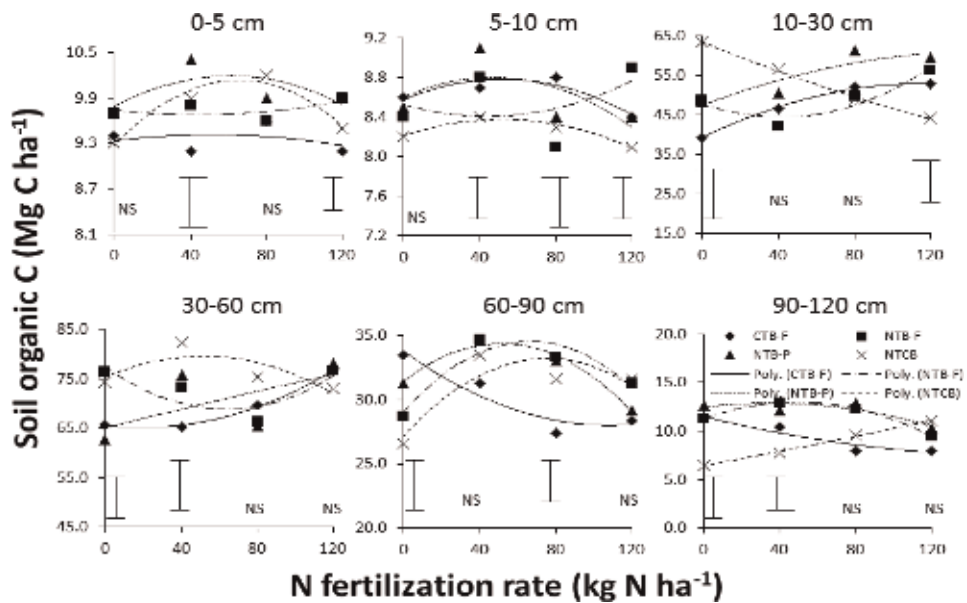


Figure 5. Soil organic C at the 0–120 cm depth as affected by 6 years of N fertilization rates to malt barley in various cropping systems in eastern Montana, USA. CTB-F denotes conventional-till malt barley-fallow; NTB-F, no-till malt barley-fallow; NTB-P, no-till malt barley-pea; and NTCB, no-till continuous malt barley. Vertical bars denote least significant difference between tillage and cropping sequence treatments within a N rate at $P = 0.05$ [9].

conventional till malt barley-fallow (CTB-F), the trend of soil organic C with N rates varied at various depths. Soil organic C at these depths was greater with NTB-P and NTCB than other treatments at most N rates due to greater amount of crop residue returned to the soil. Soil organic C at 5–10, 30–60, and 60–90 cm were greater with 40 kg N ha⁻¹ than other N rates. Sainju [9] also found that C sequestration rate at 0–10 cm was 83 kg C ha⁻¹ year⁻¹ with 40 kg N ha⁻¹ that was close to 94 kg C ha⁻¹ year⁻¹ at 0–15 cm with 45 kg N ha⁻¹ for dryland cropping systems in Colorado [36].

Under perennial grasses, several researchers [40, 41] did not find a significant effect of N fertilization on soil organic C at 0–30 cm after 2–5 years in Alabama and Colorado, USA. Only after 4–12 years, N fertilization increased soil organic C at 0–90 cm by 0.5–2.4 Mg C ha⁻¹ year⁻¹ compared with no N fertilization under switchgrass in USA and Canada [42, 43]. Rice et al. [43] reported that N fertilization to cool-season grasses increased C sequestration rate at 0–30 cm by 1.6 Mg C ha⁻¹ year⁻¹ compared with no N fertilization after 5 years in Kansas, USA. In Alberta, Canada, Bremer et al. [42] observed that N fertilization to perennial grasses increased C sequestration rate at 0–5 cm by 0.5 Mg C ha⁻¹ year⁻¹ compared with no N fertilization after 6–12 years. In South Dakota, USA, Li et al. [44] noted C sequestration rate of 2.4 Mg C ha⁻¹ year⁻¹ at 0–90 cm under switchgrass after 4 years. Sainju et al. [45] found increasing trend of soil total C at 30–60 cm with increased N rate under intermediate wheatgrass and smooth brome grass and a declining trend with switchgrass after 5 years in eastern Montana (**Figure 6**). At 60–90 cm, the trend reversed with grasses. They suggested that longer than 5 years is needed to observe the effect of N fertilization on soil total C under perennial grasses.

Nitrogen fertilization has less impact on soil total N than soil organic C. Sainju and Singh [46] reported that soil total N at 0–15 cm under cotton and sorghum was greater with 60–65 than 0 kg N ha⁻¹, but not at lower depths in the

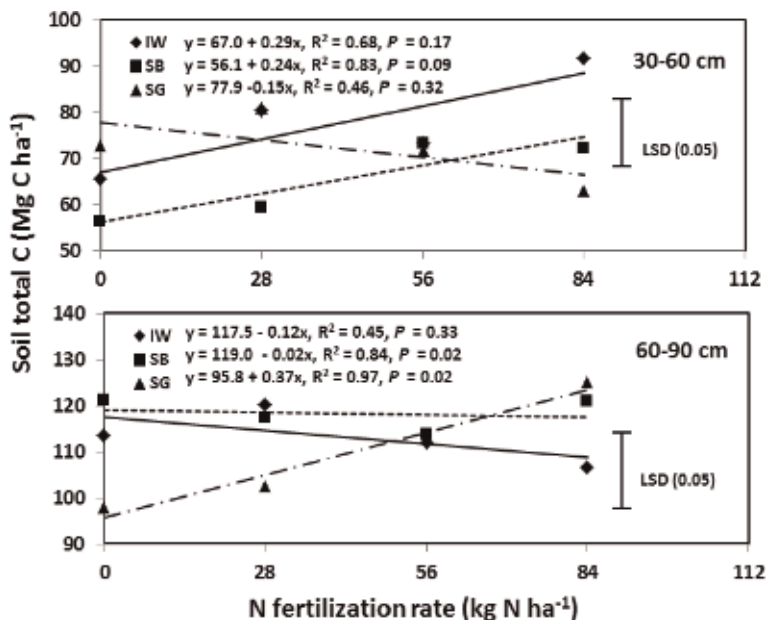


Figure 6. Soil total C at 30–60 and 60–90 cm depths as affected by 5 years of N fertilization rates to perennial grasses in eastern Montana, USA. Perennial grasses are IW, intermediate wheatgrass; SB, smooth bromegrass, and SW, switchgrass. LSD (0.05) is least significant difference between grasses within a N rate at $P = 0.05$ [45].

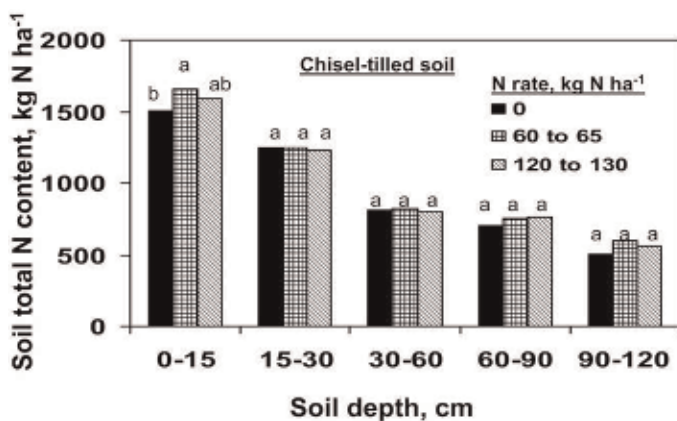


Figure 7. Soil total N at 0–120 cm in the chisel-tilled soil as affected by 6 years of N fertilization rates to cotton and sorghum in central Georgia, USA. Bars with the same letter at the top are not significantly different among N rates at a depth at $P \leq 0.05$ [46].

chisel-tilled soil in central Georgia, USA (**Figure 7**). Ghimire et al. [29] observed that soil total N at 10–20 cm increased with increased N rates after 70 years of N fertilization to winter wheat, but the trend varied with different tillage practices at higher N rates in eastern Oregon, USA (**Figure 8**). At 0–45 kg N ha^{-1} , soil total N was greater with subsurface sweep than a moldboard plow. At 90–180 kg N ha^{-1} , soil total N was lower with disc plow than other tillage practices. Increased N substrate availability due to N fertilization along with tillage may have increased microbial activity and N mineralization and therefore reduced soil total N over time.

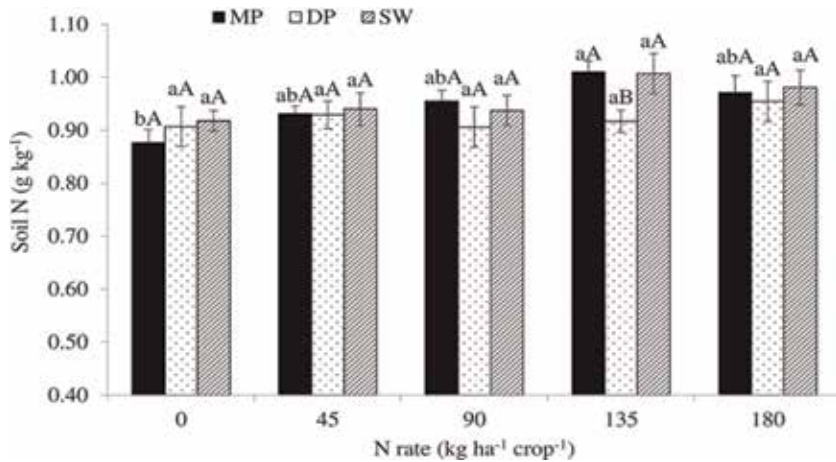


Figure 8. Soil total N as affected by 72 years of N fertilization rates to spring wheat and tillage in eastern Oregon, USA. Tillage practices are DP, disk plow; MP, moldboard plow, and SW, subsurface sweep. Bars with different lowercase letters at the top are significantly different among tillage practices within a N rate at $P \leq 0.05$. Bars with different uppercase letters at the top are significantly different among N rates within a tillage practice at $P \leq 0.05$ [29].

5. Soil residual nitrogen and nitrogen leaching

Soil residual N refers to inorganic N ($\text{NH}_4\text{-N} + \text{NO}_3\text{-N}$) accumulated in the soil profile after crop harvest. This occurs because crops cannot take up all applied N fertilizer from the soil [5, 47]. Accumulation of soil $\text{NO}_3\text{-N}$ increases with depth and is directly related to N fertilization rate [47, 48]. Deep accumulation of $\text{NO}_3\text{-N}$ in the soil profile increases the potential for N leaching to shallow water tables [49]. Nitrogen fertilization rates that exceed crop requirement can increase $\text{NO}_3\text{-N}$ accumulation in the soil profile and N leaching [50].

Soil inorganic N			
Treatment	0–10 cm	10–30 cm	0–30 cm
(kg N ha ⁻¹)			
Cover crop			
Winter weeds	19.6b ^a	32.9b	52.5c
Rye	19.1b	34.1b	53.2c
Hairy vetch	23.6a	38.4a	62.0a
Hairy vetch/rye	21.6a	34.8b	56.4b
N fertilization rate (kg N ha ⁻¹)			
0	19.6b	33.5b	53.1b
60–65	20.8b	35.3ab	56.1ab
120–130	22.5a	36.4a	59.9a

^aNumbers followed by the same letter within a column in a set are not significantly different at $P \leq 0.05$.

Table 4. Effect of cover crop and N fertilization rate on soil residual inorganic N ($\text{NH}_4\text{-N} + \text{NO}_3\text{-N}$) content at the 0–30 cm depth in central Georgia, USA [16].

N fertilization rate kg N ha ⁻¹	NH ₄ -N content at the soil depth kg N ha ⁻¹										
	0-5 cm	5-10 cm	10-30 cm	30-60 cm	60-90 cm	90-120 cm	0-10 cm	0-30 cm	0-60 cm	0-90 cm	0-120 cm
0	2.4b [†]	2.5a	10.4a	15.8a	19.4a	23.8a	4.9b	15.3a	31.2a	50.2a	72.0a
40	2.3b	2.3a	10.6a	15.4a	19.7a	25.0a	4.7b	15.2a	30.6a	49.7a	72.7a
80	2.5b	2.5a	10.3a	15.5a	19.7a	25.1a	5.0ab	15.4a	30.8a	49.1a	72.2a
120	2.9a	2.6a	10.8a	16.2a	19.6a	25.7a	5.5a	16.1a	32.0a	50.8a	73.6a

[†]Numbers followed by the same letters within a column are not significantly different at $P \leq 0.05$.

Table 5. Effect of N fertilization rate on soil residual NH₄-N content at the 0-120 cm depth from 2006 to 2011 in eastern Montana, USA [55].

N fertilization rate kg N ha ⁻¹	NO ₃ -N content at the soil depth kg N ha ⁻¹										
	0-5 cm	5-10 cm	10-30 cm	30-60 cm	60-90 cm	90-120 cm	0-10 cm	0-30 cm	0-60 cm	0-90 cm	0-120 cm
0	6.7c [†]	3.7c	13.3c	15.5c	13.7c	16.7b	10.2c	23.6d	39.0d	52.7d	68.7c
40	8.1c	4.3bc	14.6c	17.5bc	17.1b	21.4ab	12.5c	27.1c	44.6c	61.6c	82.3b
80	10.1b	5.1b	16.7b	19.8b	17.7b	21.0ab	15.2b	31.9b	51.8b	69.4b	89.6b
120	12.2a	6.2a	20.0a	23.4a	21.7a	24.7a	18.3a	38.2a	61.7a	83.3a	107.0a

[†]Numbers followed by the same letters within a column are not significantly different at $P \leq 0.05$.

Table 6. Effect N fertilization rate on soil residual NO₃-N content at the 0-120 cm depth from 2006 to 2011 in eastern Montana, USA [55].

One of the ways to reduce N fertilization rates to crops while maintaining yield goals is to account for N mineralized from soil organic matter during the crop growing season and soil residual N at crop planting [6]. Since the measurement of N mineralization requires a long time, N fertilization rates to dryland crops are adjusted by deducting soil NO₃ content to a depth of 60 cm after crop harvest in the previous year or at planting of the current year from recommended N rates [51]. Producers are increasingly interested in reducing the amount of N fertilizer applied to crops because of the higher cost of N fertilization and the associated environmental degradation.

Nitrogen fertilization rates to crops can be higher in the no-till than the conventional till system due to greater accumulation of surface crop residue that can enhance N immobilization [52]. On the other hand, N rates can be reduced in crop rotations containing legumes compared to monoculture nonlegume cropping systems [53]. Nonlegume monocropping can have higher soil residual NO₃-N content than legume-based crop rotations due to increased N fertilization rate [5, 27]. Increased cropping intensity can reduce soil profile NO₃-N content due to greater N immobilization, less summer fallow, and a greater amount of N removed by crops [54]. Sainju et al. [16] and Sainju [9] found that both soil NH₄-N and NO₃-N contents increased with N rates and depths (**Tables 4–6**).

It is well known that excessive N fertilizer application can increase N leaching in the groundwater, which is a major environmental concern [50]. Nitrate-N concentration >10 mg L⁻¹ in the drinking water poses a serious threat to human and animal health [56]. Nitrate-N is soluble in water and moves down the soil profile with percolating water [47, 57]. Increased application of N fertilizer to crops during the last several decades has increased NO₃-N contamination of groundwater [56]. This occurs because of excessive NO₃-N accumulation in the soil profile [57] due to N fertilization rates that exceed crop requirements, accompanied by poor soil and crop management practices [56]. Nitrate-N accumulation and movement in the soil profile depend on soil properties, climatic conditions, and management practices [58]. For example, N leaching is greater in sandy than clayey soils due to the presence of a large number of macropores and leaching is higher in the humid than arid and semiarid regions due to differences in annual precipitation [56, 58]. Nitrate-N leaching occurs mostly in the fall, winter, and spring seasons in the northern hemisphere when evapotranspiration is low, crops are absent to uptake soil N, and precipitation exceeds the water holding capacity of the soil [59].

6. Greenhouse gas emissions and global warming potential

Management practices on croplands can contribute about 10–20% of global greenhouse gases (GHGs: carbon dioxide [CO₂], nitrous oxide [N₂O], and methane [CH₄]) [60]. Quantitative estimate of the impact of the GHGs to global radiative forcing is done by calculating net global warming potential (GWP) which accounts for all sources and sinks of CO₂ equivalents from farm inputs, farm operations, soil C sequestration, and N₂O and CH₄ emissions [61, 62]. The net GWP for a crop production system is expressed as kg CO₂ eq. ha⁻¹ year⁻¹. Net GWP is also expressed as net greenhouse gas intensity (GHGI) or yield-scaled GWP, which is calculated by dividing net GWP by crop yield [61]. These values can be affected both by net GHG emissions and crop yields. Sources of GHGs in agroecosystems include N₂O and CH₄ emissions (or CH₄ uptake) as well as CO₂ emissions associated with farm machinery used for tillage, planting, harvesting, and manufacture, transportation, and applications of chemical inputs, such as fertilizers, herbicides, and pesticides, while soil C sequestration rate can be either a sink or source of CO₂

[62, 63]. In the calculations of net GWP and GHGI, emissions of N_2O and CH_4 are converted into their CO_2 equivalents of global warming potentials which are 310 and 28, respectively, for a time horizon of 100 years [60]. The balance between soil C sequestration rate, N_2O and CH_4 emissions (or CH_4 uptake), and crop yield typically controls net GWP and GHGI [61, 62].

Nitrogen fertilization typically stimulates N_2O emissions when the amount of applied N exceeds crop N demand [51, 61]. Nitrogen fertilization, however, can have a variable effect on emissions of other GHGs, such as CO_2 and CH_4 [64, 65]. Sainju et al. [65] found that the application of 80 kg N ha^{-1} to dryland malt barley increased CO_2 emissions, but not N_2O and CH_4 emissions (Table 7). Because N_2O emissions has a large effect on net GWP and GHGI, practices that can reduce N fertilization rates without influencing crop yields can substantially reduce net GHG emissions [61, 62]. Other factors that can influence N_2O emissions are the type, placement, time, and method of application of N fertilizers. Applying N fertilizer in the spring compared with autumn and using split application compared with one single application at planting can reduce N_2O emissions in some cases [66]. Applying N fertilizer at various depths can have a variable effect on N_2O emissions [67]. Anhydrous ammonia can increase N_2O emissions compared with urea [67, 68]. Similarly, chemical additives to reduce nitrification from N fertilizers, such as polymer-coated urea and nitrification inhibitors, can substantially reduce N_2O emissions compared with ordinary urea and non-nitrification inhibiting fertilizers [69]. Some N fertilizers, such as urea, emit both CO_2 and N_2O . Nitrogen fertilizers also indirectly emit N_2O through NH_3 volatilization and NO_3-N leaching [68].

Increased N fertilization rate can enhance net GWP and GHGI due to increased N_2O and CO_2 emissions associated with the manufacture, transport, and application of N fertilizers, regardless of cropping systems and calculation methods [61, 70]. In a meta-analysis of 12 experiments, Sainju [71], after accounting for all sources and sinks of CO_2 emissions, reported that net GWP decreased from 0 to $\leq 45 \text{ kg N ha}^{-1}$ and net GHGI from 0 to $\leq 145 \text{ kg N ha}^{-1}$ and then increased with increased N fertilization rate (Figure 9). Using partial accounting, net GWP decreased from 0 to 88 kg N ha^{-1} and net GHGI from 0 to $\leq 213 \text{ kg N ha}^{-1}$ and then increased with increased N rate. These N rates probably corresponded to crop N demand when crops used most of the soil available N. The cropping systems that left little residual N in the soil reduced N_2O emissions, and therefore net GWP and GHGI, whereas net GWP and GHGI increased linearly with increase in N application rates that exceeded crop N demand, suggesting that excessive N fertilizer applications can induce global warming. Similar results have been reported by Li et al. [44]. Therefore, N fertilizers should be applied at optimum rates to reduce net GWP and GHGI while sustaining crop yields. The optimum N rates, however, depended on net GWP measured either per unit area or per unit crop yield.

N fertilization	CO_2 flux	N_2O flux	CH_4 flux
kg N ha^{-1}	Mg C ha^{-1}	g N ha^{-1}	g C ha^{-1}
0	1.15b [†]	308a	-314a
80	1.23a	329a	-291a

[†]Numbers followed by different letters within a column are significantly different at $P \leq 0.05$ by the least square means test.

Table 7. Effect of N fertilization on total soil surface greenhouse gas fluxes (from March to November) averaged across years from 2008 to 2011 under rainfed malt barley in eastern Montana, USA [65].

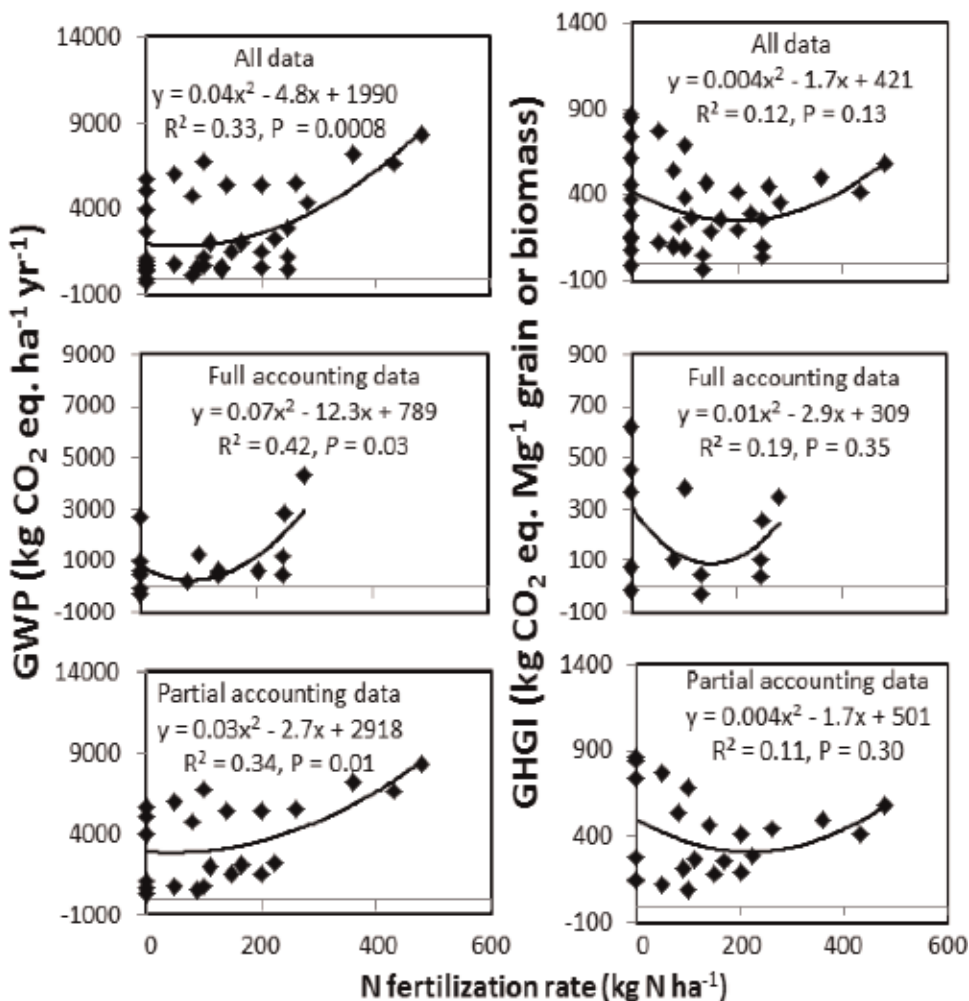


Figure 9. The relationship between N fertilization rate and net global warming potential (GWP) and greenhouse gas intensity (GHGI). Full accounting data denote calculations of GWP and GHGI by accounting all sources and sinks of CO₂ (N₂O and CH₄ emissions, farm inputs, operations, and soil C sequestration). Partial accounting data denotes partial accounting of sources and sinks (N₂O and CH₄ emissions and/or soil C sequestration). All data denotes inclusions of full and partial accounting data [71].

Sainju [71] observed that the relationships between net GWP, net GHGI, and N rate were further improved when the duration of the experiment and soil and climatic conditions were taken into account in the multiple linear regressions. Duration of experiment and annual precipitation had positive effects, but air temperature and soil texture had negative effects on net GWP when all sources and sinks of CO₂ emissions were accounted for. With partial accounting, only air temperature had a positive effect on net GWP, but other factors had negative effects. For net GHGI, the factors having negative effects were air temperature using the complete accounting of CO₂ emissions and annual precipitation and soil texture using the partial accounting. Sainju et al. [70] reported that net GWP and GHGI calculated from soil respiration and soil C sequestration methods were lower with 80 than 0 kg N ha⁻¹ (Table 8). They noted that, although CO₂ equivalents from N fertilization and soil respiration were higher with 80 kg N ha⁻¹, the amount of plant residue returned to the soil, soil C sequestration rate, and grain yields were greater

Cropping sequence ^a	N rate	Farm operation (A)	N fertilizer (B) ^b	Soil respiration (C)	N ₂ O flux (D)	CH ₄ flux (E)	Annualized crop residue (F) ^c	SOC (G) ^d	GWP _R (H) ^e	GWP _C (I) ^f	Annualized grain yield (J)	GHG _R (K) ^g	GHG _L (L) ^h	kg CO ₂ kg ⁻¹ grain yield	
														kg N ha ⁻¹	kg CO ₂ kg ⁻¹ grain yield
CTB-F	182	77	2722b ¹	425a	-16a	3476b	-114c	-89a	778a	1408b	-0.06a	0.55a			
NTB-P	124	91	3303a	469a	-16a	5980a	554a	-2005c	115b	1649a	-1.22c	0.07b			
NTCB	124	103	3547a	394a	-15a	5411a	268b	-1259b	337b	1683a	-0.75b	0.20b			
	0	143	3093b	416a	-16a	4421b	-94b	-787a	635a	1399b	-0.56a	0.45a			
	80	143	3288a	443a	-15a	5487a	566a	-1448b	185b	1761a	-0.82b	0.11b			

^aCropping sequences are CTB-F, conventional-till malt barley-fallow; NTB-P, no-till malt barley-peas; and NTCB, no-till continuous malt barley.
^bTotal CO₂ equivalents from direct and indirect sources of N fertilization.
^cTotal above- and below-ground crop residue.
^dCarbon sequestration rate calculated from linear regression of change in soil organic C at the 0–10 cm depth from 2006 to 2011.
^eColumn (H) = Column (A) + Column (B) + Column (C) + Column (D) + Column (E) – Column (F) [61]. Negative values indicate GHG sink.
^fColumn (I) = Column (A) + Column (B) + Column (D) + Column (E) – Column (G) [61, 62]. Negative values indicate GHG sink.
^gColumn (K) = Column (H)/Column (I) [61]. Negative values indicate GHG sink.
^hColumn (L) = Column (I)/Column (J) [61]. Negative values indicate GHG sink.
ⁱNumbers followed by the same letters within a column in a set are not significantly different at P ≤ 0.05.

Table 8. Net global warming potential (GWP_R and GWP_C) and greenhouse gas intensity (GHG_R and GHG_L) based on soil respiration and organic C (SOC) methods as influenced by cropping sequence and N fertilization rate in eastern Montana, USA [70].

with 80 than 0 kg N ha⁻¹, thereby resulting in lower net GWP and GHGI with N fertilization than without, regardless of the method used for calculation.

7. Conclusions

Nitrogen fertilization is one of the most commonly used practice to increase crop yields throughout the world because of abundant availability of N fertilizers and their great effectiveness to increase yields compared with other organic fertilizers, such as manure and compost. Excessive application of N fertilizers in the last several decades, however, has resulted in undesirable consequences of soil and environmental degradations, such as soil acidification, N leaching to the groundwater, and greenhouse gas (N₂O) emissions. Crop yields have declined in places where soil acidification is high due to unavailability of major nutrients and basic cations and toxic effect of acidic cations. Other disadvantages of excessive N fertilization include increased cost of fertilization, reduced N-use efficiency, and negative impact on human and livestock health. To reduce excessive N fertilization, composited soil sample to a depth of 60 cm should be conducted for NO₃-N test prior to crop planting and N fertilization rate be adjusted by deducting soil NO₃-N content from the desirable N rate.

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Advancement of Nitrogen Fertilization on Tropical Environmental

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Abstract

The nitrogen (N) fertilization synthetic or biological is primordial for food production worldwide. The consumption of N fertilizers in agricultural systems increased in exponential scale, mainly in developing countries. However, some negative points are associated to industrial N consumption; consequently the industry promoted ways to minimize N losses in production systems of tropical agriculture. Biological nitrogen fixation is a very important natural and sustainable process for the growth of leguminous plants, in which many micronutrients are involved, mainly as enzyme activators or prosthetic group. However, other mechanisms in the rhizosphere and molecular region still need to be clarified. Therefore, the aim of this chapter is to compile information about the historical and current affairs about the advances in N fertilization in tropical environments through a history from N fertilization world-wide, N balance in the main agricultural systems, introduction of alternative ways to avoid N losses, advances between BNF and micronutrients, as well as the effects of N absence in plant metabolisms. Biological nitrogen fixation is a very important natural process for the growth of leguminous plants, in addition many metallic nutrients, micronutrients, are involved in BNF metabolism, mainly as enzyme activators or prosthetic group. But other mechanisms in the rhizosphere and molecular region still need to be clarified.

Keywords: ammonia synthesis, biological N fixation, humic substances, N balance, volatilization

1. Introduction

Hellriegel and Wilfarth showed definitive evidence for N_2 fixation by microbes in legumes in 1886, but the industrial process to fertilizer production known as the Haber-Bosch was established just in 1906, which uses a catalytic agent at high pressure and high temperature [1].

Actually, the world population has now been increasingly relying on nitrogen (N) fertilizers in order to keep up with the demands of food and economic growth rates; on the other hand, less than 30% of synthetic fertilizers would actually be

utilized; the unused chemicals sprayed on crops would be lost in the field and could subsequently cause serious environmental problems.

Urea is a popular N source in developing countries due to its advantages of a high N content, safety, and easy transportation [2]. However, the increase of pH and surface soil NH_4^+ concentrations resulting from urea hydrolysis can exacerbate NH_3 emission.

This causes low N use efficiency, especially in alkaline soils or soils with low sorption capacity, which limits the use of urea fertilizer in Europe [3]. In tropical areas, increasing the adoption of no-tillage systems also induces to high N losses from urea fertilization, in tropical soils, due to high temperatures and moisture; NH_3 losses exceeding 40% of the surface-applied urea N have been reported, especially under no-till or perennial crops where plant residues are kept on the soil surface [4].

Nitrogen losses by NH_3 emission not only brings economic loss to farmers, but also detrimental effects to ecosystems and human health, while the biological nitrogen fixation (BNF) has the advantage of being environmentally friendly and therefore would be ideal for sustainable agriculture.

Enormous progress in almost all aspects of BNF has been made in the past century, especially in the recent two decades, in genetics and biochemistry, culminating in the determination of the crystallographic structures of both nitrogenase components and micronutrients metabolism.

These information collaborated to elucidate N assimilation routes in plants clarifying further its essentiality and allowing to infer that plants can be affected negatively in molecular even genetic level in N absence.

Therefore, the aim of this chapter is to compile information about the historical and current concerns about the advances in N fertilization in tropical environments through a history from N fertilization worldwide, N balance in the main agricultural systems, introduction of alternatives ways to avoid N losses, advances between BNF and micronutrients, as well as the effects of N absence in plant metabolism.

2. History of nitrogen fertilization on tropical environmental

Nitrogen is an essential element to all organisms, because it is part of protein, acids, and other organic compounds [5]. The importance of this nutrient for plants is already known since the 1660s; however, only at 1804 De Saussure received credits for N essentiality after observations of nitrate uptake from soil solution. In this same period, other researchers, as Liebig at 1840, fortified the idea of plants absorb N from atmosphere [6, 7].

Around 78% of the atmosphere gas is compound for N however in gaseous form chemically unavailable. In front of the increased demand by food production and need of N restitution after crop harvests, Fritz Haber at 1909 synthesizes the gaseous element to ammonia (NH_3) through a reaction with hydrogen and iron on high pressure and temperatures, which posteriorly was industrially developed by Carl Bosch in 1912–1913, resulting at the known Haber-Bosch process [8].

The N sources used on agricultural activities, even at the end of the eighteenth century, were from crop residues and animal manure modified or not through composting. The production and management of N fertilizers to increase crop yield, as well as corn [9, 10] and wheat [11] around the world [12] have begun at the Green Revolution of the nineteenth century, followed by ammonia synthesis in the beginning of the twentieth century and the increased need of high yield on agricultural areas [13].

World	2015	2016	2017	2018	2019	2020	Reference
Total capacity NH ₃	174.781	181.228	185.222	186.804	186.920	188.310	[11]
Africa							
Total capacity NH ₃	8.310	9.545	10.739	10.700	10.700	11.000	
Americas							
Total capacity NH ₃	24.301	27.618	28.688	29.304	29.320	29.346	
Asia							
Total capacity NH ₃	99.959	101.188	101.703	101.734	101.734	102.799	
Europe							
Total capacity NH ₃	40.378	41.044	42.338	43.211	43.311	43.311	
Oceania							
Total capacity NH ₃	1.833	1.833	1.854	1.854	1.854	1.854	

Table 1.
Estimative of supply capacity of N (NH₃) in continents (in thousand tons) of 2015–2020 (adapted of FAO [12]).

World	1960	1980	2000	Reference
Animal manure applied in soil	22%	16%	14%	[14]
Animal feces	56%	40%	40%	
Synthetic fertilizers	22%	44%	46%	
Africa				
Animal manure applied in soil	4%	4%	4%	
Animal manure left in pasture	91%	84%	84%	
Synthetic fertilizers	5%	12%	12%	
Americas				
Animal manure applied in soil	16%	13%	13%	
Animal manure left in pasture	60%	50%	47%	
Synthetic fertilizers	24%	37%	40%	
Asia				
Animal manure applied in soil	20%	13%	12%	
Animal manure left in pasture	61%	34%	30%	
Synthetic fertilizers	19%	53%	58%	
Europe				
Animal manure applied in soil	40%	28%	30%	
Animal manure left in pasture	27%	17%	17%	
Synthetic fertilizers	33%	55%	53%	
Oceania				
Animal manure applied in soil	2%	3%	4%	
Animal manure left in pasture	96%	91%	77%	
Synthetic fertilizers	2%	6%	19%	

The percentual represents averages from the 1960s, 1980s, and 2000s (adapted of FAO [12]).

Table 2.
Global cumulative of N fertilization from animal manure and fertilizers between 1961 and 2014.

Data from the FAO [14] estimated that the global capacity of N ammonia offer increases annually of 1.5% in average, with production of 174,781–188,310 thousands of tons of 2015–2020 (**Table 1**).

In addition, during this period, Africa, Oceania, Europe, and the Americas increased the capacity to 32.4, 1.1, 7.3, and 20.8%, respectively, however, stands out to Asia continent with the highest productive capacity estimated to 102,799 thousands of tons of N to 2020 (**Table 1**).

Estimates in global scale from FAOSTAT [15] show N inputs from animal manure increased from 66 to 113 million from 1961 to 2014, while N fertilizers applied in soils increased from 18 to 28 million of tons of N, respectively.

The use of N fertilizer at Europe continent increased 33% (about 5 million of tons of N), as a similar tendency observed in others regions (**Table 2**).

Brazil is one of the biggest fertilizer consumers in the world. The significant increase in fertilizer consumption occurred between 1988 and 2010 [16] as consequence of public policy implementation and Brazilian agriculture modernization.

Nitrogen had a higher growth consumption among the nutrients from NPK in the analyzed period, around 250%, from 814,952 to 2,854,189 tons; however, N fertilizers consumption was 12,211,855 ton from 2010 to 2013 and to around 15,469,549 tons from 2014 to 2017 [17, 18].

3. Nitrogen balance in the tropical agricultural systems

Nitrogen balance in the systems becomes a concern for tropical agriculture as a result of the high scale of N fertilizer production. Nutrient balance is a parameter that analyzes the relation between quantity of vegetable biomass produced and nutrient applied. Besides, nutrient balance is a tool with easy application and able to guide the management to efficient fertilization [19].

Nitrogen balance as a management technique accounts the nutrient exportation by crops, residual in soil and the N losses [20]; thus it is essential to a balanced fertilization strategy aiming to maximize the economic return and ensure the environmental quality.

The calculations of nutrient balance evaluation must account for the input and output of N because this nutrient can be distributed by soil, plant, and animal (**Table 3**). Between 95 and 100% of the total N input into soil is from the surface through rainfall or dust and aerosols, irrigation, runoff and groundwater, biological fixation by phototrophic and heterotrophic organisms, organic and inorganic fertilization, and seed reserves. Besides the plants exports, the N output occurs by erosion, leaching and drainage, ammonia volatilization, denitrification, and senescence plants [21, 22].

Brazilian crop exports 50% of N in harvested product mainly by the largest exportations of soybean (70%), corn (15%), sugarcane (8%), rice (2%), and wheat (2%) [17]. However, these N quantities have contribution from the N biologic fixation (NBF), mainly from soybean with 82% of the total N input in crops production.

Soybean occupied the largest area of agriculture in Brazil between 2013 and 2016 and also was responsible for the largest nutrient exportation, although N is not applied in this crop, it comprised 70% of the total N exported by all crops, while phosphorus and potassium reached 57.5 and 56.8%, respectively [23]. Analyze nutrient exportation for area unity in this period was found out the largest nutrient exporters were soybean (181 kg ha⁻¹), tomato (159 kg ha⁻¹), and cotton (129 kg ha⁻¹).

Source	Amount	References
N input		[3]
Total N fertilization rates	A	
Total manure applied	B	
N symbiotic fixation	C	
Atmospheric deposition of N	D	
Irrigation water	E	
N input by seed in harvest	F	
N nonsymbiotic fixation	G	
Total N input	$X = A + B + C + D + E + F + G$	
N output		
N exportation in crops and/or biomass	I	
N losses by denitrification	J	
N losses by ammonia volatilization	K	
N losses by plants senescence	L	
Gaseous losses of N (except NH ₃ volatilization)	M	
N losses by surface runoff	N	
N leaching	O	
N losses by soil erosion	P	
Total N output	$Y = I + J + K + L + M + N + O + P$	
Total N in soil		
Total N in beginning of the experiment	Q	
Total N in end of the experiment	R	
Total N changes in soil	$Z = R - Q$	
N balance		
During experiment performance	$N_b = X - Y - Z$	
N balance for year (kg N ha⁻¹ yr⁻¹)	$N_b / \text{Years of experiment}$	

Table 3. N balance from total of inputs and outputs and N in soil in the beginning and final of agricultural experiments, modified from [3].

4. Ways to avoid N losses from agricultural systems

In agricultural systems there are losses in general; however, N losses are considered highly relevant [24, 25]. Nitrogen losses are a potential contaminant and can impact production cost. Nitrogen is a dynamic element in soil and can be lost to the atmosphere by denitrification and ammonia volatilization [24, 25].

Ammonia volatilization is a concerning problem because it represents high N losses in soil–plant system besides to be a threat for global environmental [26], while the N losses by denitrification in tropical areas are less significant in consequence of its restriction in the use of nitrate as fertilizer due its explosive potential [25–28].

Global agricultural production is responsible for 50% of N losses by ammonia volatilization meaning 37 tons of N for year; however, the losses can be higher according to the N source, application way, soil management, climate, soil temperature, and humidity [29–34].

	Rate of applied (kg N ha ⁻¹)	Mean % N volatilized	Location	Reference
Grassland soils	180	22.8	Argentina	[16–22]
	15–200	17.6	New Zealand	
	50	36.0	USA	
	30–150	26.7	UK	
	25	7.5	New Zealand	
Arable soils	50	55	Brazil	[11, 17, 23–29]
	150	30	Brazil	
	120	77	Brazil	
	90	17.8	Denmark	
	200	30	India	
	60	79	Argentina	
	46	23	Australia	

Table 4.

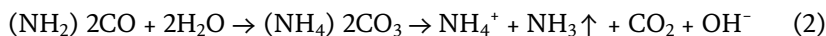
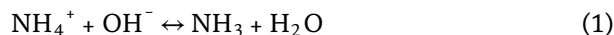
Examples of ammonia volatilization due to urea application in different soils, modified from [20].

Urea is the most N source used in the world; however, also it has high susceptibility to be lost in agricultural systems [24, 25]. The high presence of urease enzyme in soil causes a rapid hydrolysis of urea and, consequently, ammonia losses to the atmosphere [35].

Variable quantities of ammonia lost to the atmosphere were related by urea use in agriculture [35–37] according to the exemplified in **Table 4**.

Urease is an extracellular enzyme naturally presents in soil, plants, and microorganisms acting as a catalyzer of urea in the hydrolysis process [30–32]. This chemical process induces excess of protons (H⁺); consequently it rises pH in soil around the fertilizer granules of 6.5–8.8 or until 9.0 causing unbalance between ammonium (NH₄⁺) and ammonia (NH₃) [33, 34].

During hydrolysis ammonium carbonate is formed, which is dissociated to produce ammonia ions and hydroxide; however, the relative concentration of ammonia and ammonium is determined by the pH in soil solution, and ammonia is favored under high pH condition according to equations [28].



Researches about urease inhibition in soil have begun over than 70 years ago, resulting in many compounds evaluated and patented as urease inhibitors [38]. Urease has a great effect on the soil-plant system through plant N efficiency, as well as being a versatile enzyme, presenting technological, biotechnological and transgenic applications [39].

Nitrogen losses can be avoided or reduced through organic or inorganic chemical compounds included in urea as an able technology to increase the efficiency of N fertilization at low cost [40–42]. Urea with urease inhibitor can cost around 30% higher than conventional urea [43].

The phosphorotriamides, hydroquinone, catechol, copper, boron, and zinc are the most evaluated additives as urease inhibitor [44]. There are more than

40 phosphorotriamides synthesized considered the most effective compound to urease inhibition because its composition comprises a functional group containing P=O or P=S bonded for at least one free amide (NH₂) to react with urease active sites and they are considered [45].

Urease inhibitor known as NBPt (N-(n-butyl) thiophosphoric triamide) has been the most used additives in Brazil, in which urea is the most used N source.

This additive is dissolved in a nonaqueous solvent to adding characteristics as (i) larger stability to NBPt molecule under temperature, humidity and transportation variances, and (ii) higher solubility; (iii) improves adherence of mix solvent + NBPt to urea granule, (iv) low toxicity, and inflammable potential; and (v) acts as buffer agent to keep alkaline pH similar to hydrolysis environment of urea in soil providing NBPt stability [43].

The largest of compounds used along with urea are low efficient when applied in soil [43]. NBPt aim is to retard the ammonia volatilization peak [46]. Generally, chemical compounds with similar structure as urea can be more efficient to retard the volatilization; thus, the bond sites and length of amide of phosphoryl triamide are similar to urea; however, there are no substrates for urease [45].

Recently, lab researches reported beneficial and/or synergic effects of the humic substances use with urea [47–49]; however, the action mechanism is still unknown [49]; also depending of humic substances, the results can be contradictory [50, 51], but there are hypotheses that urease enzymes reduce with the association of humic acid and urea [48]; besides it minimizes N losses, it can improve buffer effect in soil pH [52].

Urease inhibitor and humic substances with urea at adjusted pH (pH = 7) provided reduction of 50% from total N volatilization on a Latossolo Vermelho on sugar cane [53].

5. Interaction between biological N fixation (BNF) and micronutrients to higher plants

Biological N fixation (BNF) is an important process to global agricultural systems. This phenomenon was discovered in the mid of the nineteenth century by the German chemist Hermann Hellriegel (1831–1895); however, factors on root nodules were unknown, until the Dutch microbiologist and botanic Martinus Beijerinck (1851–1931) identifies microorganisms on root nodules able to realize chemical process to transform atmospherically N to ammonia allowing fixation and absorption by plants, proving the symbiosis between legumes and bacterial [54].

Fixation biological of N₂ (BNF) through the bacteria from genus *Bradyrhizobium* can supply N quantity necessary in legume crops as soybean, besides it is currently observed for many researchers as a clean technology contributing to replace mineral N fertilizers in legume crops [55].

Nitrogen fixation by bacteria already is well described [56]; however, currently studies are focused in nutrients involved in this metabolism, especially micronutrients [57, 58]. Among the micronutrients able to influence the BNF are boron, copper, zinc, cobalt, iron, nickel, manganese, and molybdenum, essential as structural components and enzyme activators in plants [56–59].

Iron is necessary to the production of cofactor FeMo that acts along with nitrogenase enzymes, which can affect significantly the BNF [60]. Excess or default of zinc and nickel can affect the established bacteria inside of the nodules and its symbiosis with plants [57].

There was an increase in BNF and N uptake as a result of the growth of nodules in number and mass with boron foliar application, and these results were attributed

to the role of boron in the induction of nitrate assimilation by increasing protein synthesis by plant [58].

Manganese has direct role on many enzymatic processes on the BNF, including amide hydrolase enzyme which is directly dependent of Mn^{+2} , and it is responsible for ureide degradation being able to control the BNF under hydric deficiency [61].

Low copper affects the nodule formation and reduces the quantity of fixation bacteria; this element is essential for both bacteria and plants; however, its direct role on BNF is still unclear [59].

Molybdenum is an essential nutrient to BNF taking part on nitrate reductase with the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and on the nitrogenase process in conversion of dinitrogen (N_2) to ammonia (NH_3) by fixation bacteria. The low quantity demand of molybdenum allows its application on soil and foliar or even by seeds treatments, which is a form of quality aggregation to the seeds by affecting positively on germination [62].

Cobalt is a component of cobalamin and leghemoglobin synthesis, which is controlling their levels on nodules and avoiding nitrogenase enzyme inactivation; thus this element can be considered essential to N_2 fixation [63].

Nickel can affect directly the presence and quantity of fixation microorganisms because it is a hydrogenase component (Ni-Fe), which can recycle H_2 that is

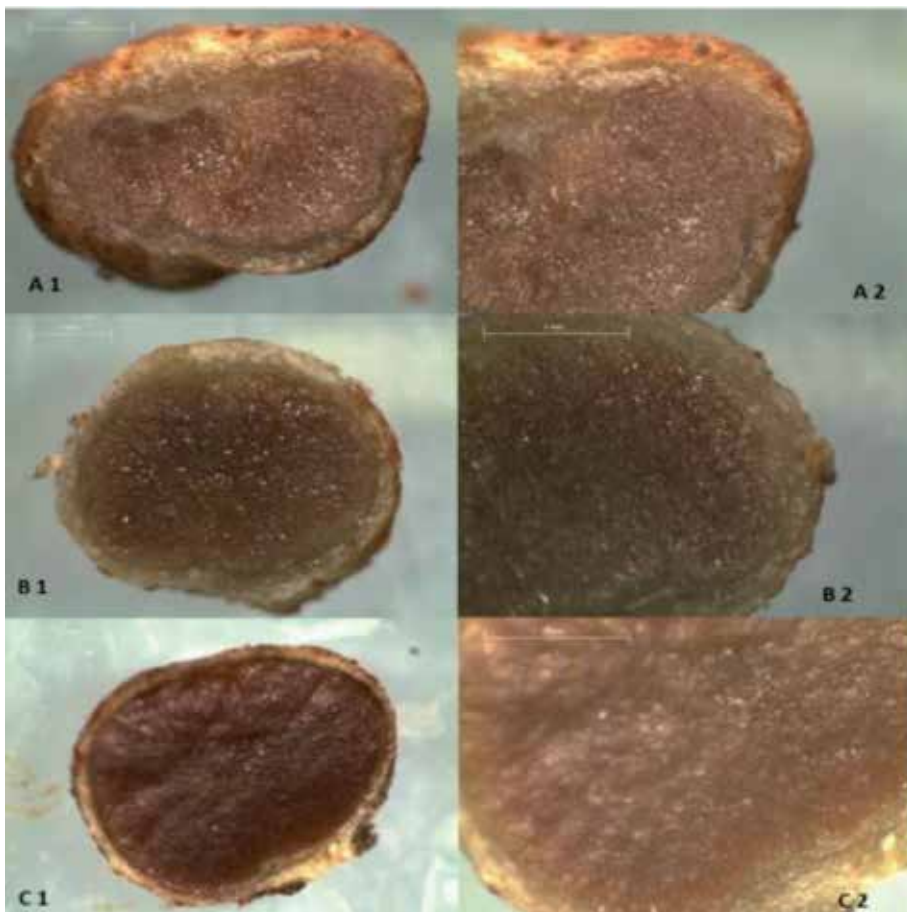


Figure 1. Root nodules from legume. A1, longitudinal section; A2, approximated image on nodules developed with no Ni; B1, longitudinal section; B2, approximated image on nodules developed with 0.5 g dm^{-3} of Ni; C1, longitudinal section; C2, approximated image on nodules developed with 10 g dm^{-3} of Ni [64].

generated from N reduction and could affect positively or negatively the legume metabolism [64]. Nickel balance on BNF can be seen on fixation nodules where in its absence causes large formation of internal cells according to **Figure 1**.

6. Recent reports about N absence on plants metabolism

Even though the essentiality had been established for N at higher plants, there are still remained doubts about how the N absence can affect the metabolism. Recently, by modern techniques and sensible equipment, it was possible to determine clearly as N absence affects plant metabolism and production.

The N deficiency exposure of *Olea europaea* plants was described as a significant decrease on chlorophyll a and net photosynthetic rate (**Figure 2**). Photosynthesis is a process that involves light absorption by the photosynthetic pigments present in

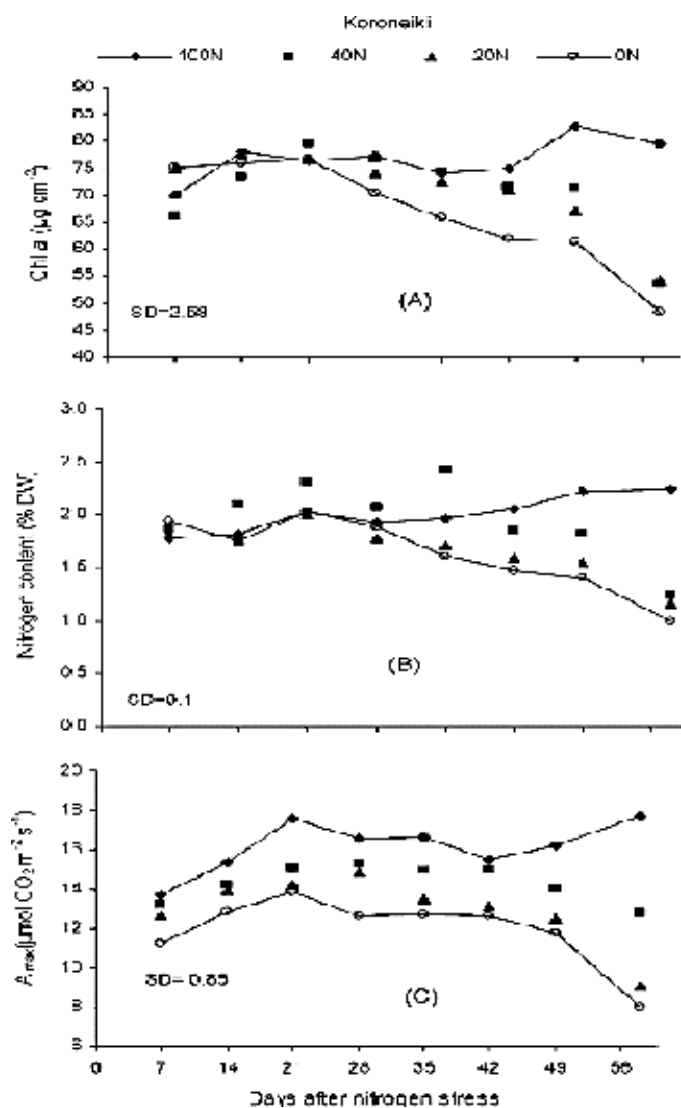


Figure 2. Chlorophyll a (Chl a), nitrogen content, and net photosynthetic rate (A_{max}) in *Olea europaea* plants exposed to nitrogen deficiency [65].

light-harvesting complexes, being crucial for plant development and largely dependent on the leaf N content, because N composes the chlorophyll molecules [65].

The effects of N deficiency in the leaves of *Oryza sativa* seedlings were verified that the fluorescence parameters were negatively modulated in N-deficient plants [66]. While **Figure 2** presents few modifications until the fifth day in N-deficient plants, when compared with control plants, however as nitrogen deficiency continued, chlorophyll fluorescence of N-deficient plants was significantly impacted, in comparison with control plants.

The decrease in the ratio Fv/Fm of plants under water deficit indicates reduction in the photochemical activity, leading to the inhibition of the photosynthetic rate and the generation of reactive oxygen radicals in the chloroplast, causing damages to PSII components. Additionally, the decline in ETR values of plants under water deficit is due to the deficiency of plastoquinone (PQ) used in oxidation-reduction reactions.

7. Concluding remarks

Nitrogen fertilizer consumption follows the increasing demand by food, fiber, and energy production. The quantification of nitrogen inputs and outputs on agricultural system has been a useful and efficient tool to the evaluation of managements, mainly to the tropical agricultural.

Biological fixation is an important nitrogen input to productive systems comprising benefits in economic and environmental concerns, mainly for tropical agriculture; however, the narrow relation among this process and micronutrients and its metabolic routes still needs to be clarified.

Advances of the N fertilization on tropical environment reported at this chapter are focused mainly in an attempt to reduce ammonia volatilization from urea in consequence of its largest use as N source.


Among urease inhibitors used in tropical agriculture, NBpt has been highlighted; however, humic substances have been shown as a future alternative to reduce ammonia volatilization that still requires knowledge about its origin, molecular composition, and environmental questions.

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Comprehensive Account of Inoculation and Coinoculation in Soybean

Muhammad Jamil Khan, Rafia Younas, Abida Saleem, Mumtaz Khan, Qudratullah Khan and Rehan Ahmed

Abstract

This chapter elaborates dependency of leguminous plants on rhizobia to carry out dynamic process of nitrogen fixation. Soybean, an extensively grown leguminous crop with 30% share in world's vegetable oil, is taken into account to understand its symbiotic relationship with plant growth-promoting rhizobacteria (PGPRs). This chapter narrates colonization of PGPRs on soybean roots and single and mixed inoculation and coinoculation of certain strains of specialized bacteria with rhizobia. PGPRs' coinoculation seemed more effective than mono-inoculation and is discussed in Ref. to nodulation rate. Moreover, dynamic linear models for quantification of leguminous biological nitrogen fixation (BNF) are reviewed. This chapter further uncoils the relevance of foliar application to the release of phytohormones by PGPRs, resulting in situ biosynthesis of active metabolites in phyllosphere. Inoculation of phytohormones is compared to their exogenous application for nodule organogenesis. Finally, the influence of coinoculation on enhanced micronutrient bioavailability is relayed. The chapter is concluded with technical and economic aspects of coinoculation in soybean.

Keywords: legumes, nodulation, BNF, phytohormones, mixed inoculation

1. Introduction

Better plant growth is ensured by the balanced availability of essential nutrients in soil. Each nutrient has its own function and is required in different amount depending on the plant demand. Nitrogen (N), one of the most essential macronutrients, is routinely applied through chemical fertilizer as most field crops require large amounts of it. Nitrogen, the fifth most abundant element in the universe, was first discovered in 1772 by a Scottish physician, Daniel Rutherford. Due to its essentiality for survival of life on earth, it was called as "azote," meaning "without life," by Antoine Lavoisier about 200 years. Nitrogen is essential for the sustenance of life on this planet as it serves as building block for the synthesis of proteins. The inevitable role of N is well acknowledged in several biochemical processes such as cell division, growth promotion, and photosynthesis, as part of vitamins and carbohydrates and energy reactions in the plant body [1, 2]. Deficiency of N in plants is

recognized by the symptom of delaying maturity of plant which leads to the late blooming. Deficiency symptoms also include chlorosis of leaves (light green or yellowing of leaves) and retarded plant growth. Due to high mobility of N, these deficiency symptoms first appear in older leaves of the plant [3].

The gaseous form of N is termed as dinitrogen (N_2) which accounts for 78% of the total gaseous content of the atmosphere. This form of N is unavailable for plants until it is fixed and converted into ammonium and nitrates, the forms in which plants can uptake N [4]. Soils contain both organic and inorganic N; however, organic form constitutes a major part of total soil N content. Plants, on the other hand, can use only specific inorganic forms of N like nitrate and ammonium. Like phosphorus (P) and carbon (C), N undergoes biogeochemical conversion from gaseous state to mineralized form in soil followed by its return to the atmosphere in the gaseous phase. The net concentration of N_2 per year was estimated to be 3×10^9 tons on global basis [5]. Nitrogen cycle is considered to be a biogeochemical cycle, where the N changes into different chemical forms and shifts to different ecological spheres of the earth. The fundamental components of N cycle are decomposers and N-fixing bacteria. Nitrogen cycle initiates with microbial fixation of N in the soil, where mineralization of N takes place by conversion of atmospheric or organic N into ammonium, a process known as ammonification. Further, ammonium is converted into nitrate by soil microbes and nitrifying bacteria, e.g., *Nitrobacter* and *Nitrosomonas* species. Denitrification is the ultimate step carried out by the denitrifying bacteria such as *Pseudomonas* and *Clostridium*, which decompose nitrate and convert it into N_2 , thus returning N_2 back to the atmosphere.

2. Nitrogen fixation

The fixation of N involves conversion of N_2 into various nitrogenous compounds such as ammonium and nitrate, so that they may become more reactive and plant available.

2.1 Industrial N fixation

Industrial N fixation involves the Haber-Bosch process which is an energy-inefficient method for making nitrogen fertilizers:



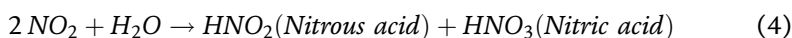
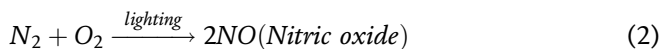
2.2 Natural N fixation

N fixation can be biological and nonbiological in natural environment.

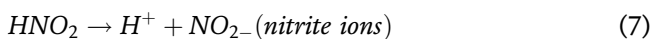
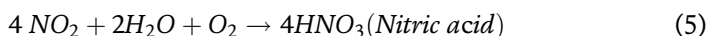
2.2.1 Nonbiological N fixation (lightning)

In nonbiological fixation, a relatively small amount of N is fixed by a spontaneous reaction that occurs during lightning. It is estimated that about 10% of the world's supply of fixed N comes from lightning [6]. Lightning can be described as occurrence of a sudden electrostatic discharge during a thunderstorm. During lightning, atmospheric nitrogen reacts with oxygen to form nitric oxide (NO). In the presence of excessive O_2 , nitric oxide oxidizes to nitrogen dioxide (NO_2). In the presence of water, NO_2 may react to form nitrous (HNO_2) and nitric acid (HNO_3) or may react with rainwater and oxygen to produce nitric acid. These acids find their way to reach the soil with rainwater, interaction with alkaline substrates

occurs, and hydrogen is released forming nitrate (NO_3^-) and nitrite ions (NO_2^-). The nitrate ions can be readily consumed by microbes and plants. However, soil microbes are not directly involved in this kind of N fixation. The chemical reactions involved in such N fixation are presented below:



OR



2.2.2 Biological N fixation

Biological fixation of N_2 is carried out by N-fixing bacteria in soil. This fixation accounts for approximately 60% of fixed N in soil. Fixation of N_2 by microbes is termed as biological N fixation (BNF). Soil microbes are diazotrophs (bacteria and archaea) that contain enzyme nitrogenase, capable of converting N_2 into ammonium and nitrates, a process termed as nitrification. Common diazotrophs are rhizobia, blue-green algae (cyanobacteria), *Azotobacter*, *Frankia*, and green sulfur bacteria. Diazotrophs usually have a symbiotic relationship with leguminous family of plants. The major legumes are flowering plants like soybean, peanuts, clover, and lupines, tea plants like rooibos, and grasses such as alfalfa. The roots of legumes contain small protrusions called as nodules. These nodules are anchored by diazotrophs, providing anaerobic conditions for diazotrophs, further necessary for nitrogen fixation. Plants in turn use this fixed N for different functions. Upon death of the plants, this fixed N is released to the soil and acts as a nitrogen source for soil and non-leguminous plants. Nitrogen fixation is an energy-intensive process. One molecule of nitrogen gas breaks into its atoms and combines with hydrogen to form 2 molecules of ammonia at the expense of 16 molecules of ATP and a complex set of enzymes. Its reduction reaction can be written as:



2.3 Classification of biological nitrogen fixation (BNF)

BNF can be classified into nonsymbiotic (free-living) and symbiotic (in association).

2.3.1 Nonsymbiotic biological nitrogen fixation

Microorganisms that fix atmospheric nitrogen independently are known as free-living diazotrophs. This type of fixation is carried out by free-living microorganisms. Examples of free-living organisms, which fix N, are cyanobacteria (blue-green algae, e.g., *Anabaena*, *Calothrix*, *Gloeothece*, and *Nostoc*), aerobic (*Azotobacter*, *Azospirillum*, *Beijerinckia*, *Derrxia*), facultative (*Bacillus*, *Klebsiella*), and anaerobic

(non-photosynthetic such as *Clostridium* and *Methanococcus* and photosynthetic such as *Chromatium* and *Rhodospirillum*).

2.3.2 Symbiotic biological nitrogen fixation

Symbiotic nitrogen fixation, carried out by specialized soil bacteria as discussed above, is the good source of N for plants. In return, plants provide required nutrients and energy for bacterial growth. Upon the death of nitrogen-fixing bacteria, nitrogen is released to the environment, and some non-leguminous plants may benefit from that nitrogen. In leguminous plants, nitrogen-fixing bacteria colonize on plant roots forming nodules. Within these nodules, nitrogen fixation is carried out by the bacteria, and the end product, NH_3 , produced is absorbed by the plant [7].

2.4 Legumes

Legumes belong to Fabaceae or Leguminosae family and are primarily grown for human consumption, as forage and silage for livestock, and act as a green manure for enhancing soil fertility. Some common legumes include alfalfa, soybeans, chick peas, pigeon peas, clovers, cow peas, kidney, lentils, mung beans, peanuts, peas, and vetches. These are native to tropical rain forests and dry forests in America and Africa [8]. Legumes consist of 750 genera and 19,000 species of herbs, shrubs, trees, and climbers.

Legume seeds (pulses or grain legumes) are the major part of human diet. Nutritionally, legume seeds are rich in protein contents as compared to cereal grains. The combined use of legumes and cereals may provide necessary dietary proteins. Legumes are also used as pasture and animal fodder in which soybeans are most commonly used. Legumes, as green manure, improve soil quality by adding nitrogen and organic matter. Legumes are used in crop rotation for the sustainable crop production. About 2500 species of Leguminosae produce root nodules.

2.5 Soybean

The soybean (*Glycine max* L.), commonly called soja bean or soya bean, is a legume species native to East Asia. It is enormously grown for edible seeds and oil extraction. The major countries involved in cultivation of soybean are the United States, Brazil, and Argentina. Soybean is the most economical source of vegetable protein around the world. It is also involved in the production of several chemical products. Many botanists believed that soybeans were first cultivated in central China earlier in 7000 BC and in the United States in 1804 [9]. Soybean appears to be an erect branching plant with length more than 2 m. It is a self-pollinated plant with adoption to various cultivable lands. This plant conveniently cultivate in fertile, well-drained, and sandy loam with relatively warm conditions. The vital source of N in legumes is nodulation prevailed by N-fixing bacteria. Soybean can fulfill 50–70% of its N demand from the air by establishing root nodules through adequate population of N-fixing bacteria.

2.6 Nodule formation

Nitrogen fixation in legumes starts with the formation of small, knob-like protuberances called nodules. The bacteria get all the necessary nutrients and energy from the plants. The roots of legumes release chemicals known as flavonoids to attract the bacteria [10]. In response to flavonoids, the soil bacteria produce nod

factors. Nod factors are signaling molecules which are sensed by the roots. As a result, a series of biochemical modifications lead to cell division in the root to create the nodule. Lectins, a sugar-binding protein in root hairs of legumes, are activated by nod factors. This helps in the recognition and attachment of rhizobial cells to the root hairs whose tips in turn become curved. The growing root hair curls around the bacteria in several attempts until one or more bacteria are enclosed. The enclosed bacteria colonize and eventually enter the developing nodule through infection thread. Infection thread is a structure extended through the root hair into the epidermis cell and then comes out of the root cortex. The bacteria are then surrounded by plant-derived membrane. Rhizobial multiplication starts in cortical cells which results in the formation of nodule on the surface. In side nodules, the bacterial cells continue multiplication and colonization until host cells are completely filled. After that bacterial cell becomes dormant bacteroids and starts floating in leghemoglobin. Leghemoglobin is a reddish pigment in cytoplasm of host cells which efficiently scavenges O_2 so that maintenance of the steady state of oxygen and stimulation of ATP production is possible. Plants provide shelter and organic compounds to the rhizobia, and in turn rhizobia provide fixed nitrogen to the plant. Among leguminous crops, soybean takes great consideration due to higher contribution of BNF. Normally, nodulation occurs after 4 weeks of plantation. The small nodules become visible after 1 week of the infection. The color of nodule appears white or gray when nitrogen fixation is insufficient, whereas color changes to pink or reddish as N_2 fixation progresses. This color change is attributed to the occurrence of leghemoglobin which is similar to blood hemoglobin that regulates the flow of oxygen to the rhizobia.

Perennial legumes such as alfalfa, clover, etc. develop nodule about half an inch capable of fixing N throughout the growing season. Annual legumes like beans, soybeans, and peanuts have short-lived nodule, round in shape with size of pea. These nodules are continuously replaced during the growing season. Annual legumes provide nourishment to developing seed instead of nodules; therefore, nodules cannot fix N anymore. The number of nodules varies per plant species, e.g., on average beans comprised of <100 nodules per plant, soybean can have several 100 nodules per plant, and peanut may have >1000 nodules per plant. Nodules on annual legumes, such as beans, peanuts, and soybeans, are short-lived and round in shape and can reach the size of a large pea and will be replaced constantly during the growing season. At the time of pod fill, nodules on annual legumes generally lose their ability to fix nitrogen because the plant feeds the developing seed rather than the nodule. Beans have less than 100 nodules per plant, soybeans will have several hundred per plant, and peanuts may have 1000 or more nodules on a well-developed plant.

Nodulation is regulated by both external and internal processes. Soil temperature, soil N mineral content, acidity of soils, and water scarcity can be categorized as external factors, whereas autoregulation and ethylene are the most influential internal factors. Autoregulation of nodule (AON) specifies the number of nodules per plant. Leaf tissue via chemical signal can sense the onset of nodulation and inhibit it in the developing root. Such chemicals are leucine-rich repeat (LRR) receptor kinases that are crucial for autoregulation of nodule formation. The mechanism for nodule formation is coded by *enod40* gene also called nodulin 40. Its expression leads to relocalization of nuclear proteins.

Microbes inhabiting soil can be termed as plant growth-promoting rhizobia (PGPR) due to their multifunctionality in symbiotic relationship with plant. PGPRs play role in plant nutrition by mineralizing nutrients in rhizosphere. PGPRs as indicated by name actively participate in phosphate solubilization and production of siderophore, phytohormones, and several enzymes. The biochemical

characteristics of PGPR, for instance, lipopolysaccharides (endotoxins), homoserine lactones (signaling molecules), acetoin (preventing over-acidification in cytoplasm), and flagella (locomotive and sensory organs) help plants to develop systematic resistance against pests and pathogens. The PGPRs enhance tolerance against extremity of environmental conditions such as drought, nutrient deficiency, and prevalence of organic (pesticides) and inorganic (heavy metals) toxicity. PGPR, therefore, are considered as biofertilizers for sustainable agricultural practices.

3. Inoculation and coinoculation of PGPRs

Soybean develops symbiotic relationship with a range of PGPRs to fix nitrogen (N) and improve plant growth [11–13]. Establishment of symbiotic relationship between roots of the host plant and symbiont is a two-step process. In first step, host tissue is infected with rhizobacteria and in second nodule formation occurs. Plant roots contribute in the symbiotic relationship by releasing flavonoids, while rhizobacterium produces nodulation factors. Rhizobacterium is entrapped in plant hairs' curls, and infection threads are formed at the root hair curls, permitting bacterial invasion of the root tissue. The process of nodulation is initiated just below the infected point. Rhizobacterium may be restricted to infection threads, but mostly, they are released into nodule cells where nitrogen fixation occurs.

3.1 Inoculation

Inoculation and coinoculation of PGPRs have become a popular research area in recent crop production. The interest in rhizosphere microbiology was developed due to the beneficial effects of some free-living strains of bacteria on plant growth and disease control and maintaining good soil health. Initial studies were focused on bacterial genera including *Pseudomonas*, *Rhizobium* spp. *Azotobacter*, *Bacillus*, and *Azospirillum* to enhance plant growth by fixing atmospheric nitrogen [14, 15]. However, later research was shifted to elucidate the role of PGPRs in promoting plant growth by mineralizing organic phosphorous, solubilizing inorganic soil phosphorous, modulating plant hormones, and rendering plant tolerance to adverse environmental conditions [11, 16]. This has triggered diversified application of PGPRs' inoculation and coinoculation in various field crops. The term “inoculation” may refer to “natural or deliberate application of certain beneficial strains of bacteria to plant seeds or soil to enhance plant growth.” Inocula, the strain of bacteria used in inoculation and coinoculation, may be native or alien, with inherent or engineered ability to colonize plant roots and promote plant growth. Plant growth-promoting genera may include different strains of *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Rhizobium*, *Mycobacterium*, etc. Bacterial inoculation has increased yield in many crops using *Azotobacter* and *Bacillus* strains. Steadily, the research focus has been shifted to *Azospirillum* from *Azotobacter* due to better crop yields reported with the later. Similarly, *Bacillus subtilis* and *Pseudomonas* spp. have been proven to be effective in controlling plant pathogens of soil origins. One major positive effect of inoculation is the solubilization of inorganic phosphate in the soil to make it plant available. Root exudates greatly influence the colonization of rhizobacteria. However, one major challenge in successful inoculation is the colonization of PGPRs in the rhizosphere where indigenous microbes may limit survival of the introduced bacteria. This has been addressed through introduction of antibiotic-resistant *rhizobacteria*. Besides these, soils are complex heterogeneous environments with great variations in particle size distribution, pH, organic matter

content, temperature, water, and availability of nutrients that may greatly influence inoculation success.

3.2 Coinoculation

To overcome some limitations of inoculation and increase PGPRs' efficiency, coinoculation is now commonplace in experimental and field trials. The objective is to increase the consistency and frequency of nodulation rate in various plant species. By definition, coinoculation is the combined application of PGPRs and other bacteria, bestowed with some specialized functions, to increase the nodulation rate, plant growth, and plant tolerance to adverse environmental conditions. For example, coinoculation of PGPRs with nitrogen-fixing bacteria has caused earlier nodulation and greater intensity, better uptake of nutrients and water, and improved plant growth [11]. In another study, coinoculation of soybean plants with strains of *Pseudomonas* and *Bacillus*, in combination with *Sinorhizobium meliloti*, has improved plant phosphorous uptake [16]. Moreover, *Azospirillum* has been used to increase the rhizobia-legume symbiotic relationship in soybean to improve its nutritive value [12]. For coinoculation, in vitro strain selection or genetically engineered strains are commonly used [17]. It is generally assumed that one rhizobacterium may be less effective in diverse environmental conditions. Thus, mixtures of various rhizobacterial species are promising in enhancing plant growth. But the coexistence of different bacterial strains under normal and adverse field conditions may be a challenge. Nowadays, coinoculation of PGPRs with mycorrhizal fungi is being practiced to promote growth in various plant species [13, 18–19]. Moreover, some studies have been focused on combining free-living bacteria, PGPRs, and mycorrhizal fungi [20].

3.3 Efficiency of coinoculation for enhancing nodulation rate

Inoculation and coinoculation of plants with single or multiple PGPRs may bring changes in the number of root hairs, nodule formation, root exudation, and release of phytohormones in addition to several physiological and metabolic changes. Generally, the potential of a specific PGPR strain to enhance nodulation rate can be best judged in a single experiment; however, consistent performance needs multiple field trials. The initial study on the role of PGPRs in enhancing nodulation rate was conducted on *Rhizobium trifolii*. The efficiency of coinoculation may also be dependent on the hormones and enzymes produced by PGPRs. For example, *Azospirillum* produces indole-acetic acid and pectinase which affect the development of symbiotic relationship and ultimately the nodulation efficiency [21]. Corporate research is focused on developing commercial inocula; however, several challenges need to be overcome before the product can make sense to the users. These include but are not limited to explaining exact mode of action of PGPRs under individual circumstances, persistency of performance over different ecological environments, and the optimization of the fermentation systems.

4. Phytohormones released by PGPRs

Rhizosphere is the soil adjacent to the growing roots of a plant. A strong interaction exists between the roots and soil. The microbial activity in the rhizosphere makes the interaction even stronger. The interaction between the plants and microbes can be symbiotic, nonsymbiotic, neutral, and parasitic. There are a number of microbes that are found in the rhizosphere; these include bacteria, fungi,

actinomycetes, protozoa, and algae. Among these the most common is the bacterial population. Plant growth-promoting rhizobacteria are the bacterial biomass that colonizes the plant roots in the rhizosphere [22]. PGPRs have been reported to play many important functions in plants; these include nitrogen fixation and uptake, tolerance under stress conditions, and production of certain phytohormones, i.e., plant growth regulators, siderophores (iron-binding protein compounds), volatile substances, and also certain enzymes, i.e., glucanase and chitinase to protect plants against disease [23, 24].

Phytohormones are produced in low concentration but have greater influence on the biochemical, physiological, and morphological functions of plants. They function as chemical messengers to transfer cellular activities in higher plants [25]. During the abiotic stress condition, these phytohormones play vital roles through communicating different transducing signals, which may control the external and internal stimuli [26]. Also some of the phytohormones are identified as stress hormones like abscisic acid (ABA). These phytohormones have a significant role in various plant processes. ABA besides facilitation during biotic and abiotic stress also is critical for maintaining seed dormancy, growth regulation, inhibiting germination, controlling the stomatal closure, and fruit abscission [27]. The plant growth regulators produced include auxin, gibberellic acid, cytokinins, and ethylene. Ahmad and Hasnain (2010) [28] have reported that *Bacillus* spp. producing auxin showed positive effect on the growth of potatoes. Earlier research work has revealed that PGPRs inoculation improved plant tolerance to stress condition due to enhanced production of growth regulators [29, 30].

4.1 Effect of synthetic PGRs on release of phytohormones

Plant growth regulators (PGRs) are synthetically available and are used in commercial agriculture extensively. Through various investigations, it has been found that application of growth regulators at pre-sowing stage to the seeds may enhance the nutrient reserves, tissue hydration, growth, and yield of crops [31]. Khan et al. (2018) [32] found synergistic effects of PGPRs and PGRs on different qualitative parameters of crops, i.e., chlorophyll, sugar, and protein contents. They concluded that application of PGRs to the plants inoculated with PGPRs helped plants under stress conditions. Also the amount of PGRs applied exogenously to the plants may be stored as reversible conjugates, and they also release phytohormones as required by the plants at different growth stages. Also these PGRs are found effective in transferring accumulates from source to the sink [33, 34].

Also some of the researchers have reported that the release of phytohormones may be enhanced several times by the applications of some suitable precursor of the plant hormones. These precursors are utilized by the rhizobacteria and converted into active phytohormones, and they are continuously used by the plants [35]. Among these precursors, L-methionine is an important precursor of ethylene (C₂H₄), a gaseous plant hormone that positively affects at almost all stages of growth and developmental processes [36]. Application of L-methionine to the rhizosphere enhanced the ethylene production and has shown significant increase in the growth and yield traits of soybean [36].

4.2 Effect of coinoculation on release of phytohormones

The bacterial population in the rhizosphere sometimes modifies the formation of nodules when they are coinoculated. The mechanism behind this process is that the coinoculation may directly enhance the growth and development of plant by the

increase in microbial biomass, extending the root system by release of phytohormones, solubilization of phosphate in the rhizosphere, etc. Moreover, development of roots provides additional sites for nodule formation [37, 38]. Indole-acetic acid (IAA) is an important metabolite of auxin group produced by the *Azospirillum brasilense* bacteria in the presence of tryptophan. Also the *A. brasilense* may produce the IAA in the absence of tryptophan under aerobic condition in the presence of NH_4 [39, 40].

Some PGPRs produce allelochemicals which are phytotoxic in nature. Production of these allelochemicals may adversely affect the soil health [41], by having negative effect on the enzymatic activity and plant functions, and may also hamper the nutrient availability to plants. The number of allelochemicals has been isolated from the bacterial strain present in the rhizosphere. It has been reported that a single strain of bacteria may produce a wide range of allelochemicals, e.g., *Streptomyces hygroscopicus* may produce nigericin and geldanamycin; these may be isolated and utilized as herbicides [42].

5. Influence of coinoculation on bioavailability of micronutrients

An exponential increase in the world's population will demand a higher production of food crops. By 2050, it is projected that the world's food demand will reach up to 3 billion tons. This high demand for food has been resulted in the excessive use of chemical fertilizer (nitrogen, phosphorus, potassium) in combination with advancements in technology to enhance the plant growth and production. Nitrogen is a vital nutrient in plant growth and productivity. Unfortunately, when a recommended dose of fertilizers is applied to crops for an average yield, less than 50% of applied nitrogen fertilizer is consumed by plants [43]. This low use efficiency of N causes the high fertilizer consumption and nitrate contamination of groundwater and soil which finally resulted in environmental degradation and health problems. Inoculation with microbes has been considered as an environmentally friendly alternative to minimize the use of synthetic nitrogen fertilizer without compromising the crop growth and yield [44, 45]. By biological nitrogen fixation, atmospheric nitrogen is converted to plant-utilizable forms, which is performed by microorganisms which convert the nitrogen to ammonia [46]. These microorganism generally is categorized into two groups: (i) nitrogen-fixing bacteria which generally includes the Rhizobiaceae family members and forms symbiotic associations with legume plants [47] and other non-leguminous plants and (ii) nonsymbiotic nitrogen-fixing bacteria (free-living, associative, and endophytic) such as *Cyanobacteria*, *Azotobacter*, *Gluconacetobacter diazotrophicus*, *Azococcus*, *Azospirillum*, etc. [48]. Rhizobia (including *Sinorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*) are considered as symbiotic partners of legume plants and known by their role in the formation of N-fixing nodules in plant rhizosphere [49], while the nonsymbiotic N-fixing bacteria deliver only a small amount of fixed N which is required by the associated plant [50]. N-fixing PGPB strains and their effects on leguminous plants have been tabulated in **Table 1**.

Plant growth-promoting bacteria (PGPB) comprise a group of microorganisms that colonize the internal plant tissue and root surface and provide many benefits to host plants [51, 52]. These microorganisms can improve plant growth by contributing several mechanisms and processes including synthesis of hormones such as cytokinins, auxins [53], ethylene [54], gibberellins [55], and a variety of other molecules [56], biological control of pathogens [57, 58], and solubilization of phosphate [59]. Combinations of these mechanisms finally benefit the plant by

Bacterial strains	Plant	Effect	References
<i>Azotobacter chroococcum</i>	<i>Avena sativa</i> L.	Improved growth and yield	Devi et al. [70]
<i>Azospirillum brasilense</i>	Maize	Improved N use efficiency and improved yield	Morais et al. [71]
<i>Bradyrhizobium</i> spp. + <i>Azospirillum brasilense</i>	Soybean	Promoted growth and yield with N application	Hungria et al. [79]
<i>Rhizobium</i>	Chickpea	Promoted growth in combination with N application	Namvar et al. [80]
<i>Bradyrhizobium</i> , <i>Azospirillum</i>	Soybean	Significantly improved nodule biomass	Chibeba et al. [81]
<i>Rhizobium</i> sp. BARIRGm901	Soybean	Increased nodule weight and crop yield, improved the activity of nitrogenase enzyme and nitrogen assimilation	Alam et al. [82]
<i>Diazotrophic bacteria</i>	Rice	Increased grain yield	Araujo et al. [83]
<i>Ochrobactrum ciceri</i> Ca-34, <i>Mesorhizobium ciceri</i> TAL-1148	Chickpea	Improved nodule biomass and crop yield	Imran et al. [84]

Table 1.
N-fixing PGPB strains and their respective effect on leguminous plants.

improving growth [60, 61] and biological nitrogen fixation and increase the activity of nitrate reductase when growing as plant endophytes [62]. These bacteria also produce the siderophores and synthesize enzymes, antibiotics, or fungicidal compounds that protect the plants against phytopathogenic microorganisms [63, 64]. There are several factors such as agricultural practices, plant genotype, bacteria species, and strain that may affect the success of inoculation and plant response to these PGPB [65, 66]. Chickpea and *Rhizobium leguminosrum* subsp. *Cicero* associations, for instance, produce up to 176 kg/ha annually depending on environmental factors, cultivars, and bacterial strain [67]. *Azorhizobium caulinodans* is root- and stem-nodulating nitrogen-fixing bacterium which has been isolated from the stem nodules of *Sesbania rostrata* (*Bremek and Oberm.*) [68]. By endophytic colonization of non-legume roots, i.e., wheat, it can stimulate root growth and increase nitrogen content and yield [69]. Devi et al. [70] reported that the growth and yield of oats (*Avena sativa* L.) were increased due to the seed inoculation with *Azotobacter chroococcum* combined with the nitrogen fertilizer as compared to control and nitrogen fertilizer alone. Highest yield (239.02 quintal per hectare) was observed in *Azotobacter* seed inoculated +80 kg N as compared to control (111 q/ha) and nitrogen 80 kg/ha (205 q/ha) alone. In another study, Morais et al. (2016) [71] reported the effects of *Azospirillum brasilense* (inoculated in seed furrow) on maize growth and yield. Average maize grain productivity was observed to be 12.76 and 13.06 ton/ha when nitrogen is applied at the rate of 100 and 200 kg/ha, respectively. However, with the addition of seed furrow inoculation at the rate of 200 ml/ha, average grain productivity of maize was increased up to 13.21 and 14.0 ton/ha under the nitrogen application at the rate of 100 and 200 kg/ha, respectively. This PGPR improves the growth and yield by increasing the N and P content in plant, higher phosphate solubilization, ammonia, indole-acetic acid (IAA), and siderophore production [72]. Inoculation of seeds with *Rhizobium* increases the protein, chlorophyll content, nitrogen uptake, and growth parameter in legume crops [73, 74].

Now scientists have developed new microbial associations to avoid such negative interrelations and increase the effectiveness of biofertilizers. Consortia of PGPR with mycorrhizal algae [75] or fungi [51] can show a better performance as a result of cumulative or synergistic interactions between beneficial mechanisms of different microorganisms. Mycorrhiza is a symbiotic interaction between plants and soil fungi called as arbuscular mycorrhizal fungi (AMF). Both associates get benefits for this relationship by improving nutritional status, which reduces the needs of fertilizers for crops [76, 77]. Vesicular-arbuscular mycorrhizal fungi improved the availability of nitrogen and phosphorus to support the plant to survive in different environmental severe conditions [78].

6. Quantification of nodulation process by dynamic linear models

The symbiotic relationship between N_2 -fixing bacteria and leguminous plants is a core factor in enhancing soybean crop yield around the world. The atmospheric nitrogen captured by these bacteria is enzymatically reduced to ammonia. This ammonia is assimilated by plant tissues in the form of nitrogenous compounds. Around 20–22 million tons of N is fixed by symbiotic rhizobia [85], while 17 million tons is removed or assimilated by aerial biomass of legumes [86]. The fixed N can serve as an inevitable resource of N depending on net N fixation in soil as compared to its removal or assimilation in aerial parts of legumes which is estimated to be 45–75% [87]. Nonetheless, the cropping systems with legumes have high crop yield as compared to non-legumes [88]. The fixation of N_2 can be maximized by sustainable and organic farming practices. However, legume specie, soil type and climatic conditions can also impact fixation rate of N_2 [89]. The production of soybean as cash crop is evident in Brazil, Argentina, Russia, Ukraine, and the United States [90]. In Asia, North China and Japan chiefly cultivate soybean along with wheat [91].

Quantification of leguminous biological nitrogen fixation (BNF) can be beneficial for sustaining N demand and supply which can increase productivity and ability to combat environmental stresses. The techniques available for quantifying legume BNF are costly and protracted. Moreover, the data provided by such techniques are pertinent to limited time and space. Simulation of legume BNF is attainable by empirical and dynamic modeling. Empirical modeling is based on observation and experiment, while dynamic modeling is capable of representing a pattern or behavior over a time period. In case of legume BNF simulation, dynamic modeling can be desirable as it can correlate various environmental factors and legume growth status with N fixation. Broadly, legume BNF is discussed in relation to demand, uptake, and assimilation of N in biomass of root, nodule, and aerial parts of leguminous plants. Moreover, concentration of N accumulated in soil, along with soil's environmental parameters such as water content, N mineral concentration, internal substrate, C substrate and supply, and temperature are essential to quantify N fixation. Last but not the least growth rate of leguminous plant is a dynamic indicator in estimation of fixed N [92–95].

6.1 Estimation of N fixation by considering economic yield or aboveground biomass

During growing period, N fixation can be estimated by considering economic yield or dry matter of aerial biomass [96–98]. For this purpose, the equation can be:

$$N_{fix} = \alpha \cdot DM \cdot f_{leg} \cdot N_{con} \cdot \%Ndfa \cdot (1 + R_{root}) \quad (9)$$

where DM represents dry matter of aerial biomass or yield, f_{leg} is proportion of legume crop in intercropping system, N_{con} is concentration of N assimilated in legume plant, and %Ndfa indicates proportion of N in crop which is derived from fixation of N_2 , whereas R_{root} is a ratio of N fixed in belowground parts to the N fixed in aerial parts of legumes. α is a parameter which can have different definition depending on the researcher. For example, α can be used to represent correlation between decline in %Ndfa and high soil N content. In order to estimate total N input, α can be calculated as:

$$\alpha = 1 - \beta \cdot N_{net.inorg} \quad (10)$$

where β evaluates the responsiveness of legume for N fixation to already present mineral N (nitrate and ammonia) in the soil [98]. This method can directly estimate N fixation. Its parameter values can be taken both as estimated values from literature or measured values from on-site analysis. This method can work in the absence of previous data from past years. In these equations, environmental and weather conditions are not considered; therefore, this method can only be suitable for soils with similar properties and with exposure to moderate weather conditions. Moreover, the parameter values can be accustomed according to soil condition.

6.2 Linear empirical model

The empirical model can be used to explicit correlation between amount of N fixed in legumes and the total harvested part of legumes. In the case of intercropping system, fixed N in legumes can be correlated to the present fraction of legumes in the field. The equation is devised to calculate N fixation in $kg\ N\ ha^{-1}$, such as:

$$N_{fix} = c + d \cdot Leg \quad (11)$$

where Leg denotes excess in harvested biomass ($kg\ ha^{-1}$) while c and d comprise the selected parameters.

The empirical model is based on statistical correlation with speculation of strong linear relationship between N fixation and variables. The applicability of this model is on wide variety of soils. This model requires adequate amount of data to constitute a correlation study and to determine the values for the selected parameter. The linear empirical model, however, does not account environmental conditions [99, 100].

6.3 Crop models as example of dynamic models

Leguminous N fixation in soybean was first simulated by Duffy et al. (1975) [101]. He estimated rate of N fixation by measuring root growth rate after specific days of planting. Crop models being dynamic in nature involve the potential impacts of soil environmental conditions for estimating N fixation. However, soil salinity, pH and availability of other nutrients are exempted in such models. Examples of crop models are Sinclair [102, 103], EPIC [104–106], Hurley Pasture model [107–110], Schwinning model [111, 112], CROPGRO [113–115, 93, 116], SOILN [117], APSIM [95, 118], Sousanna model [94] and STICS [119–121]. These crop models are applicable in varying environmental conditions; therefore, each model can have different versions for calculating N fixation. Thus, Liu et al. (2011) [122] devised a general equation for these crop models:

$$N_{fix} = N_{fixpot} f_T f_W f_N f_C f_{gro} \quad (12)$$

where N_{fixpot} indicates the potential rate of N to be fixed by legumes (g N fixed day⁻¹), f represents the influence function of environmental conditions, f_T is impact of soil temperature, f_W can be taken as impact of water deficiency or flooding in soil, f_N can estimate impact of availability of mineral N (nitrate and ammonia) in soil or N availability in root substrate, f_C represents effect of C concentration in root and aerial parts of legume plant, and f_{gro} is the effect of plant's growth stage on potential rate of N fixation. In the case of Environmental Policy Integrated Climate (EPIC) model and Simulateur multidisciplinaire pour les Cultures Standard (STICS), the equation is generalized as:

$$N_{fix} = N_{fixpoint} f_T \min (f_W, f_N) f_{gro} \quad (13)$$

where \min indicates the minimum value that can be assumed between f_W and f_N . If applying STICS model, the limitation by anoxia is represented by extra function, i.e., f_a .

6.3.1 Potential N fixation

In dynamic models, the potential rate of N fixation is estimated on the basis of demand or uptake of N by legume plant or on the ability of root nodules to fix atmospheric N₂. In EPIC, the potential rate of N fixation is equal to the demand of N by legume plant [107]. The higher the demand of the N in legume plant, the higher will be the potential of N fixation. In contrast, according to Agricultural Production Systems simulator (APSIM), the internal concentration of N in plant tissues governs the N demand of legume plant, which in turn defines the potential rate of N fixation in legumes. However, APSIM is applicable when plant has sufficient N concentration which can fulfill N demand of new tissues by uptake N from the soil [122]. N uptake is relatively passive and much preferable than N fixation; therefore, N fixation is only estimated when plant's demand for N is not fulfilled by N uptake [123]. Potential rate of N fixation, therefore, can be defined as difference between N demand and uptake [95, 118]. On the other hand, some researchers claim that the potential of N fixation is dependent on size and biomass of root and nodules, i.e., above- and underground biomass [124, 125]. However, estimation of N fixation using aboveground biomass is more convenient to handle than underground biomass [126].

6.3.2 Soil temperature

Soybean being plant of tropical and sub-tropical regions requires warm conditions for growing. The favorable temperature for soybean root zone ranges from 25–30°C [127]. Crop models such as Hurley Pasture model, CROPGRO, SOILN, and STICS estimated the effect of soil temperature on rate of N fixation by specifying certain temperature range. The generalized forms of equations are:

$$f_T = \begin{cases} 0 & (T < T_{min} \text{ or } T > T_{max}) \\ \frac{T - T_{min}}{T_{optL} - T_{min}} & (T_{min} \leq T \leq T_{optL}) \\ 1 & (T_{optL} \leq T \leq T_{optH}) \\ \frac{T_{max} - T}{T_{max} - T_{optH}} & (T_{optH} < T < T_{max}) \end{cases} \quad (14)$$

where T represents soil temperature in °C, T_{\min} is the minimum temperature below which N fixation can stop, T_{\max} is the maximum temperature above which N fixation can stop to occur, and T_{optL} and T_{optH} indicate low and high values of optimal temperature range. In optimal condition, the optimum response to soil temperature becomes equal to the unit. Depending on location and legume species, the temperature range can vary in different models [109].

6.3.3 Soil water content

The excessive and deficient amount of soil water in the *Rhizobium* can negatively impact N fixation by the nodule. In STICS model, water deficit point is defined as segment of soil layers with water content above permanent wilting point [119]. Sinclair model, on the other hand, correlated transpirable water with nitrogenase activity of nodules [103, 104]. The nitrogenase activity is assessed by the reduction of acetylene which is used to explicit the proposed mechanism of BNF. The reduction in transpiration rate < 10% determines the transpirable water in soil, which in turn is stipulated by comparing the field capacity of soil and soil water content [102]:

$$f = -1 + \frac{2}{1 + e^{(-m * f_{TSW} + n)}} \quad (15)$$

where f_{TSW} represents the fraction of transpirable water in soil, whereas m and n are constants defining responsiveness of legumes for N fixation in low soil water content. APSIM, EPIC, and SOILN formulated linear function, which is expressed as:

$$f_{\omega} = \begin{cases} 0 & (W_f \leq W_a) \\ \phi_1 + \phi_2 \cdot W_f & (W_a < W_f < W_b) \\ 1 & (W_f \geq W_b) \end{cases} \quad (16)$$

where W_f is the ratio of relative availability of water content in soil at a given field capacity, W_a is the minimum value of water content below which N fixation cannot occur, ϕ_1 and ϕ_2 are the coefficients, and W_b is the threshold value of W_f above which N fixation is not impeded by water content of soil.

However, researchers with special focus on water stress conditions revealed that the top layer of soil around 30 cm is susceptible to dryness or wetness during dry spell or irrigation period. This can influence the access of water to root nodules [128]. Therefore, the presence of water within the roots is a more reliable factor in quantifying N fixation in limited water supply. Contrarily, in Hurley Pasture model, the chemical activity in the roots is assumed to control N fixation, wherein the chemical activity indirectly relies on probable water content in the root and temperature of soil [107]. So the effect of water is correlated with the thermal condition of soil such as:

$$f_W = e^{20 * \left[\frac{18 * \phi_{rt}}{8314 * (T_s + 273.15)} \right]} \quad (17)$$

where Φ_{rt} , probable water content in the root ($J \text{ Kg}^{-1}$) and T_s is termed as thermal value of water content in soil (°C).

Excessive water can cause anoxic conditions in soil. In such condition, N fixation is assumed to be at zero in Sinclair model [103]. In anaerobic conditions, pore spaces become occupied with water; therefore, N fixation cannot occur.

6.3.4 Mineral N/internal substrate

The availability of N in the form of nitrates and ammonia is said to be mineral N in soil. In SOILN model, mineral N is incorporated for estimating N fixation in nodules such as:

$$f_N = \begin{cases} 1 - 0.0784 \ln N_s & (N_s \geq 1) \\ 1 & (N_s < 1) \end{cases} \quad (18)$$

where N_s is mineral N content of soil (mg N m^{-3}). The N uptake can be influenced by mineral N in soil; therefore, Schwinning model estimates potential of N fixation as:

$$f_N = \varepsilon \times (1.0 - f_{N_{up}}) = \varepsilon \times \left(1.0 - f_{max} \frac{1}{1 + K_N/N_s} \right) \quad (19)$$

where ε is the efficiency of legume BNF, f_{max} is the maximum amount of N derived from the uptake of mineral N from soil, K_N indicates the concentration of nitrate in soil (g N m^{-2}) with N uptake reaching at half of its maximum rate, and N_s is the actual concentration of nitrate in soil (g N m^{-2}). In the given soil conditions, if nitrate concentration (N_{sNitra}) lies between 10 and 30 $\text{g (Nm}^{-3})$ within 30 cm topsoil layer, the EPIC model can be represented as:

$$f_N = \begin{cases} 1 & (N_{sNitra} \leq 10) \\ 1.5 - 0.05N_{sNitra} & (10 < N_{sNitra} < 30) \end{cases} \quad (20)$$

In STICS model, high nitrate concentration in soil is assumed to inhibit nodulation progress which ultimately reduces potential rate of N fixation. If the concentration of nitrate in soil is higher than critical value, N_{fixpot} is set at baseline value; otherwise, N_{fixpot} is set at normal value [119]. In Hurley Pasture and Soussanna models, the plant substrate N concentration is included, such as:

$$f_N = \frac{1}{1 + N_{inter}/K_r} \quad (21)$$

where N_{inter} ($\text{g N g}^{-1}\text{r.wt}$) is assumed to be the N concentration in the root substrate (in Hurley Pasture model), or N concentration in plant substrate (in Soussanna model), and K_r is the coefficient for stating inhibition of N fixation at high nitrate concentration level in soil.

6.3.5 C in plant substrate or C supply

In plants, C is the source of energy for N fixation. Carbohydrate supports nodule biomass accumulation. The effect of C in estimating potential rate of N fixation is incorporated in Hurley Pasture and CROPGRO models such as:

$$f_C = \frac{1}{1 + K_C/C_r} \quad (22)$$

where C_r indicates concentration of C and K_C stands for Michaelis–Menten constant.

6.3.6 Plant growth stage

The impact of seasonal change on N fixation is incorporated in EPIC and STICS [106] such as:

$$f_{gro} = \begin{cases} 0 & (g < g_{min} \text{ or } g > g_{max}) \\ \frac{g - g_{min}}{g_{optL} - g_{min}} & (g_{min} \leq g \leq g_{optL}) \\ 1 & (g_{optL} \leq g \leq g_{optH}) \\ \frac{g_{max} - g}{g_{max} - g_{optH}} & (g_{optH} < g < g_{max}) \end{cases} \quad (23)$$

where G_{min} is indicating the time period before which N fixation does not occur. This happens because of insufficient nodulation (expressed as % of total time period required for growing); g_{optL} is the initial time of growth and g_{optH} is the final time of growth. The time period between g_{optL} and g_{optH} represents N fixation by legumes, which is independent of growth stage. g_{max} is the growth time where N fixation stops due to deterioration of nodule.

The influence of symbiosis on metabolic fluxes and plant growth is quantified by a flux balance analysis. A genome-scale compartmentalized model for the clover (*Medicago truncatula*) as model plant has been devised by Pfau et al. (2018) [129]. The model predicted that nitrate uptake is significantly inhibited by the presence of ammonium in soil. When both nitrate and ammonium are available in soil, the uptake of ammonium is much favorable due to its integration into amino acids with fewer reductants and energy than nitrates.

The simulation of BNF by the abovementioned models included various biotic and abiotic factors to simulate and predict N fixation. Nodule biomass is more reliable to estimate N_{fixpot} than root and aerial biomass. C supply is considered to be the prominent factor in estimating N_{fixpot} . High concentration of nitrate in soil as mineral N can act as inhibitor for N fixation by nodules. Although empirical and dynamic models incorporated several factors such as soil temperature, water content, C, and other mineral contents, all the models lack information regarding the influence of soil pH and O_2 permeability. Therefore, adequate experimental work is required to cumulate the effect of such factors on biological fixation of N in legumes.

7. Technical and economic aspects

The impact of inoculation and coinoculation with elite strains such as *Azospirillum* species (*A. brasilense*) and *Bradyrhizobium* species (*B. japonicum*, *B. elkanii*, and *B. diazoefficiens*) has been extensively studied [130, 131]. Inoculation of *Azospirillum* spp. directly influences grain yield by improving N availability and its uptake. Moreover, this strain is helpful in the synthesis of phytohormones and developing pest resistance [132]. Crop yield is considered to be a primal factor for estimating profitability; therefore, increments in revenue are based on increments in grain yield [133, 134]. Coinoculation, regardless of cultivar, is reported to increase profitability by 14.4% as compared to non-inoculated treatments [134]. The economic evaluation of soybean plant is based on variables such as number of pods per plant, 100 grain weight, and yield. The data is usually quantified in $kg\ ha^{-1}$ at wet basis [134].

The production of soybean crop can be estimated by total operating cost (TOC) method [135]. TOC is the sum of cost of fertilizers, heavy machinery, labor, pesticides, interests, etc. The major expenses are contributed by mechanization and fertilizers besides the cost of desiccation, control of weeds, pests, and pathogens. The inoculation of *Azospirillum brasilense* has increased the TOC, whereas the lowest TOC was reported with inoculation of *Bradyrhizobium* strain. However, the highest soybean yield was obtained with coinoculation of *A. brasilense*, leading to higher financial returns. Inoculation with *Bacillus* and *Pseudomonas* led to significant improvement in protein and nitrogen contents in grains in addition to high yield [136]. Similar results were reported when *Rhizobium* and *Pseudomonas fluorescens* improved yield and protein content when inoculated in beans [137].

In some studies, foliar inoculation of PGPR is found to be more effective than inoculation or coinoculation. For instance, foliar inoculation of *Azospirillum* in later stages of plant growth is correlated to high N content in developing grains [12]. This is because of the release of IAA by *Azospirillum*, which instigated nodulation in secondary nodules, thereby facilitating N fixation and its uptake in growing soybean plants. Likewise, foliar inoculation of *Azospirillum brasilense* at advanced growing stage of soy plant proved to be much more effective than its inoculation and coinoculation with *Bacillus japonicum* at sowing stage [12, 138]. However, foliar application at sowing stage is unable to produce any noticeable improvement in grain yield [12]. Moreover, coinoculation of *A. brasilense* and *B. japonicum* is reported to increase leghemoglobin by 39%, leading to high proportion of active nodules which in turn increased N fixation [139].

Organic and inorganic fertilizers such as NPK fertilizer and farmyard manure used along with PGPR, i.e., *Azotobacter* and *Trichoderma*, are reported to produce the highest biomass yield [140]. However, the inoculation of *Bradyrhizobium japonicum* on seeds increased grain yield of soybean by 8.4% (222 kg/ha), while its coinoculation with *A. brasilense* in furrow yielded 16.1% (427 kg/ha) without applying any external N source [141]. Similarly, Hungria et al. (2015) [79] coinoculated seeds of soybean with *Azospirillum* and *Bradyrhizobium* which resulted in high crop yield (388 kg/ha) without using any N fertilizer. The onset of earlier nodulation in soybean crop has been observed by the coinoculation of *Bradyrhizobium* and *Azospirillum* [81]. Moreover, these researchers claimed that the presence of *Azospirillum* after 18 days after emergence (DAE) facilitated plants to environmental stresses. Phosphorus as an essential nutrient for root growth is also necessary for rhizobia to convert N₂ into mineralized N [142]. Depending on the genotype of soybean, other nutrients like P can be influential in nodulation [143]. These researchers carried out coinoculation of rhizobia with arbuscular mycorrhizal fungi (AMF) in deep and shallow root genotypes of soybean. Regardless of soil N content, P was found to be a limiting factor in increasing nodulation, with low P colonization of AMF increased, whereas with high P, nodulation progressed in deep root soybean. Microbial inoculants are quite economical, making inoculation as a sustainable approach in soybean production [144]. Hence, the introduction of PGPR at appropriate stage of plant cycle can be a beneficial and reliable procedure for low-cost investment and sustainable agriculture.

8. Future prospects and conclusions

The reliance on N fixation is inevitable in spite of application of inorganic N fertilizer in huge amount (18 million tons/year) [86]. Legume plants being highly nodulated have high potential for N fixation which can be further facilitated by sustainable agricultural practices for high crop production. Inoculation and

coinoculation with different strains not only positively impact crop yield but also improve nutrient value of grains. PGPRs are natural source of plant growth hormones especially IAA, prompting nodule growth whether applied at sowing or in later stages of plant growth. In some cases, foliar inoculation was more effective for nitrogen and protein assimilation in soybeans than inoculation and coinoculation at sowing phase. Among PGPRs, certain strains of *Azospirillum* have a great potential of replacing inorganic sources of N, making inoculation a more economical approach toward sustainable agriculture.

The viability of PGPR inoculants is susceptible to rhizospheric conditions of soil, for which the compatibility studies are a compulsion [145]. When applied in the field, certain bacterial species (endophytes and rhizosphere-restricted bacteria) become VBNC, i.e., viable but not cultivable [63]. This might occur due to stress encounter by bacteria while colonizing host cell. The reason for VBNC is still unknown, but it is common to most rhizobial species. The research at molecular and genetic levels might solve this mystery. Soils with high mineral N content (ammonium and nitrate ions) are more prone to N reduction, as PGPR can readily consume it. Therefore, the viability of an applied farming approach can indicate the accessibility of organic N content in soil [98]. Moreover, the soils with common physico-chemical features and exposure to similar climatic conditions may differ in net reduction of N content. This may be due to probable surface or drainage runoff of organic N during agricultural practices [146]. However, the estimation of soil N mass balance (input and output) requires long-term study which in turn will be helpful in the election of suitable cropping system. The use of economical viable PGPR inoculants along with efficient cropping systems can increase the probability of stable N retention in soils. In the case of developing countries, the lack of knowledge and relevant technological restrains demand an immediate implication of research (i.e., PGPR inoculation at sowing or spraying on leaves) in field conditions, thus providing cost reduction benefits to farmers and empowering local communities.

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N-Fertilization Adjustment in Sugarcane Crop Cultivated in Intensive Mechanization

Sérgio G. Quassi de Castro
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Abstract

Currently sugarcane is cultivated in Brazil in intensive mechanization during all cultural practices, since planting, harvesting until fertilizer applications. In this sense, to increase the sugarcane yield which it was stagnant along the last 5 years, it is necessary looking for alternatives to management the crop and maximize the yield gain. One alternative is through the adjustment the N-fertilization in green cane crop according to the IPNI guidelines—“4R Nutrient Stewardship System” (International Plant Nutrition Institute—IPNI) program which seeks to apply fertilizer in the right location, at the right time, in the right amount and the right source. Therefore, the main goal of this chapter is shows these alternatives and the yield gains that it was obtained in researches during 2013–2017.

Keywords: nitrogen, *Saccharum* spp., green cane, fertilizer

1. Introduction

According to statistical surveys conducted in the last decade by the International Plant Nutrition Institute [1], the largest consumer market for NPK fertilizers was Asia (57%), followed by the Americas (25%), Europe (13%), Africa (3%), and Oceania (2%). In 2015, approximately 183.2 million tons of NPK fertilizers were consumed, being the most consumed nitrogen fertilizers with 60% of total (110.4 million tons), followed by phosphorus (22% (40.7 million tons)) and potassium (18% (32.1 million tons)). In the Americas, the largest consumers were the United States and Brazil, with Brazil showing a significant increase in fertilizer consumption during the last decade, representing a consumption of approximately 14 million tons of NPK fertilizers in 2015. This value was 50% higher than obtained in the previous decade [1]. This large demand resulted in a financial movement of approximately R\$ 19.5 billion per year, originating from the internal sale of NPK fertilizers [2].

In Brazil, the main crops (soybean, maize, and sugarcane) use approximately 6.2 million tons of NPK fertilizers during 2015. The sugarcane crop represents 22.6% of this amount, with consumption of 1.4 million tons of NPK fertilizers, which generated investment higher than R\$ 2 billion per year. Within this billion-dollar market of fertilizers for sugarcane, potassic fertilizers were the most used (609 thousand tons), followed by nitrogen fertilizers (573 thousand tons) and phosphates fertilizers with 195 thousand tons used [2].

Most of the NPK fertilizer market in the Brazilian sugarcane sector is based on four sources, such as potassium chloride (source of K), urea and ammonium nitrate (N sources), and simple superphosphate (P source). Although the industry is a large and efficient waste recycler, such as filter cake that is rich in phosphorus and potassium-rich vinasse, the high demand for the importation of these raw materials shows the risk in our food and energy security.

Brazil is the world's largest producer of sugarcane with approximately 640 million tons [3], grown in an area of 9 million hectares, followed by India and China, respectively, with 352 and 126 million tons [4]. Despite this, the increase in the sugarcane area, which was 5.8 million hectares during the last 10 years, not took place increase in stalk productivity, which remains stagnant (72 Mg ha^{-1} —crop season 2016/2017) compared to the Brazilian historical series, which has already reached an average of 80 Mg ha^{-1} [3].

The IPNI has established a Best Practices Fertilizer Management (BPFM) program. This program shows the practical actions necessary to provide a better economic, social, and environmental performance of crops, in order to adapt the supply of nutrients to the needs of the crop minimizing the losses of its nutrients in the soil-plant-atmosphere continuum [5]. In the focus of plant nutrition and fertilizer use, BPFM encompasses the 4R principle: (1) applying the right nutrient source, (2) taking the right amount, (3) at the right place, and (4) in the correct time (right time). In this context, the present text aims to address aspects related to the nutrition of the sugarcane crop, focusing on nitrogen fertilization, showing the challenges and bottlenecks for increasing the efficiency of use of these nutrients by sugarcane.

2. Nitrogen fertilization: definition of the correct source, applied time, application mode, and right dose

The N is the nutrient that has the highest interaction in the environment due to the numerous reactions, mediated by microorganisms, that occur in the soil, being affected by temperature and humidity [6]. In addition, there are several routes of N losses (leaching, volatilization, and immobilization), promoting only about 26% of the N applied by fertilizer that is used by the plant [7]. For other crops this value usually is higher than 50% [8]. The large N stock in the soil, representing more than 95% of the total N, comes from organic matter. However, organic N is not directly utilized by plants, requiring their mineralization to produce ammonium, which can be absorbed or transformed into nitrate (nitrification process) that will be absorbed by plants [6].

In general, it is recommended to apply $30\text{--}60 \text{ kg ha}^{-1}$ of N in plant cane, applied in the planting furrow. In ratoon the dose can vary from 80 kg ha^{-1} N, for yields between 60 and 80 tons of stalks per hectare (TSH), up to 140 kg ha^{-1} N, in areas where it is expected to produce above 140 TSH [9]. There is no doubt about the importance of nitrogen fertilization for productivity gains in sugarcane cultivation, especially in ratoon areas. In a very good literature review made by Otto et al. [7], the authors found that in 75% of the total number of papers reviewed, there was an increase in TSH due to nitrogen fertilization. In 30% of papers, the gain in stalk yield was higher than 25% in relation to the control.

The most common nitrogen fertilizers used in Brazilian sugarcane fields are ammonium nitrate (33% N), urea (45% N), and ammonium sulfate (21% N). It is interesting to note that each option has positive and negative aspects, such as (i) ammonium nitrate has N in two forms: nitrate (NO_3^-) and ammonium (NH_4^+), in which ammonium has been reported to be a mineral preferential form uptake by

sugarcane [10]. In addition, there is not losses due the volatilization of ammonia by this N source (ammonium nitrate) in tropical soils. However, it can lead to nitrate leaching losses [11]. Also since it is a raw material for explosive devices, it must be restricted in its commercialization in the coming years around the world; (ii) urea is the fertilizer with the highest N content in its composition, making it possible to reduce the relative cost of its acquisition (R\$ per kg of N). Nevertheless, with the advent of mechanized harvesting of sugarcane, which generated straw covers in sugarcane fields and currently 85% of all sugarcane cultivated area in Brazil is harvested this mode [12], the application of urea over the straw implies in higher losses of NH_3 by volatilization; (iii) ammonium sulfate, although it is the option with lower concentration of N, presents in its composition approximately 24% of sulfur (S). This fact makes this fertilizer an excellent option, especially in areas where there is no application of vinasse, as it is able to supply nitrogen and sulfur at the same time to the plant. However, it has a high salinity index. When it is applied locally, in high amounts and in periods of low soil moisture, it can cause problems in the plant growth.

One of the major limitations to increase the productivity of sugarcane in Brazil is the availability of adequate amounts of nutrients in soils, especially N [13]. This is due to the many factors that affect nitrogen utilization efficiency (EUN), such as soil characteristics (pH, cation exchange capacity (CEC), organic matter, texture, clay, aeration, and compaction), climatic conditions (temperature and rainfall), and agronomic practices (cultivation, soil preparation, and crop rotation) [14]. Due to the difficulty to define the dose of N to be applied in sugarcane due to the lack of a laboratory methodology that allows quantification of N available for plants into the soil, the dose of N is often defined according to the expected productivity [15]. This fact causes sub or super estimates of the N doses to be applied to the crop. In this scenario, new works [7, 16] seek the development of strategies for the management of nitrogen fertilization in sugarcane plantations, aiming to increase nitrogen fertilization efficiency.

In addition, the presence of straw over the soil surface and the harvesting season (wet or dry season) should be considered for the proper management of sugarcane. This can be justified by analyzing the average productivity obtained over four harvests—2015, 2016, 2017, and 2018 (**Figure 1**), where the same variety of sugarcane (IACSP95-5000) was harvested at different crop season (beginning, middle, and end of the harvest corresponding to the months of April, August, and October, respectively). Comparing the fertilizations (same dose) made in wet season and dry season, the results show that the fertilizations carried out in the humid season always promoted the highest yields (**Figure 1**). In general, the area harvested at the beginning of the harvest had the highest average yield of stalks (107 TSH), followed by the middle area (95 TSH) and the final harvest (77 TSH). Therefore, the best time to apply the N in the sugarcane ratoon should be when there is moisture in the soil, regardless of the period in which the harvest was performed. This contrasts with the traditional management of sugarcane, in which fertilization was done when the plant had a significant canopy (number of green leaves) and tillering [15].

Other possibilities for increasing the efficiency of nitrogen fertilization in sugarcane, together with increases in yield of stalks, are related to the application forms. Regarding the forms of nitrogen fertilizer application, this can be done in several ways (**Figure 2**): (1) application incorporated to the soil by cultivation in the interrow (“triple operation”), (2) superficial application in band, (3) application under the straw in band, (4) application made in both sides of the ratoon, (5) surface application in total area, and (6) surface application with liquid fertilizer (a) and foliar application (b).

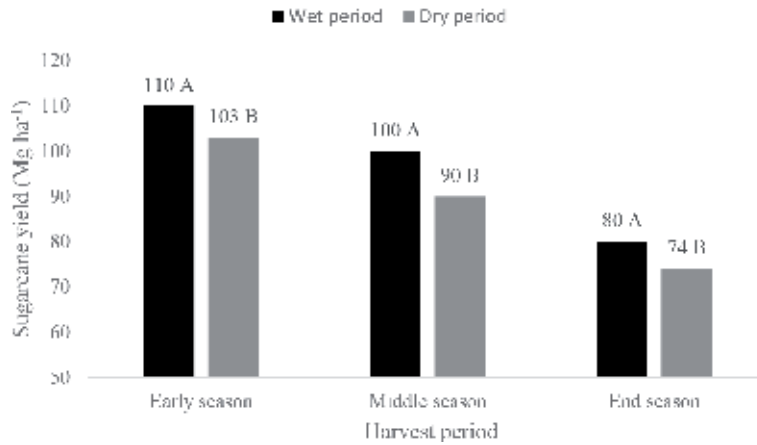


Figure 1. Effect of the nitrogen fertilizer application time according to the sugarcane harvest season. Source: Castro [17]. Note: Capital letters differ from each other within each harvest season.



Figure 2. Alternatives of N-fertilizer application methods in sugarcane ratoon. Source: Castro et al. [18].

The N-fertilizer applications made through the interrow are the most traditional in Brazil sugarcane fields. This is because the cultivation had been widely used in the burned cane—in the past. Regardless, in the currently years, with the adoption of the green cane cultivation (without fire in the harvest), the surface application has greater operational in sugarcane fields, where the straw layer resulting from the harvest can disrupt the scarification of the interweaving when opted for cultivation. Castro et al. [19] showed that no effect was obtained on crop productivity by the adoption of interlaced cultivation (**Table 1**).

The absence of alterations in TSH in sugarcane yield shows that the cultivation operation may not be necessary, mainly because sugarcane does not exploit the interleaving region [20, 21], even in areas where the traffic of machines in this region did not occur [22]. The application performed by the triple operation (number 1 in **Figure 2**), despite being incorporated in the soil, presents lower yields of TSH (**Figure 3**) due precisely to the distance at which the fertilizer is positioned in relation to the stump of the plant, and the area covered the root system, which in the middle of the interline is very small [20].

On the other hand, some studies show increases in TSH when incorporating the nitrogen fertilizer at 0.1 m depth [23], on both sides of the sugarcane ratoon: on average 0.2–0.3 m of the cane row (**Figure 2** (no. 4)). The deposition of N-fertilizer near the root

Treatments	2008	2009	2010	2011	Mean
With tillage	125 ^{b*}	112 ^a	85 ^a	81 ^a	101 ^a
Without tillage	131 ^a	112 ^a	88 ^a	81 ^a	103 ^a

* Means with the same letter in column did not differ according to the "Tukey" test ($p > 0.05$)

Table 1. Sugarcane yield (TSH) associated to the performance or not of the mechanical cultivation of the interrow of crop (adapted Castro et al. [18]).

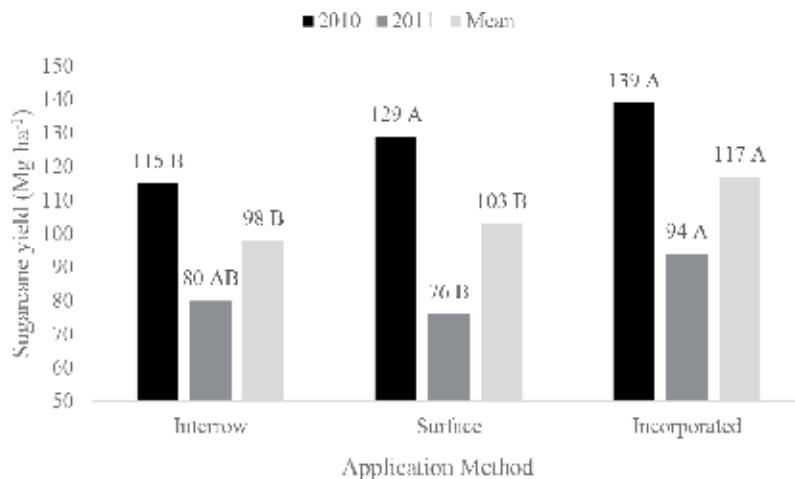


Figure 3. Effect of the N-fertilizer application method in sugarcane yield. Source: Castro et al. [23]. Note: Capital letters differ from each other within each treatment evaluated in each year.

system of the plant can facilitate its absorption, improving the TSH of the crop. This aspect also explains the difference between the incorporated application and the surface application; because when applied on the soil surface, the fertilizer needs to transpose the layer of straw which often occurs with the rains, and because it is not constant, a temporal difference in N uptake and plant development when compared to the incorporated application. In general, the incorporation of N-fertilizer presented an increase in sugarcane yield of 14% (28 TSH) and 19% (38 TSH) when compared, respectively, to the superficial application and the interrow, which did not differ between them.

Due to the large extension of sugarcane cultivated areas and the short interval of time for fertilization (adequate soil moisture conditions), alternatives that allow higher operational yield (hectares h⁻¹) are necessary. In this sense, one of the options is the application of the nitrogen fertilizer by means of machines such as the Uniport of the Jacto manufacturer, which deposits the fertilizer below the layer of straw (Figure 3 (n°3)), due to the fertilizer granules were applied with a pressure which these granules are able to transpose the straw layer. In a research conducted by Brazilian Bioethanol Science and Technology Laboratory (CTBE) [24], three forms of application of nitrogen fertilizer in sugarcane ratoon were compared, being application under straw and in strip and surface application in strip and application incorporated to the soil. The results showed that the yield of the crop was higher when the fertilizer was applied in an incorporated form or under the straw compared to the surface application (Figure 4). Considering the average productivity obtained in the two agricultural years (Figure 4), the application incorporated to the soil and under the straw provided increases in the TSH of 16% (26 TSH) and 13% (21 TSH), respectively, when compared to surface application.

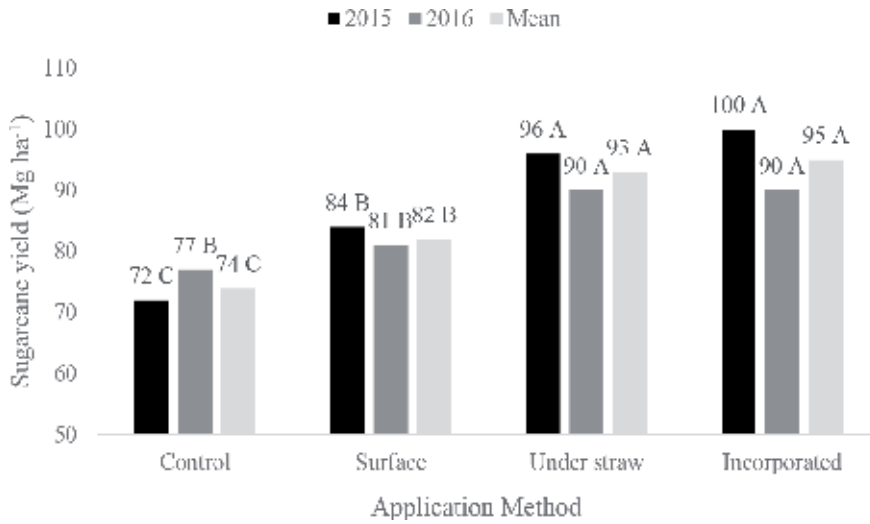


Figure 4. Effect of N-fertilizer application forms in sugarcane yield. Source: Franco et al. [24]. Note: Letters compare the forms of application in each of the years.

Therefore, in nitrogen fertilization, one must consider the choice of a source that allows lower losses or minimized if such losses exist, for example, by adjusting the mode of application. It is also convenient to adapt the time of application of N-fertilizer, in which, if there are operational conditions, nitrogen fertilizer should be applied when there is moisture in the soil, recommending the application incorporated in both sides of the cane row or application under the straw. Considering the management of the time and method of application of the nitrogen fertilizer in sugarcane, associated to the fact that in the south central region of Brazil, crop harvest occurs from March to December, thus an extended period for the accomplishment of cultural

Sugarcane Price (U\$\$ Mg ⁻¹)	N fertilizer price (U\$\$ Mg ⁻¹)					
	200	225	250	275	300	325
N economical rate (base line 120 kg N ha⁻¹)						
	Early	Middle	End	Early	Middle	End
10						
13	-25%	-13%	-25%	-30%	-25%	-30%
15						
18						
20	+10%	0%	+15%	-15%	-5%	-15%
23						

Ps: Price of sugarcane according to Consecana Brasil; Nitrogen fertilizer price according to Anda [2].

Table 2. N-economic rate to be applied in the sugarcane crop according to the harvesting season associated with the price of the raw material and the N-fertilizer.

treatments; it is possible to adjust the N dose to be applied (variation of 30%) without loss of productivity (**Table 2**). In this sense, the investment made in the acquisition of the nitrogen fertilizer must be removed by increasing productivity, then that high doses can obtain a gain which is not enough to pay off the investment [17].

3. Conclusion

The adoption of the best technologies (IPNI—Best Practices Fertilizer Management (BPFM)) to apply the N-fertilizer in sugarcane crop cultivated in intensive mechanization is possible to have an increase in the yield and a reduction in the application cost. It is possible with the choice of the right N rate, associated with the right application method and right time to perform this operation. In this context, the average yield gain is near 30%, as well as the production cost reduced near 15%.

Considering the sugarcane (green cane) cultivated in central south region of Brazil, where the harvest season occur between March and November, the best time to apply the N-fertilizer is in wet period. The best application method is incorporated at 0.1 m depth in both sides of the sugarcane row. With this adoption is possible have a reduction in the N rate applied in the sugarcane ratoon, as well as, there is an environmental sustainability in the nitrogen fertilization in sugarcane crop, due there is not adopt the high N rate in the fields.

The absence of alterations in TCH in sugarcane yield shows that the cultivation operation may not be necessary, mainly because sugarcane does not exploit the interleaving region [20, 21], even in areas where the traffic of machines in this region did not occur [22]. The application performed by the triple operation (number 1 in **Figure 2**), despite being incorporated in the soil, presents lower yields of TCH (**Figure 3**) due precisely to the distance at which the fertilizer is positioned in relation to the stump of the plant and the area covered by the root system, which in the middle of the interline is very small [20].

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Effects of Pesticides, Temperature, Light, and Chemical Constituents of Soil on Nitrogen Fixation

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Dennis Goss-Souza, Ritesh Singh, Arti Shanware
and Lalit Kharbikar*

Abstract

Nitrogen is a vital component of atmosphere and plays important roles in the biochemistry of all life forms on the earth. Various mechanisms of biological nitrogen fixation and recycling in the environment have been evolved in all known ecosystems. For example, symbiotic nitrogen fixation is the major N₂-fixing mechanism in the agroecosystems. Symbiotic nitrogen fixation is dependent on the biotic factors, such as host plant genotypes and the microbial strains. However, the interaction of these biotic factors is influenced by abiotic factors, such as climate and environmental conditions. The effects of various environmental variables, such as pesticides, temperature, and light as well as acidity, alkalinity, salinity, phosphorus, and water content status of the soils on the nitrogen fixation have been discussed briefly in this chapter.

Keywords: nitrogen fixation, salinity, ammonification, nitrite, denitrification

1. Introduction

The nutrient nitrogen is an essential element required by all life forms including the plants due to the presence in essential molecules such as proteins, amino acids, and enzymes. Although, this nutrient is in a large amount in the atmosphere, a small group of microorganisms is able to fix it becoming them available to plants.

The biological and physical processes in an ecosystem convert the nitrogen into multiple chemical forms and the phenomenon is called as nitrogen cycle. Biological nitrogen fixation accounts for about 70% of the atmospheric nitrogen. The four steps involved in a nitrogen cycle, namely nitrogen fixation, ammonification, nitrifications, and denitrification are described in **Figure 1**.

This process of nitrogen fixation is influenced by several aspects of biotic and abiotic variables. In this chapter, the effects of various factors especially the pesticide applications, temperature, and light as well as acidity, alkalinity, salinity, phosphorus, and water content status of the soils on the nitrogen fixation have been discussed briefly.

This chapter has addressed each environmental variable and discussed the effect of each one on the nitrogen fixation process and brings information related to it, for

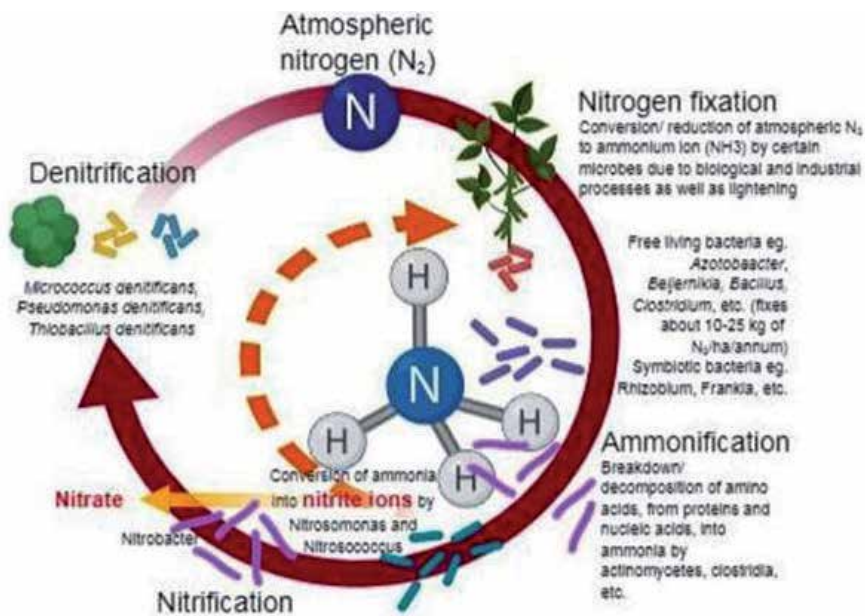


Figure 1.
The nitrogen cycle.

example, regarding the use of pesticides in an indiscriminate way. Usually, when the pesticides are used in the recommended dose, it does not harm the soil microbiota, but when the pesticides are overused, it may affect the physiological and growth characteristics of both rhizobia and legume plants. They also inhibit more or less the symbiotic nitrogen fixation by blocking biochemical communication and dialogues between the legumes and rhizobial symbionts.

2. Effects of pesticides on nitrogen fixation

In the current scenario of modern agriculture, agrochemicals play a key role in improving plant protection and production of food crops. Chemical pesticides are extensively used against a range of pests infesting agricultural crops worldwide. Due to the indiscriminate use of pesticides, the issue of the impact of these chemicals on the environment and soil has gained attention. Only about 0.1% of the total applied pesticides reach the target site/organism, while the remaining bulk contaminates the soil environment [1]. Consequently, sizable amounts of pesticides reach soil directly and indirectly, which affects the composition of soil microflora [2].

One of the most important and potentially limiting factors to biological nitrogen fixation is the use of chemical pesticides. Recommended doses of pesticides, in general, do not have any harmful effect on nitrogen fixation.

At higher doses, various chemical pesticides affect the physiological and growth characteristics of both rhizobia and legume plants. They also inhibit more or less the symbiotic nitrogen fixation by blocking biochemical communication and dialogues between the legumes and rhizobial symbionts [3].

Herbicides applied to leguminous crops constitute a potential hazard to the establishment and performance of the N₂-fixing root nodules. Soil and foliar application of herbicide at the recommended rates altered the morphology of root hairs and reduced nodule numbers and nitrogenase activity [4]. Some rhizobium strains also shown resistance towards herbicides when isolated and grown in laboratory media.

Legume seeds are inoculated with specific rhizobium to increase yield and at the same time they are also treated with fungicides to disinfect and guard it against seed and soil-borne pathogens. It is imperative to know the effect of seed disinfectants on the efficacy of rhizobia. Earlier studies show fungicidal seed treatment is safe as far as nitrogen fixation by rhizobium in symbiosis with legumes is concerned. Contradictory, Osman et al. reported a very strong negative effect of the dose of the thiram on *Rhizobium meliloti* [5].

3. Effects of temperature and light on nitrogen fixation

Temperature has a significant influence on survival and persistence of N-fixing microbes in soils [6]. Elevated temperatures may delay nodule initiation and development, and interfere with nodule structure and functioning in temperate legumes. Whereas in tropical legumes, competitive ability and N fixation efficiency of legume symbionts are mainly affected. Similarly, low temperatures reduce nodule formation and nitrogen fixation in temperate legumes.

In the extreme environment (-40 to -68°C) of the high arctic, native legumes can nodulate and fix nitrogen at rates comparable to those observed with legumes in temperate climates [7]. This indicates that both the plants and their rhizobia have successfully adapted to arctic conditions.

At constant temperature (20°C) under long photoperiod (14 hours), plants may not show a significant decrease in N fixation in terms of fixation per plant or per unit nodule mass [8]. However, under a short photoperiod (6 hours) at the same temperature, plants show a significant decrease in N fixation. Under short photoperiod, plants compensate for reduced photosynthesis by maintaining only half the root nodule mass and N fixation activity as that of long photoperiod. However, similar rates of N_2 fixation per unit mass of nodule may be observed in some plants under both the long and short photoperiods. This is because the shoot reserves for sustaining nitrogenase activity may compensate for reduced N fixation ability of plants.

4. Effects of acidity, alkalinity, and salinity on nitrogen fixation

A major problem associated with many acidic soils is metal toxicity. Highly acidic soils ($\text{pH} < 4.0$) frequently have low levels of phosphorus, calcium, and molybdenum and high levels of aluminium and manganese which are often toxic for both plants and symbiotic N-fixing bacteria [7]. Nodulation by this N-fixing bacteria is more affected than the normal growth of the host-plants in such conditions. It is a well-known fact that most leguminous plants grow less favourably in acidic conditions than in neutral or slightly alkaline conditions [9]. This is mainly due to a reduced nitrogen fixation as may be concluded from the improved growth at low pH upon the addition of combined nitrogen.

Highly alkaline soils ($\text{pH} > 8.0$) tend to be high in sodium chloride, bicarbonate, and borate, and are often associated with high salinity which reduces symbiotic nitrogen fixation [4]. However, the symbiotic N-fixing rhizobia, showing significantly higher salt tolerance, have been isolated from alkaline soils [10]. This suggests that the possession of high salt tolerance trait might be of some evolutionary significance for the survival of rhizobia in alkaline soils. Phosphate solubilising, alkaline tolerant rhizobia have also been isolated from wild legumes grown in dune systems of the southwest coast of India [11]. The stress tolerance traits of these rhizobia are of potential value in strain improvement of symbiotic bacteria for efficient N-fixing ability.

Soil salinity, which extremely increases in the protected cultivation, may occur when there are irregular irrigation schedules, inadequate drainage systems, wrong fertiliser applications, etc. [12]. The specific sensitivity of leguminous crops, where the N fixation is predominated by symbiotic bacteria, to soil salinity is well documented for initiation, development, and function of nodules [13]. An increase in salinity decreases the nodule permeability in these crops. This decrease is associated with a contraction of nodule inner-cortex cells and an increase in abscisic acid content of the nodule [14]. This phenomenon may lead to less survival of the symbiotic N-fixing bacteria associated with these crops. Even if the bacteria are survived, they may be less viable with decreased N-fixing ability. However, survival of the viable *Rhizobium* sp. under extreme conditions of salinity has been reported recently in root nodules of *Sesbania aculeata* [10].

5. Effects of phosphorus (P) status of soil on nitrogen fixation

Phosphorus deficiency in soils affects nodule functioning; however, it does not inhibit the other aspects of plant growth and metabolism. There exists an interaction between the plant's growth and N fixation in response to increasing P supply [15]. This interaction may be positive, zero, or negative. A negative interaction suggests a greater need for P by N₂-fixing plants; a zero interaction indicates that the plants have the same P requirement for growth and N fixation, while a positive interaction indicates that higher P supply may be inhibitory to N₂ fixation (Figure 2).

Legume tissues do not appear to have higher P content than those of other plants. Therefore a positive interaction exists between legume's growth and N fixation in response to P supply (Figure 2). For example, in both soybean and lucerne (alfalfa), nodules from plants grown under P deficiency may have a higher concentration of P than those grown with sufficient P and may fix more N₂ per unit of P [16].

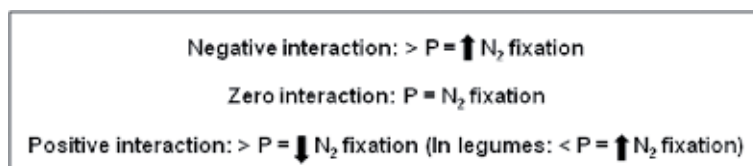


Figure 2.
Effects of phosphorus (P) status of soil on nitrogen fixation.

6. Effects of soil water availability on nitrogen fixation

Water influences the growth of soil micro-organisms through processes of diffusion, mass flow, and nutrient concentration [17]. Soil water retention is related to soil pore space, and soils containing larger pores and pore spaces retain less water. Thus, soil aggregates having smaller internal pore spaces offers more favourable environments for the growth of N-fixing microbes.

There is a high degree of correlation between soil water content and N-fixing activity [16]. Maximum N fixation occurs at about field capacity of soil and above this, the N-fixing activity may be reduced due to water logging. Slow natural drying of soil over a 6 week period may result in a progressive reduction of N-fixing activity, which can be restored by irrigation. This indicates that soil water availability is

the major environmental factor affecting nitrogen fixation. It is plausible that water stress directly affects nodule activity. This effect may further be aggravated by reduced supplies of photosynthate due to wilted plant leaves [18].

7. Conclusion

Environmental variables such as pesticides, temperature, and light as well as acidity, alkalinity, salinity, phosphorus, and water content status of the soils have a significant impact on nitrogen fixation. Despite many decades of progress and the acquisition of ample information, the physiological and molecular bases of the effects of these environmental variables on the symbiotic and non-symbiotic N₂ fixation systems remains largely unknown and empirical in nature. Although understanding these processes was originally thought to be straightforward and tractable, we now have learned that the flavonoid nod-gene inducers are specific for a particular N₂ fixation system [19]. Needless to say that, the production of these inducers is influenced by environmental variables. Therefore, more work needs to be done to understand the underlying molecular bases for tolerance to environmental variables in both N₂ fixation systems. Further, the genomics and proteomics tools need to be combined with traditional plant breeding and microbial selection studies in order to rapidly define and utilise their genetic loci involved in tolerance to environmental stresses.

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Distribution and Characterization of the Indigenous Soybean-Nodulating Bradyrhizobia in the Philippines

Maria Luisa Tabing Mason and Yuichi Saeki

Abstract

The research about the indigenous soybean bradyrhizobia in the Philippines is scarce, and this greatly influences the improvement of soybean production in the country. Thus, soil samples were collected from 11 locations in the country and were used to isolate the indigenous bradyrhizobia in the soil. Through the use of polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP) and sequence analysis of the 16S rRNA gene, 16S-23S rRNA gene internal transcribed spacer (ITS) region and *rpoB* housekeeping gene, the most abundant and dominant indigenous bradyrhizobia in the country were identified. Then, the representative isolates of the most dominant species per location were used to test their symbiotic efficiency and N-fixation ability with the local soybean cultivars. The results showed that among all the tested indigenous strains, the *B. elkanii* IS-2 is the most effective and efficient microsymbiont of the Philippines' local soybean cultivars. This report was able to provide necessary information on the distribution of soybean bradyrhizobia in the Philippines and characterized the symbiotic performance of the indigenous strains.

Keywords: tropical rhizobia, genomic diversity, N-fixation, symbiotic performance

1. Introduction

Soybean (*Glycine max* [L.]) is a leguminous plant that can form a symbiotic relationship with the nitrogen-fixing group of bacteria living in the rhizosphere, which are generally termed as rhizobia. In the Philippines, soybean production has been limited by the poor grain yield which leads to the importation of more than 90% of the country's demand. Thus, it is essential to look for an alternative way to increase the volume of production per unit area.

The research about tropical bradyrhizobia indicated a high diversity of species and their distribution has been reported to be due to several abiotic and biotic factors such as soil acidity [1–3], alkalinity [3, 4], temperature [1, 5–11], climate [12, 13], soil water status [14, 15], soil type [2, 14, 16–18], and soil management or cultural practices [2, 14, 19–22]. In case of the Philippines, the pioneer research that was able to identify the most dominant species of bradyrhizobia in the country reported that *B. elkanii* species was the most abundant, followed by the

B. diazoefficiens, *B. japonicum*, and some yet unclassified *Bradyrhizobium* sp. [14]. In this later study, it was identified that the distribution of these indigenous species of bradyrhizobia were influenced mainly by the water status of the soil, followed by soil pH, nutrient content, and soil type.

Previous studies have reported that aside from the various agro-environmental factors, the competition with the native rhizobia is a hindrance for a successful inoculation [23, 24]. The utilization of inoculants for legumes had shown promising results for the increase in grain yield as evidenced by recent reports [25, 26]. The role of the biological nitrogen fixation (BNF) in providing the N requirement of the plant in a natural way has been deemed necessary especially these times that the soil has become more degraded due to over-fertilization. The indiscriminate use of NPK fertilizer could cause soil pollution and less crop production [27]. Therefore, it is essential to select and evaluate the symbiotic competitiveness of the indigenous strains which are native and existing in high density in the country. The use of different genetic markers to accurately identify the rhizobia for taxonomic purposes has been proposed [28] and so we have used three genetic markers such as the 16S rRNA gene, 16S-23S rRNA gene internal transcribed spacer (ITS) region, and the *rpoB* housekeeping gene.

Thus, this study was formulated with the aim to utilize the recently identified indigenous bradyrhizobia in the Philippines and characterize their symbiotic performance with the local soybean cultivars.

2. Materials and methods

2.1 Collection of soil and soybean cultivation

The soil samples were collected from 11 locations in the Philippines, where some basic information on the sites are listed in **Table 1**. The collection of soil was conducted by first removing the surface litters then, obtaining a bar of soil with a dimension of approximately 20 cm in depth and 3 cm in thickness that weighs about 1 kg. A total of 10 subsamples per location were obtained and were mixed thoroughly until a 1 kg of composite soil sample was taken. A 0.5 kg soil was air-dried for the chemical analyses while the remaining 0.5 kg of the fresh soil was used for the soybean cultivation.

Location	Rep. Isolate	pH ^a	N ^a (%)	P ^a (mgkg ⁻¹)	K ^a (mgkg ⁻¹)	CEC ^b (meq)
1. Isabela1 (IS)	IS-2	5.90	0.13	1.86	51.80	17.1
2. Isabela 2 (GI)	GI-4	5.52	0.17	2.30	58.60	27.4
3. Benguet (BA)	BA-24	5.22	0.24	22.22	51.00	29.3
4. Nueva Ecija1 (NE1)	NE1-6	6.21	0.13	6.74	73.90	30.1
5. Nueva Ecija 2 (NE2)	NE2-37	5.81	0.22	21.63	49.40	27.7
6. Sorsogon (SO)	SO-1	5.26	0.22	2.57	55.80	20.3
7. Leyte (LT)	LT-3	5.80	0.15	6.39	174.20	40.6
8. Negros Occ. (NR)	NR-2	5.62	0.07	20.44	74.10	14.1
9. Bohol (BO)	BO-4	5.82	0.06	2.80	47.80	9.7
10. Maguindanao (SK)	SK-5	6.64	0.19	4.53	59.60	40.5
11. South Cotabato (SC)	SC-3	5.52	0.14	31.18	47.20	9.7

^aMason et al., 2018

^bThis study

Table 1. Result of the soil chemical analysis on the 11 locations in the Philippines.

The cultivation of soybean was performed using a 1-L capacity culture pots (n = 3). Each pot was filled with vermiculite and a N-free solution [29] was added at 40% (vol/vol) water content. The culture pots were sterilized by autoclaving for 20 min at 121°C. Meanwhile, the soybean seeds were surface-sterilized by soaking into a 70% EtOH for 30 s, then by a diluted sodium hypochlorite solution (0.25% available chlorine) for 3 min and followed by washing with sterile distilled water for about 6–8 times. Then, a 2–3 g of soil sample was placed on the vermiculite at a depth of about 2–3 cm, the seeds were sown on the soil and the pot was weighed and recorded. The plants were grown inside a growth chamber for 28 days at 28°C (8 h, night) and 33°C (16 h, day) then were supplied weekly with sterile distilled water until the initial weight of the pot was reached.

2.2 Isolation of soybean rhizobia and DNA extraction

After 28 days, approximately 20 random nodules were collected from the roots of each soybean plants and were sterilized with 70% EtOH and sodium hypochlorite solution as previously described [29]. Each nodule was homogenized with sterile distilled water in a microtube and streaked on to a yeast-extract mannitol agar (YMA) plate [30]. The YMA plate was incubated in the dark at 28°C for about 1 week until a single colony was formed. After then, the single colony was streaked on to a YMA plate containing a 0.002% (wt/wt) bromothymol blue (BTB) [31] and was incubated as above. Repeated streaking was done until a pure single colony was obtained which was cultured for about 3–4 days in a HEPES-MES (HM) broth culture [32, 33] at 28°C in a shaker for 120 rpm. After then, the bacteria cells were collected by centrifugation at 9000×g and washed with sterile distilled water. The DNA was extracted by using BL buffer as described [34] from the method reported by Hiraishi et al. [35].

2.3 PCR amplification of the 16S rRNA gene, ITS region, and *rpoB* gene

For the amplification of the 16S rRNA gene, the primer set: 16S-F: 5' AGAG TTTGATCCTGGCTCAG-3' and 16S-R2: 5'- CGGCTACCTTGTTACGACTT-3' [36]. The PCR tubes were then placed in the PCR Thermal Cycler (TaKaRa Co. Ltd.) with the following conditions: pre-run at 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Final extension was set at 72°C for 10 min and indefinite preservation at 4°C.

On the other hand, the PCR amplification of the ITS region was conducted using the following primer set: Bra-ITS-F: 5-GACTGGGGTGAAGTCGTAAC-3' and Bra-ITS-R1: 5'-ACGTCCTTCATCGCC TC-3' [6]. The PCR cycle for the ITS region was almost the same with the 16S rRNA gene except for a shorter denaturation and annealing periods which were conducted at 30 s for each step.

For the *rpoB* gene, simplification was done using the following primer sets: *rpoB*83F: 5'-CCTSATCGAGGTTTCAC AGAAGGC-3' and *rpoB*1540R: 5'-AGCTGCGAGGAACCGAAG-3' [37]. The PCR cycle conditions were as follows: pre-run at 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Final extension was set at 72°C for 5 min and indefinite preservation at 4°C.

2.4 Restriction fragment length polymorphism (RFLP) of the 16S rRNA gene, ITS region, and *rpoB* gene

The successfully amplified products were subjected to the RFLP treatment using four restriction enzymes which were *HhaI*, *HaeIII*, *MspI*, and *XspI*. For the *rpoB* gene, the enzymes that were used for RFLP are *HaeIII*, *MspI*, and *AluI*.

The reference strains that were used in this study are the *Bradyrhizobium* USDA strains (*B. japonicum* USDA 4, 6^T, 38, 62, 115, 122, 123, 124, 125, 127, 129, 135, *B. diazoefficiens* USDA 110^T, *B. elkanii* 31, 46, 61, 76^T, 94, 130, and *B. liaoningense* 3622^T) which were previously described [38]. This was done in a 10- μ L reaction mixture containing a 2.5- μ L amplified PCR product and was incubated in a 37°C for 16 h. Afterward, a 3–4% agarose gel was used in a submerged gel electrophoresis for about 60 min, stained with ethidium bromide and the patterns were visualized using a Luminiscent Image Analyzer LAS-4000 (FUJIFILM Tokyo, Japan).

2.5 Single-strain inoculation test

After the amplification and the RFLP treatment of the 16S rRNA gene, a single-strain inoculation test was conducted for all the amplified isolates that shared the same restriction enzymes' fragment patterns with the USDA *Bradyrhizobium* reference strains. This was done to confirm the strain's capability to nodulate soybean and was tested on two local varieties which are the PSB-SY2 and Collection 1 which are both commercially available across the country.

The cultivation of soybean was conducted as described above, but without soil. Each isolate was cultured in a YM broth (YMB) [30] at 28°C for about 1 week on a shaker. After then, the cultures were diluted with sterile distilled water at about 10⁶ cells mL⁻¹ and were inoculated on the cultivated soybean at a rate of 1.0 mL per seed. This was done with three replications. After inoculation, the weight of the pot was recorded and it was placed inside a growth chamber with a condition set to mimic the average temperature in the Philippines at 26°C (8 h, night) and 33°C (16 h, day). The same condition was used for the cultivation of an uninoculated control and a positive control pot that was inoculated with *B. diazoefficiens* USDA110. The pots were kept inside the growth chamber for 28 days and were supplied weekly with sterile distilled water until the initial weight of each pot was reached.

2.6 Sequence analysis of the 16S rRNA gene and ITS region

According to the similarities of the band patterns through the RFLP treatment, a representative of the most abundant isolates was chosen for each location. In total, there were 11 isolates that were selected to confirm the nucleotide sequence of the 16S rRNA gene and the ITS region. The sequence primers that were used were reported previously [22]. From the PCR amplified product, the samples were purified according to the protocol of the manufacturer (Nucleospin® Gel and PCR Clean-up; Macherey-Nagel, Germany). Then, the samples were sent to the company for the sequence analysis (Eurofins Genomics, Tokyo, Japan).

Then, the Basic Local Alignment Search Tool (BLAST) program in DNA Databank of Japan (DDBJ) was used to determine the nucleotide homology of the isolates. Only the sequences with a similarity of at least 99% for the 16S rRNA and 96% for the ITS region with our isolates were retrieved from the BLAST database. The alignment was performed using the ClustalW and Neighbor-Joining [21] method was used to construct the phylogenetic trees. The genetic distances were computed using the Kimura 2-parameter model [39] in the Molecular Evolutionary Genetic Analysis (MEGA v7) software [40]. Subsequently, the phylogenetic trees were bootstrapped with 1000 replications. All the nucleotide sequences determined in this study were deposited in DDBJ at <http://www.ddbj.nig.ac.jp/>.

3. Results

3.1 Soil analysis and characterization of the indigenous bradyrhizobia

The soil samples that were used in this study were all slightly to moderately acidic (5.22–6.64) with non-saline condition (0.05–0.20 dS/m), low nutrient status as evidenced by low amounts of NPK and CEC (**Table 1**). These values are generally typical of agricultural soils that are used for crop production all throughout the year. These results showed that the soils used in this study have low fertility status that indicated the need for soil restoration strategies.

The growth morphologies of the pure single colony for each strain of bradyrhizobia were characterized and listed in **Table 2**. All the isolates were slow growers which were able to form single colonies measuring about 2 mm between 5 and 7 days upon streaking on YMA plates and incubation in a dark room. Based on the morphology, the isolates were grouped into three. Group I include the isolates IS-2, NE1-6, NR-2, and BO-4 which were translucent and the colonies are circular

Isolate	No. of isolates	Colony morphology	Formation of acid / alkaline substances
IS-2	40	Translucent, circular, liquid, slightly-convex elevation with entire margin	Alkaline
GI-4	30	Translucent, circular, slightly-mucoid, convex elevation with entire margin	Alkaline
BA-24	31	Translucent, circular, mucoid, convex elevation with entire margin	Alkaline
NE1-6	49	Translucent, circular, liquid, slightly-convex elevation with entire margin	Alkaline
NE2-37	31	Translucent, circular, slightly-mucoid, convex elevation with entire margin	Alkaline
SO-1	44	Translucent, circular, mucoid, convex elevation with entire margin	Alkaline
LT-3	42	Translucent, circular, mucoid, convex elevation with entire margin	Alkaline
NR-2	23	Translucent, circular, liquid, slightly-convex elevation with entire margin	Alkaline
BO-4	24	Translucent, circular, liquid, slightly-convex elevation with entire margin	Alkaline
SK-5	29	Translucent, circular, mucoid, convex elevation with entire margin	Alkaline
SC-3	31	Translucent, circular, mucoid, convex elevation with entire margin	Alkaline
Total	374		

Table 2. Characterization of the morphology of the indigenous bradyrhizobia isolated from Philippines' soil according to their growth on Yeast-Extract Mannitol Agar plate medium [30].

in shape with slightly convex elevation and an entire margin. When they were manipulated with a needle, the colony was liquid. Group II include the isolates BA-24, SO-1, LT-3, and SK-5 were translucent with circular colonies, convex elevation with entire margin. When manipulated with a needle, the colonies have mucoid viscosity. On the other hand, last group (III) are the isolates GI-4 and NE2-37 which have similar growth morphology with Group II except that their viscosity was intermediate between liquid and mucoid. All the isolates produced alkaline substances when grown on YMA plate with BTB which is an indication of the *Bradyrhizobium* genus.

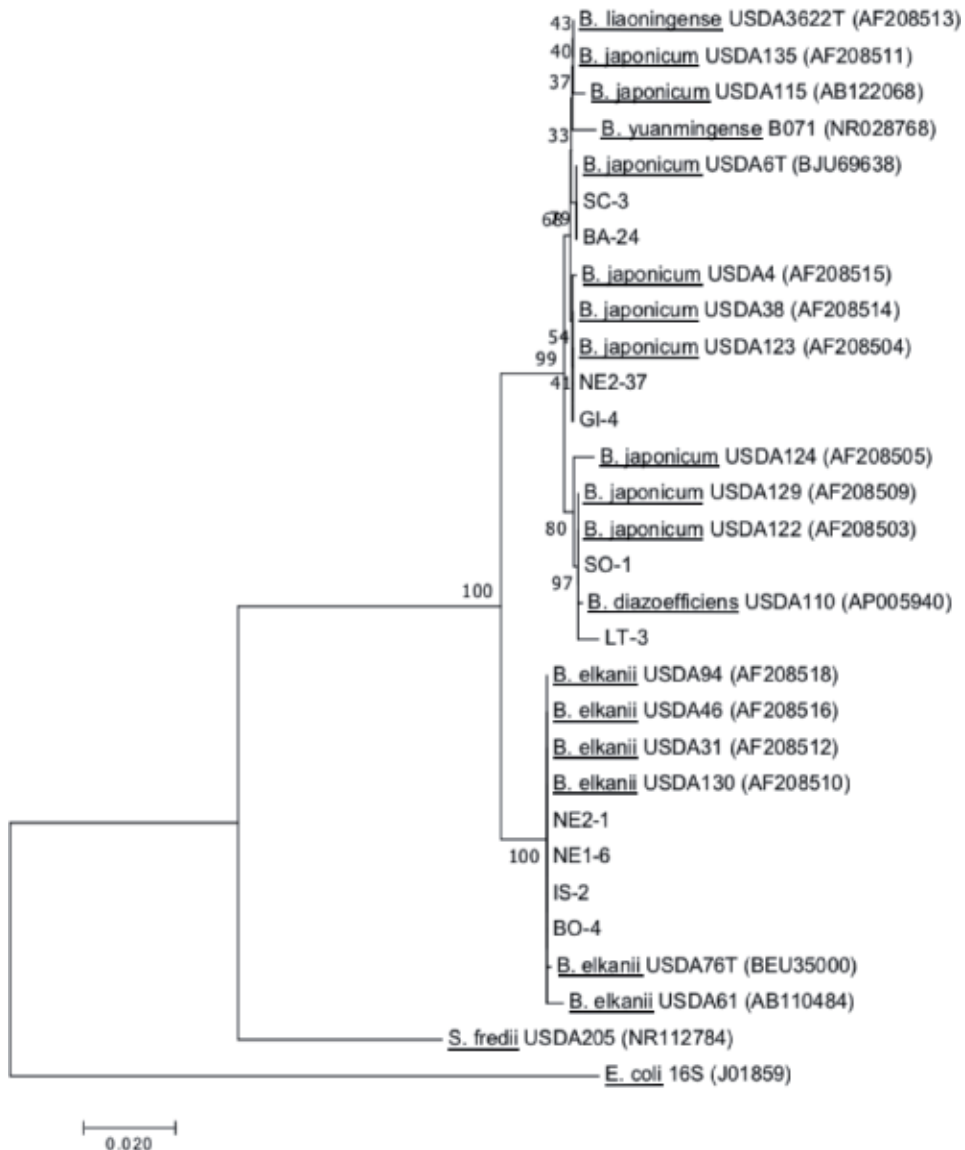


Figure 1. Phylogenetic tree based on the sequence analysis of the 16S rRNA gene. The tree was constructed using the Neighbor-Joining method with the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications in MEGA v.7 software. The accession numbers are indicated only for sequences obtained from BLAST. The isolates in this study are indicated with letters and number combinations, for example: BO-4— isolate no. 4 collected from Bohol.

phylogenetic tree from the sequence analysis of the 16S rRNA gene. To further confirm the classification of the indigenous bradyrhizobia, the phylogenetic trees constructed from the ITS region and the *rpoB* gene are presented in **Figures 2** and **3**, respectively. For the ITS region and the *rpoB* gene, the isolates were distinctly grouped into three species, *B. elkanii*, *B. japonicum*, and *B. diazoefficiens*. Additionally, an independent cluster composed of the representative isolates GI-4



Figure 3. Phylogenetic tree based on the sequence analysis of the *rpoB* housekeeping gene. The tree was constructed using the Neighbor-Joining method with the Kimura 2-parameter (K2P) distance correlation model and 1000 bootstrap replications in MEGA v7 software. The accession numbers are indicated only for sequences obtained from BLAST. The isolates in this study are indicated with letters and number combinations, for example: BO-4-isolate no.4 collected from Bohol.

and NE2–37 that are seen in the ITS region and *rpoB* phylogenetic trees were treated as *Bradyrhizobium* sp. due to their nucleotide divergence with the known species from the BLAST engine.

Meanwhile, the distribution of the most abundant soybean bradyrhizobia in the country is shown in **Table 3**, which was classified according to the results of the sequence analysis of the three genetic markers used in this study. From here, it can be seen that 4 of the 11 locations were dominated with *B. elkanii* species (37.74%), 3 locations were dominated by the isolates under the *B. diazoefficiens* (28.54%), whereas 2 locations each were dominated by the species of *B. japonicum* (16.98% and *Bradyrhizobium* sp. (16.74%). This indicated that in the Philippines, the species of *B. elkanii* is the most prevalent in terms of population and the most widespread in terms of location as its presence was detected even in minor populations on all the locations except for one, which was Sorsogon.

3.3 Symbiotic efficiency and N-fixation ability of the indigenous bradyrhizobia

Upon classification, it is important to determine the capability of the indigenous bradyrhizobia for their symbiotic performance and N-fixation ability. As can be seen in **Figure 4A**, although USDA110 strain has the highest N-fixation ability, it should be noted that the amount of N that was fixed by *B. elkanii* IS-2 is the highest among all the indigenous bradyrhizobia isolated from the Philippines' soil on *Rj₄* plants. However, the N-fixation ability of IS-2 was comparably similar with other strains (GI-4, NE2–37, and SK-5) with the non-*Rj* plants. The lowest N-fixation ability was observed from the strain LT-3 which was classified under the *B. diazoefficiens* species. This suggested that the process of biological N-fixation is a mutual relationship that is influenced by both the plant and the rhizobia and that the plant-rhizobia compatibility should be taken into consideration for inoculation strategies.

Presented in **Figure 4B** is the nodulation test performed on the strains and it can be seen for *Rj₄* plants, there was not much significant difference in the nodulation

Location	<i>B. elkanii</i>	<i>B. diazoefficiens</i>	<i>B. japonicum</i>	<i>Bradyrhizobium</i> sp.	No. isolates
Isabela1 (IS)	100.0	-	-	-	40
Isabela 2 (GI)	16.7	-	-	83.3	36
Benguet (BA)	3.0	-	97.0	-	33
Nueva Ecija1 (NE1)	87.5	1.8	-	10.7	56
Nueva Ecija 2 (NE2)	8.6	-	2.9	88.6	35
Sorsogon (SO)	-	100	-	-	44
Leyte (LT)	2.3	97.7	-	-	43
Negros Occ. (NR)	74.2	3.2	22.6	-	31
Bohol (BO)	82.8	13.8	3.4	-	29
Maguindanao (SK)	23.2	67.4	-	9.3	43
South Cotabato (SC)	8.8	-	91.2	-	34
Total isolates	160	121	72	71	424
Percentage (%)	37.74	28.54	16.98	16.74	

Table 3. Percentage distribution of the dominant *Bradyrhizobium* species in the Philippines as identified from the sequence analysis of the 16S rRNA gene, 16S-23S internal transcribed spacer (ITS) region, and *rpoB* housekeeping gene.

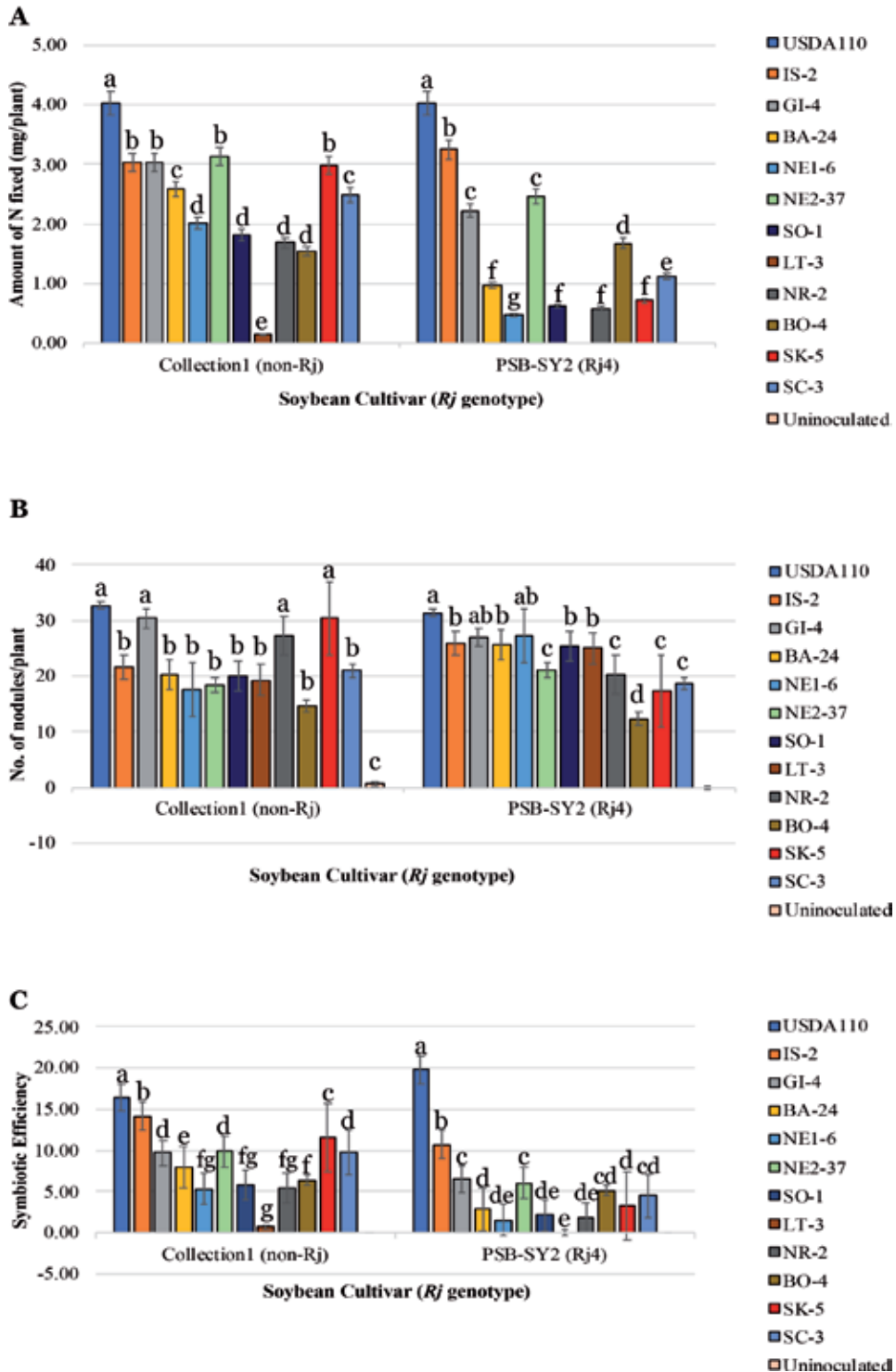


Figure 4. Characterization of the dominant indigenous *Bradyrhizobium* strains isolated from the 11 locations in the Philippines based on the (A) amount of Nitrogen fixed (B) nodulation ability and (C) symbiotic efficiency as influenced by the single-strain inoculation test against the reference strain *B. diazoefficiens* USDA110 for the two soybean cultivars from the Philippines. Different letters indicate a significant difference by Tukey's test at $p > 0.05$, $n=3$, $\text{bar}=\text{SE}$.

ability of the strains, except for the low nodulation ability that was observed for the BO-4. In contrast, a significant difference in the nodulation ability was detected on the strains upon inoculation on the non-*Rj* plants. Although all the strains were able to form nodules on both soybean cultivars, the strains GI-4, NR-2, and SK-5 obtained the highest number of nodules for the non-*Rj* plants.

On the other hand, the symbiotic efficiency of the strains used in this study is presented in **Figure 4C**. Similar with the N-fixation ability, the USDA110 still possesses the highest symbiotic efficiency. But among all the indigenous bradyrhizobia, the strain IS-2 obtained the highest efficiency regardless of the *Rj* genotype of the soybean plants. As with the N-fixation, LT-3 obtained the least efficiency for symbiosis. This result indicated that the symbiotic efficiency of the rhizobia might not be directly influenced by the *Rj* genotype of the plant.

4. Discussion

4.1 Distribution and characterization of bradyrhizobia in the Philippines

The distribution of the most dominant and abundant species of soybean bradyrhizobia in the Philippines are reported in this study along with the characterization of their growth morphology. According to our earlier reports, we have elucidated that the Philippines was dominated by the soybean-nodulating bradyrhizobia that were classified under the *B. elkanii* species and the most important agro-environmental factors that affected their diversity and prevalence in the country was the similarity of soil pH, salinity, and temperature in the study locations [5, 14]. Our observation that there are abundant and high diversity of indigenous bradyrhizobia in the Philippines is similar with previous reports in other sub-tropical and tropical regions [12, 25, 41–44]. The temperate regions of Japan and USA were studied in the past and were reported to be dominated by species of *B. japonicum* and *B. diazoefficiens* [6, 9–11, 13, 45]. Our report showed that the distribution of bradyrhizobia in a tropical region like the Philippines seemed to be different from those of temperate regions.

Meanwhile, it was included in a recent report that the distribution and abundance of *B. diazoefficiens* and *B. japonicum* at specific locations were due to the longer period of flooding conditions [14]. The effect of nutrient content and soil type were also correlated with the abundance of these two species. In a report by Shiina et al. [17], it was stated that the predominance of *B. diazoefficiens* was observed on more anaerobic condition; whereas, *B. japonicum* was predominant on aerobic soils which was supported by another study [18]. Additionally, it was reported that *B. diazoefficiens* becomes predominant with enhanced flooding condition [15]. These results confirmed that our observations for the abundant of *B. diazoefficiens*, followed by *B. japonicum* and *Bradyrhizobium* sp. on flooded areas in the country which were usually used for planting rice.

4.2 Symbiotic and N-fixation ability of indigenous bradyrhizobia

In this report, the symbiotic performance, N-fixation and nodulation ability of the indigenous soybean bradyrhizobia from the Philippines were evaluated against that of the *B. diazoefficiens* USDA110 strain. The USDA110 has been extensively used in the world as a model strain for soybean inoculation due to its high ability for N-fixation and symbiotic efficiency [25, 46, 47]. Additionally, its possession of a complete set of denitrification genes that allows the release of N₂ back into the atmosphere makes it an ideal strain also for climate change mitigation studies [17, 48–50].

Therefore, we hypothesized that the indigenous isolates SO-1, LT-3, and SK-5, which were phylogenetically clustered under the USDA110 would also prove to be as effective N-fixer and efficient microsymbiont of soybean cultivars from the Philippines. However, our results indicated that the N-fixation ability and symbiotic efficiency of LT-3 and SO-1 were very low in comparison to the other indigenous isolates. For the low performance of these two isolates, it is hypothesized that the inherent ability of these strains to fix N and establish a symbiotic relationship with soybean is low. This could be explained by the fact that their nodulation ability was comparably similar with the other strains which possess higher N-fixation ability and symbiotic efficiency. In contrast, the isolate IS-2, which was clustered under the *B. elkanii* species, showed the highest symbiotic efficiency for both *Rj*-genotypes of the soybean cultivar used and the highest N-fixation ability for *Rj*₄ plants. In a previous report, the *Rj* genes that could restrict the nodulation of soybean by some strains of bradyrhizobia was summarized [51] but in case of our present report, all the strains in this study were not restricted by the two *Rj*-plants that were used. This led us to consider that the low N-fixation and symbiotic performance of some strains were not due to the restrictions from the *Rj*-genotypes of the plants but could be attributed to the strains' intrinsic capabilities. These observations might explain the reason for low yield of soybean in the Philippines. It was reported that many strains of *B. elkanii* were relatively inefficient microsymbionts of soybean and can induce chlorosis in soybean plants [52]. In a previous report [53], the high temperatures in tropical regions can limit the nodulation which could explain the low soybean yield.

It was expected that the strains which were classified as *B. diazoefficiens* could provide a better symbiotic performance than the other strains that were collected. However, the data showed that *B. elkanii* might establish a better symbiosis with local soybean cultivars in the Philippines. This result is crucial in order to devise strategies on how to increase the local production of soybean by inoculation with the indigenous strains.

Upon considering these results with the N-fixation and symbiotic performance ability of the strains, the number of nodules that can be formed from the single-strain inoculation does not seem to influence the amount of N that each strain can fix nor their symbiotic ability.

5. Conclusion

In this report, we have revealed that the distribution of tropical soybean bradyrhizobia seemed to be different than those of temperate bradyrhizobia in terms of population dominance of *B. elkanii* on higher temperature region like the Philippines. Additionally, it is proposed that for the Philippines, the most efficient N-fixer and symbiotically efficient species of bradyrhizobia would be *B. elkanii*. Yet, our results were made under the laboratory conditions only, so the results that were obtained here might not be as expected when done in field condition. For future research, utilization of more local soybean varieties with different soil types both in a controlled environment and on natural field condition would be beneficial to target the development of a site-specific and useful potential soybean inoculant. The data generated in this report would be beneficial for the augmentation of inoculation strategies in the country.

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Conflict of interest

The authors declare no conflict of interest.

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Phylogenomic Review of Root Nitrogen-Fixing Symbiont Population Nodulating Northwestern African Wild Legumes

Mokhtar Rejili, Mohamed Ali BenAbderrahim and Mohamed Mars

Abstract

The present review discusses the phylogenomic diversity of root nitrogen-fixing bacteria associated to wild legumes under North African soils. The genus *Ensifer* is a dominant rhizobium lineage nodulating the majority of the wild legumes, followed by the genus *Rhizobium* and *Mesorhizobium*. In addition, to the known rhizobial genera, two new *Microvirga* and *Phyllobacterium* genera were described as real nodulating and nitrogen-fixing microsymbiotes from *Lupinus* spp. The promising rhizobia related to nitrogen fixation efficiency in association with some legumes are shared. Phylogenetic studies are contributing greatly to our knowledge of relationships on both sides of the plant-bacteria nodulation symbiosis. Multiple origins of nodulation (perhaps even within the legume family) appear likely. However, all nodulating flowering plants are more closely related than previously suspected, suggesting that the predisposition to nodulate might have arisen only once. The origins of nodulation, and the extent to which developmental programs are conserved in nodules, remain unclear, but an improved understanding of the relationships between nodulin genes is providing some clues.

Keywords: rhizobia, North Africa, symbiosis, legumes, phylogenomic

1. Introduction

Africa has a vast array of indigenous legumes, ranging from large rain forest trees to small annual herbs [1]. However, in recent years, there has been a tendency in agriculture and forestry to use exotic species for crops and wood. As has been pointed out several times over nearly 30 years, most recently [2], by the US National Academy of Sciences, this ignores the potential of the native species, which are arguably better adapted to their environment. For this review, the nodulated indigenous legume genera in Northwestern Africa with known uses have been selected to illustrate the problems and potential for their better exploitation.

The wild legume flora in Northwestern Africa is rich, with great specific and infraspecific diversity [3]. The overgrazing and expansion of agriculture has gradually

led to the regression and extinction of many pastoral and forage species. In addition, desertification causes disturbance of plant-microbe symbioses, which are a critical ecological factor in helping further plant growth in degraded ecosystems [4]. In this context, the establishment of indigenous pastoral legume species associated with their appropriate symbiotic bacterial partners may be of increased value for success in soil fertility restoration. Biological N₂ fixation (BNF) is the major way for N input into desert ecosystems. Rhizobium-legume symbioses represent the major mechanism of BNF in arid lands, compared with the N₂-fixing heterotrophs and associative bacteria [5, 6] and actinorhizal plants [7, 8]. Deficiency in mineral N often limits plant growth, and so symbiotic relationships have evolved between plants and a variety of N₂-fixing organisms [9]. The symbiotically fixed N₂ by the association between rhizobium species and the legumes represents a renewable source of N for agriculture. Values estimated for various legume crops and pasture species are often impressive [10]. In addition to crop legumes, the nodulated wild (herb and tree) legumes have potential for nitrogen fixation and reforestation and to control soil erosion [11]. It has been reported that a novel, suitable wild legume-rhizobia associations are useful in providing a vegetational cover in degraded lands [12].

Considering the major ecological importance of many wild legumes such as *Retama* sp., *Acacia* sp., *Lotus* sp., *Lupinus* sp., *Medicago* sp., etc. in Northwest Africa by their important role in soil fertility maintenance, coverage, and dune stabilization, the present chapter proposes to review the phylogenomic diversity of root nitrogen-fixing symbiont population nodulating Northwestern African wild legumes listed in the bibliography, some of which are common and play important ecological and pastoral roles, but others are rare and endangered. As well as the host legumes, the nodule endosymbionts also vary widely in Africa and include newly described members of both α and β branches of the Proteobacteria, now often referred to as α - or β -rhizobia, even though they do not have “rhizobium” as part of their generic names [13].

Therefore, understanding the nature of indigenous populations of rhizobia-nodulating wild legumes is of considerable agricultural significance. It is also of interest to identify a wider variety of bacterial strains in a bid to define new strains for the production of inoculants for smallholder farms.

2. Genetics and functional genomics of legume nodulation

The interaction between rhizobia and legumes in root nodules is an essential element in sustainable agriculture, as this symbiotic association is able to enhance biological fixation of atmospheric nitrogen (N₂) and is also a paradigm in plant-microbe signaling [14–16]. The knowledge of the whole genome would allow the specific features of each rhizobium to be identified. The prominent feature of this group of bacteria is their molecular dialog with plant hosts, an interaction that is enabled by the presence of a series of symbiotic genes encoding for the synthesis and export of signals triggering organogenetic and physiological responses in the plant [17, 18]. In recent years, significant progress has been made in resolving the complex exchange of signals responsible for nodulation through genome assembly, mutational and expression analysis, and proteome characterization of legumes [14, 19, 20] and rhizobia [15, 21–23].

3. Phylogenomic of wild legume root nitrogen-fixing symbionts

The known diversity of rhizobia increases annually and is the subject of several reviews, the most recent and comprehensive being that of [24]. It is not our intention to revisit this subject nor the genetic basis of nodulation [25, 26], the horizontal

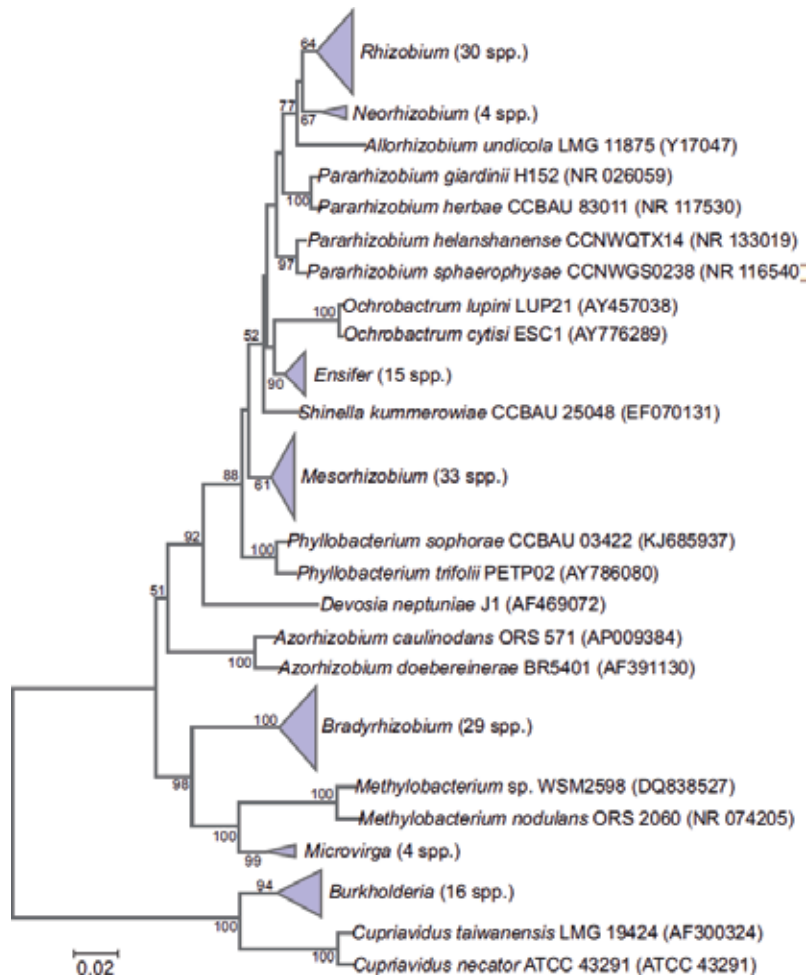


Figure 1. Phylogenetic tree showing the relationships of currently described genera and species of alpha- and beta-rhizobia, based on aligned sequences of the 16S rRNA gene (1341-bp internal region) (adapted from [28]).

transfer of symbiosis-related genes [23], or the symbiovar concept [27] but instead to attempt to link, where possible, rhizobial genotypes with their geographical locations and/or legume tribes/genera. At the time of writing, rhizobia consist of a diverse range of genera in the Alphaproteobacterial and Betaproteobacterial classes and are termed “alpha-rhizobia” and “beta-rhizobia,” respectively (**Figure 1**).

3.1 The genus *Bradyrhizobium* (*Bradyrhizobiaceae*)

The *Bradyrhizobium* genus was described by Jordan in 1982 [29]. It currently consists of nine rhizobia species.

For the *Loteae* tribe, previous studies found that *Lotus palustris* and *L. purpureus* species from Algeria were nodulated by *Bradyrhizobium lupini*, and *L. pedunculatus* by *B. japonicum* [30]. However, *L. creticus* ssp. *maritimus* is nodulated by both [30]. At Tunisia, *L. roudairei* microsymbiont is closely related to *B. japonicum* [31]. For the *Acacieae* tribe, two studies reported that rhizobial strains associated to the *Acacia saligna*, an Australian introduced species, belonged to the genus *Bradyrhizobium* genus under Algerian and Moroccan soils [32–34]. For the *Genisteae* tribe, it has been noticed that *Bradyrhizobium* is the dominant genus of

symbiotic nitrogen-fixing bacteria associated with *Retama* species in North Africa: *Retama monosperma*, *R. raetam*, and *R. sphaerocarpa* (Algeria: [35, 36]; Morocco: [37]). Recently, the novel *B. retamae* species, in which groups with *B. elkanii* and *B. pachyrhizi* and related *B. lablabi* and *B. jicamae* type strains are included in *Bradyrhizobium* group II [38], has been isolated from *R. sphaerocarpa* and *R. monosperma* in Morocco [37]. For the genus *Cytisus*, two studies reported that *Cytisus villosus* is nodulated by *B. cytisi* sp. nov. and *B. rifense* sp. nov. in Morocco [39, 40] and by genetically diverse *Bradyrhizobium* strains in Algeria belonging to *B. japonicum* and *B. canariense* and to new lineage within the *Bradyrhizobium* genus [41]. Fifty-two strains isolated from root nodules of the Moroccan shrubby legume *Cytisus triflorus* were genetically characterized, and results showed that it is nodulated by *Bradyrhizobium* strains, with 99% homology with *Bradyrhizobium* genosp. AD [42]. For the genus *Lupinus*, some endosymbiotic bacteria of *L. luteus* and *L. micranthus* from Tunisia and Algeria belonged to *B. lupini*, *B. canariense*, *B. valentinum*, *B. cytisi/B. rifense*, *B. japonicum*, *B. elkanii*, and *B. retamae* [43–45].

3.2 The genus *Mesorhizobium* (*Phyllobacteriaceae*)

The genus *Mesorhizobium* was described by Jarvis et al. [46]. Several *Rhizobium* species were transferred to this genus. It currently consists of 21 rhizobia species.

For subtribe *Astragalinae* (*Coluteinae* Clade), Guerrouj et al. [37] reported that rhizobial symbiont of *Astragalus gombiformis* in Eastern Morocco is closely related to *M. camelthorni*. A polyphasic approach analysis indicated that bacterial strains isolated from the pasture legume *Biserrula pelecinus* growing in Morocco belong to the genus *Mesorhizobium*. At Tunisia, Mahdhi et al. [47] showed that five strains isolated from *Astragalus corrugatus* were phylogenetically related to *M. temperatum* and to *Mesorhizobium* sp. From the tribe *Galegeae* (subtribe *Coluteinae*), Ourarhi et al. [48] reported that *Colutea arborescens* is nodulated by diverse rhizobia in Eastern Morocco, among them, the genus *Mesorhizobium*. For the *Loteae* tribe, *M. alhagi* as well as *M. temperatum* were isolated, at Tunisia, from *Lotus creticus* [49–51]. Zakhia et al. [31] reported that *Lotus argenteus* microsymbionts are closely related to *M. mediterraneum* in the infra-arid zone of Tunisia. Roba et al. [52] reported that *M. delmotii* and *M. prunedense* are two new rhizobial species nodulating *Anthyllis vulneraria* growing on Tunisian soils. From the *Acacieae* tribe, Boukhatem et al. [33] reported that rhizobial strains associated to the *Acacia saligna*, an Australian introduced species, to *A. ehrenbergiana* and *F. albida* belonged to *M. mediterraneum* under Algerian soils. From the *Genisteeae* tribe, the genetic diversity of *Genista saharae* microsymbionts in the Algerian Sahara reported that they belonged to *M. camelthorni* [53]. For the *Mimoseae* tribe, root-nodulating bacteria associated to *Prosopis farcta* growing in the arid regions of Tunisia were assigned to the genus *Mesorhizobium* [54]. From the *Hedysareae* tribe, Zakhia et al. [31] reported that one strain isolated from *Ebenus pinnata* root nodules is closely related to *M. ciceri* in the infra-arid zone of Tunisia.

3.3 The genus *Rhizobium* (*Rhizobiaceae*)

The genus *Rhizobium* was the first named (from Latin meaning “root living”), and for many years this was a “catch all” genus for all rhizobia. Some species were later moved in to new genera based on phylogenetic analyses [55]. It currently consists of 49 rhizobial species.

For *Galegae* tribe, Zakhia et al. [31] reported that rhizobial symbionts of *Astragalus gombiformis*, *A. armatus*, and *A. cruciatus* are closely related to *Rhizobium mongolense*, *R. leguminosarum*, and *R. galegae*, in the infra-arid zone of Tunisia. From

Genisteeae tribe, it was shown that strains from Tunisia nodulating *Argyrolobium uniflorum* are closely affiliated to *R. giardinii*, *Calicotome villosa* to *R. mongolense*, and *Genista microcephala* to *R. mongolense* and *R. leguminosarum* [31]. Mahdi et al. [56–58] reported that strains nodulating *Genista saharae* and *Retama retam* are members of the genus *Rhizobium*. Nonetheless, there are reports indicating that members of the genus *Rhizobium* nodulate *Adenocarpus decorticans* and *Cytisus arboreus* at Morocco [59]. For the *Loteae* tribe, *R. leguminosarum* and *R. mongolense* were isolated, at Tunisia, from *Anthyllis henoniana*, *R. leguminosarum* from *Coronilla scorpioides*, and *R. mongolense* from *Lotus creticus* [31]. Rejili et al. [51] reported that *Lotus creticus* microsymbiotes are closely related to *R. huautlense* in the arid areas of Tunisia. Bacterial strains isolated from root nodules of *Scorpiurus muricatus* sampled from different regions of western Algeria are affiliated to *R. vignae*, *R. radiobacter*, and *R. leguminosarum* [60]. For the *Trifolieae* tribe, *R. galegae* species was isolated, in Tunisia and Algeria, from *Medicago marima* and *M. truncatula* [31]. In Algeria, Merabet et al. [61] reported that *Medicago ciliaris* and *M. polymorpha* are nodulated by *Rhizobium* sp. Similarly, genetic diversity of rhizobia from annual *Medicago orbicularis* showed that they are affiliated to *Rhizobium tropici* [62]. For the *Vicieae* tribe, *R. leguminosarum* species was isolated from *Lathyrus numidicus* [31]. Mahdhi et al. [63] reported that *Vicia sativa* isolates from Tunisia had 16S rDNA type identical to that of the reference *R. leguminosarum*. From *Acacieae*, Boukhatem et al. [33] reported that bacteria-nodulating *Acacia saligna* and *A. seyal* under Algerian soils are affiliated to the *R. tropici* clade and *R. sullae* clade, respectively. On the other hand, the same study mentioned that five bacterial isolates, all from *A. saligna*, formed a separate clade in the vicinity of the *R. galegae*-*R. huautlense*-*R. loessense* branch [33]. The same authors showed that the *R. leguminosarum* reference strain was represented by five *A. karroo* isolates and five *A. seyal* isolates [33]. At Tunisia, the genetic diversity of root nodule bacteria associated to *Hedysarum coronarium* (*sulla*), from *Hedysareae* tribe, showed that they are closely related to *R. sullae* [64]. Similarly, Ezzakkioui et al. [65] indicated that the strains from the Moroccan *Hedysarum flexuosum* legume had 99.75–100% identity with *R. sullae*.

3.4 The genus *Ensifer* (*Sinorhizobium*) (*Rhizobiaceae*)

The genera *Sinorhizobium* and *Ensifer* were recently recognized as forming a single phylogenetic clade [66, 67] and are now united, and all species of the genus *Sinorhizobium* have been transferred to the genus *Ensifer*, in line with rule 38 of the Bacteriological Code [68, 69]. The genus currently consists of 17 species.

Bacteria belonging to *Ensifer* genus are widely distributed in arid regions of Tunisia. From the *Loteae* tribe, *E. meliloti* and *E. numidicus* were isolated, at Tunisia, from *Lotus creticus* [49–51, 69] and *Rhizobium* sp. from *Hippocrepis areolata* [47]. From the *Acacieae* tribe, genetic characterization of rhizobial bacteria-nodulating *Acacia tortilis* subsp. *raddiana*, *A. gummifera*, *A. cyanophylla*, *A. karroo*, *A. ehrenbergiana*, and *A. horrida* in Tunisia, Algeria, and Morocco reported that they belonged to the species *E. meliloti*, *E. garamanticus*, and *E. numidicus* and *Ensifer* sp. [31, 33, 70–72, 73]. At Algeria, isolates from four different host species, namely, *A. karroo*, *A. ehrenbergiana*, *A. saligna*, and *A. tortilis*, were closely related to *E. fredii*, *E. teranga*, and *E. kostiense* reference strains [33]. For the *Mimoseae* tribe, 40 isolates associated to *Prosopis farcta* growing in the arid regions of Tunisia belonged to *E. meliloti*, *E. xinjiangense*/*E. fredii*, and *E. numidicus* species [54]. For the *Trifolieae* tribe, strains nodulating different *Medicago* species in Tunisia, Algeria, and Morocco such as *M. sativa*, *M. arborea*, *M. truncatula*, *M. ciliaris*, *M. laciniata*, *M. polymorpha*, *Medicago arabica*, *M. marima*, *Medicago littoralis*, and *M. scutella* are associated to *E. meliloti*, *E. medicae*, or *E. garamanticus* [31, 61, 62, 69, 74–79]. Similarly, *Ononis natrix*

Subfamily tribe	Genus	Species	Symbiont	Geographic origin	
Mimosoideae					
Acacieae	<i>Acacia</i>	<i>A. cyanophylla</i>	<i>E. meliloti</i> , <i>E. fredii</i> , <i>Ensifer</i> sp.	Tunisia, Morocco	
		<i>A. gummifera</i>	<i>E. meliloti</i> , <i>E.</i> <i>garamanticus</i> , <i>E.</i> <i>numidicus</i> , <i>Ensifer</i> sp.	Tunisia, Morocco	
		<i>A. horrida</i>	<i>E. meliloti</i> , <i>E.</i> <i>garamanticus</i> , <i>E.</i> <i>numidicus</i> , <i>Ensifer</i> sp.	Tunisia, Morocco	
		<i>A. tortilis raddiana</i>	<i>E. meliloti</i> , <i>E.</i> <i>garamanticus</i> , <i>E.</i> <i>numidicus</i> , <i>Ensifer</i> sp.	Tunisia, Morocco	
		<i>A. saligna</i>	<i>Bradyrhizobium</i> sp., <i>Mesorhizobium</i> sp., <i>Rhizobium</i> sp., <i>Ensifer</i> sp.	Algeria, Morocco	
		<i>A. ehrenbergiana</i>	<i>Mesorhizobium</i> sp., <i>Ensifer</i> sp.	Algeria	
		<i>A. karroo</i>	<i>Rhizobium</i> sp., <i>Ensifer</i> sp.	Algeria	
		<i>A. nilotica</i>	<i>Rhizobium</i> sp.	Algeria	
		<i>A. seyal</i>	<i>Rhizobium</i> sp.	Algeria	
		<i>F. albida</i>	<i>Mesorhizobium</i> sp.	Algeria	
Mimosae	<i>Prosopis</i>	<i>P. farcta</i>	<i>Mesorhizobium</i> sp., <i>E.</i> <i>meliloti</i> , <i>E. xinjiangense</i> , <i>E. fredii</i> , <i>E. numidicus</i>	Tunisia	
Papilionoideae					
Galegae	<i>Astragalus</i>	<i>A. armatus</i>	<i>R. mongolense</i> , <i>R.</i> <i>leguminosarum</i> , <i>R. galegae</i>	Tunisia	
		<i>A. cruciatus</i>	<i>R. mongolense</i> , <i>R.</i> <i>leguminosarum</i> , <i>R. galegae</i>	Tunisia	
		<i>A. corrugatus</i>	<i>M. temperatum</i> , <i>Mesorhizobium</i>	Tunisia	
		<i>A. gombiformis</i>	<i>M. camelthorni</i> , <i>R. mongolense</i> , <i>R.</i> <i>leguminosarum</i> , <i>R. galegae</i>	Morocco, Tunisia	
		<i>Biserrula</i>	<i>B. pelecinus</i>	<i>Mesorhizobium</i>	Morocco
	<i>Colutea</i>	<i>C. arborescens</i>	<i>Mesorhizobium</i>	Morocco	
Genisteae	<i>Argyrobium</i>	<i>A. uniflorum</i>	<i>R. giardinii</i>	Tunisia	
		<i>Adenocarpus</i>	<i>A. decorticans</i>	<i>Rhizobium</i>	Morocco
		<i>Calicotome</i>	<i>C. villosa</i>	<i>R. mongolense</i>	Morocco
		<i>Cytisus</i>	<i>C. arboreus</i>	<i>Bradyrhizobium</i> sp.	Morocco
			<i>C. triflorus</i>	<i>Bradyrhizobium</i>	Morocco
			<i>C. villosus</i>	<i>B. cytisi</i> , <i>B. rifense</i> , <i>B.</i> <i>japonicum</i> , <i>B. canariense</i>	Algeria, Morocco
	<i>Lupinus</i>	<i>L. luteus</i>	<i>B. lupini</i> , <i>B. canariense</i> , <i>B. valentinum</i> , <i>B. cytisi</i> , <i>B. rifense</i> , <i>B. japonicum</i> , <i>B. elkanii</i> , <i>B. retamae</i> , <i>Microvirga</i>	Algeria, Tunisia	

Subfamily tribe	Genus	Species	Symbiont	Geographic origin
		<i>L. micranthus</i>	<i>B. lupini</i> , <i>B. canariense</i> , <i>B. valentinum</i> , <i>B.</i> <i>cytisi</i> , <i>B. rifense</i> , <i>B.</i> <i>japonicum</i> , <i>B. elkanii</i> , <i>B. retamae</i> , <i>Microvirga</i> , <i>Phyllobacterium</i>	Algeria, Tunisia
	<i>Genista</i>	<i>G. microcephala</i>	<i>R. mongolense</i> , <i>R.</i> <i>leguminosarum</i> , <i>Rhizobium</i>	Tunisia
		<i>G. saharae</i>	<i>M. camelthorni</i>	Algeria
	<i>Retama</i>	<i>R. monosperma</i>	<i>B. retamae</i>	Algeria, Morocco
		<i>R. raetam</i>	<i>B. retamae</i> , <i>Rhizobium</i>	Algeria, Tunisia, Morocco
		<i>R. sphaerocarpa</i>	<i>B. retamae</i>	Algeria, Morocco
Hedysareae	<i>Hedysarum</i>	<i>H. carnosum</i>	<i>E. meliloti</i>	Tunisia
		<i>H. flexuosum</i>	<i>R. sullae</i>	Morocco
		<i>H. coronarium</i>	<i>R. sullae</i>	Tunisia
		<i>H. spinosissimum</i>	<i>E. meliloti</i>	Tunisia
	<i>Ebenus</i>	<i>E. pinnata</i>	<i>M. ciceri</i>	Tunisia
Loteae	<i>Anthyllis</i>	<i>A. henoniana</i>	<i>R. leguminosarum</i> , <i>R.</i> <i>mongolense</i>	Tunisia
		<i>A. vulneraria</i>	<i>M. delmotii</i> , <i>M.</i> <i>prunaredense</i>	Tunisia
	<i>Coronilla</i>	<i>C. scorpioides</i>	<i>R. leguminosarum</i>	Tunisia
	<i>Hippocrepis</i>	<i>H. areolata</i>	<i>Rhizobium</i>	Tunisia
		<i>H. bicontorta</i>	<i>E. meliloti</i>	Tunisia
	<i>Lotus</i>	<i>L. argenteus</i>	<i>M. mediterraneum</i>	Tunisia
		<i>L. creticus</i>	<i>B. lupini</i> , <i>B.</i> <i>japonicum</i> , <i>M. alhagi</i> , <i>M. temperatum</i> , <i>R. mongolense</i> , <i>R.</i> <i>huautlense</i> , <i>E. meliloti</i> , <i>E.</i> <i>numidicus</i>	Algeria, Tunisia
		<i>L. palustris</i>	<i>B. lupini</i>	Algeria
		<i>L. pedunculatus</i>	<i>B. japonicum</i>	Algeria
		<i>L. purpureus</i>	<i>B. lupini</i>	Algeria
		<i>L. pusillus</i>	<i>M. alhagi</i> , <i>M.</i> <i>temperatum</i> , <i>E. meliloti</i>	Tunisia
		<i>L. roudairei</i>	<i>B. japonicum</i>	Tunisia
	<i>Scorpiurus</i>	<i>S. muricatus</i>	<i>R. vignae</i> , <i>R. radiobacter</i> , <i>R. leguminosarum</i>	Algeria
Trifolieae	<i>Medicago</i>	<i>M. arabica</i>	<i>E. meliloti</i> , <i>E. medicae</i> , and <i>E. garamanticus</i>	Morocco
		<i>M. arborea</i>	<i>E. meliloti</i> , <i>E. medicae</i> , and <i>E. garamanticus</i>	Morocco

Subfamily tribe	Genus	Species	Symbiont	Geographic origin
		<i>M. ciliaris</i>	<i>Rhizobium</i> , <i>E. meliloti</i> , <i>E. medicae</i> , and <i>E. garamanticus</i>	Algeria
		<i>M. marima</i>	<i>R. galegae</i>	Algeria, Tunisia
		<i>M. laciniata</i>	<i>E. meliloti</i> , <i>E. medicae</i> , and <i>E. garamanticus</i>	Tunisia
		<i>M. littoralis</i>	<i>E. meliloti</i> , <i>E. medicae</i> , and <i>E. garamanticus</i>	Tunisia
		<i>M. orbicularis</i>	<i>R. tropici</i>	Tunisia, Algeria, Morocco
		<i>M. polymorpha</i>	<i>Rhizobium</i> , <i>E. meliloti</i>	Tunisia, Algeria, Morocco
		<i>M. sativa</i>	<i>E. meliloti</i> , <i>E. medicae</i>	Tunisia, Algeria, Morocco
		<i>M. scutella</i>	<i>E. meliloti</i>	Algeria, Tunisia
		<i>M. truncatula</i>	<i>R. galegae</i> , <i>E. meliloti</i> , <i>E. medicae</i>	Tunisia, Algeria, Morocco
	<i>Melilotus</i>	<i>M. indicus</i>	<i>E. meliloti</i>	Algeria
	<i>Ononis</i>	<i>O. natrix</i> ssp. <i>filifolia</i>	<i>E. meliloti</i>	Tunisia
	<i>Trigonella</i>	<i>T. maritima</i>	<i>E. meliloti</i>	Tunisia
Vicieae	<i>Lathyrus</i>	<i>L. numidicus</i>	<i>R. leguminosarum</i>	Tunisia
	<i>Vicia</i>	<i>V. sativa</i>	<i>R. leguminosarum</i>	Tunisia

Table 1.

Recapitulative results of root nodule symbionts from Northwestern African wild legumes.

and *Trigonella maritima* are nodulated by *E. meliloti* [31, 63]. Nodule rhizobia of *Melilotus indicus* growing in the Algerian Sahara are affiliated to *E. meliloti* [78]. *E. meliloti* and *E. numidicus* strains were isolated from the Genisteae tribe such as *Argyrolobium uniflorum*, *Retama raetam*, and *Genista saharae* [56–58, 69]. For *Galegae* tribe, Mahdhi et al. [47] reported that rhizobial symbionts of *Astragalus corrugatus* are closely related to *E. meliloti* under Tunisian soils. From the *Hedysareae* tribe, Mahdhi et al. [35] reported that strains isolated from *Hedysarum spinosissimum* root nodules are closely related to *E. meliloti* in the infra-arid zone of Tunisia.

3.5 The genus *Neorhizobium* (*Rhizobiaceae*)

The genus *Neorhizobium* was proposed by Mousavi et al. [80] as an alternative to solve the issue of grouping the members of this genus with *Agrobacterium* and *Rhizobium* genera. The genetic diversity of the Algerian legume *Genista saharae* isolates was assessed, and results reported that they are affiliated to *Neorhizobium alkalisoli*, *N. galegae*, and *N. huautlense* [53]. Several studies reported that *N. galegae* is isolated from different legumes in Tunisia such as *Astragalus* sp. [31, 54], *Argyrolobium*

uniflorum [31], *Anthyllis henoniana* [31], *Lotus creticus* [31, 50], *Medicago marima*, and *M. truncatula* [31]. Rejili et al. [51] reported that *Lotus creticus* is also nodulated by *N. huautilense* in the arid areas of Tunisia. For *Galegae* tribe, Mahdhi et al. [47] reported that rhizobial symbionts of *Astragalus corrugatus* are closely related to *N. galegae* under Tunisian soils.

3.6 The genus *Phyllobacterium* (*Phyllobacteriaceae*)

The *Phyllobacterium* genus comprises of bacteria that are well-known for their epiphytic and endophytic associations with plants [81]. Nonetheless, root-nodulating and nitrogen-fixing *Phyllobacterium* was described in Tunisia, in the nodules of genistoid legume *Lupinus micranthus* [44, 45]. Prior to this finding, endophytic *Phyllobacterium* strains were identified on the nodules of the Tunisian legumes *Genista saharae*, *Lotus creticus*, and *L. pusillus* [51, 57], but they are lacking the ability to form nodules.

3.7 The genus *Microvirga* (*Methylobacteriaceae*)

The genus *Microvirga* which comprises soil and water saprophytes was included in the alphaproteobacterial lineage of root-nodule bacteria only in 2012, although the first symbiotic strains were detected in nodules of *Lupinus texensis* [82–84]. Recently, *Microvirga* strains were only isolated from *L. micranthus* and *L. luteus* in Tunisia, belonging to the *Genisteeae* tribe [44, 45].

Table 1 shows the root nodule symbionts from Northwestern African wild legumes.

4. Promising nitrogen-fixing rhizobia

The root nodule symbiosis established between legumes and rhizobia is an exquisite biological interaction responsible for fixing a significant amount of nitrogen in terrestrial ecosystems. The success of this interaction depends on the recognition of the right partner by the plant within the richest microbial ecosystems on Earth, the soil. Recent metagenomic studies of the soil biome have revealed its complexity, which includes microorganisms that affect plant fitness and growth in a beneficial, harmful, or neutral manner. In this complex scenario, understanding the molecular mechanisms by which legumes recognize and discriminate rhizobia from pathogens, but also between distinct rhizobia species and strains that differ in their symbiotic performance, is a considerable challenge.

By symbiotic efficiency and properties, strains isolated from wild legumes varied in their symbiosis effectiveness with their host plant of origin. A great diversity among and within isolates was reported by many authors. This symbiotic diversity within and between isolates growing in diverse geographical areas was also defined by Tinick and Hadobas [85] for other legume plants. All strains were capable of nodulation. Mahdhi et al. [55, 86] reported that two *Retama raetam* isolates RB3 and RM4 (*Rhizobium*) gave the highest nodule numbers per plant, 26 (± 2.053) and 27 (± 0.997), respectively. The effective strain LAC765 (*Ensifer*) was isolated from *Lotus creticus* with a 91.46 ($\pm 0.01\%$) dry biomass of the T_N control [50]. The dry matter of the aerial part is considered a criterion for assessing the efficiency of a given strain; a highly significant correlation between these two parameters has been reported. Results related to symbiotic efficiency showed that among 45 tested isolates, 20 isolates are highly efficient (relative effectiveness $\geq 70\%$), 20 isolates are partially effective ($60\% \leq$ relative effectiveness $< 70\%$), and 5 isolates are inefficient (relative

effectiveness <60%). The strain GN29 isolated from *Genista saharae*, affiliated to *Rhizobium* genus, is considered inefficient (relative effectiveness = 32.29%). Among the 20 isolates considered highly efficient, 5 isolates were isolated from *Retama retam*, five from *Lotus* sp., 4 from *Genista saharae*, 3 from *Vicia sativa*, 2 from *Argyrolobium uniflorum* and 2 from *Trigonella maritima*. From the 20 highly efficient isolates, 13 isolates belong taxonomically to *Ensifer* sp., 6 to *Rhizobium* sp., and one to *Mesorhizobium* sp.

5. Conclusion

The Mediterranean basin is a hotspot place of legume diversity and the center of diversification of many of them. Our review contributes to enlarge our knowledge on the LNB-legume symbioses. We evidenced the biodiversity among bacteria-nodulating wild legumes in Northwestern Africa and unknown associations were found. Several groups may represent new genospecies to be further characterized to assess their taxonomical status. This work thus opens further interesting perspectives and makes new models available for evolutionary studies and for understanding mechanisms involved in nitrogen-fixing symbiosis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Biological nitrogen fixation (BNF), the process by which gaseous N_2 is converted into ammonia (NH_3) via the enzyme nitrogenase, is crucial for the availability of nitrogen (N) in the terrestrial ecosystem. Some bacteria have the remarkable capacity to fix atmospheric nitrogen to ammonia under ambient conditions, a reaction only mimicked on an industrial scale by a chemical process. This microbiological process converts atmospheric nitrogen into a plant-usable form, thus decreasing the need to use chemical fertilizers in crop production. Chapters in this volume cover different aspects of this fantastic phenomenon, including biofertilizer, organic nitrogen in agricultural systems, nitrogen fertilization for sustainable crop production, and others. This book is designed for researchers, students and general readers.

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