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Nitrogen in Agriculture

Physiological, Agricultural and Ecological Aspects

Edited by Takuji Ohyama and Kazuyuki Inubushi

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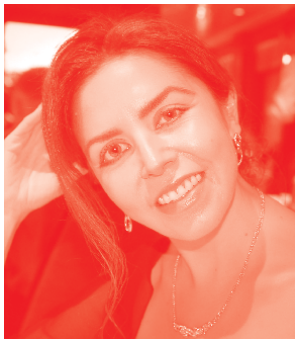

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Meet the editor

Takuji Ohyama was born in Japan in 1951. He obtained a Ph.D. with a thesis entitled "Studies on the fate of nitrogen fixed in soybean nodules" in 1980 from the University of Tokyo. Dr. Ohyama is currently a full professor in the Faculty of Applied Biosciences, Department of Agricultural Chemistry, Tokyo University of Agriculture. He was a professor in the Faculty of Agriculture, Niigata University, from 1982 to 2017. His research interests include symbiotic nitrogen fixation and metabolism in soybean plants, new technology of deep placement of slow-release nitrogen fertilizers for soybean cultivation, nitrogen and carbon metabolism in tulip, curcuma, and cucumber, and the use of stable isotopes and positron-emitting radioisotopes. Dr. Ohyama has published more than 150 papers and 20 book chapters. He has also edited five books.

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Contents

Preface

Nitrogen is an essential major element in all organisms and a component of proteins, amino acids, nucleic acids, and many indispensable metabolites. Nitrogen is the most important nutrient element for agriculture because the availability of nitrogen from the soil is generally not enough to support the yield and quality of crops.

In addition, the nitrogen stored in soil decreases with repeated cultivation because the harvested parts of the crop are taken out from the fields. To maintain soil fertility, crop rotation and application of organic matters have been practiced. After chemical nitrogen fertilizers have been produced by the industrial nitrogen fixation, farmers can use these fertilizers for agriculture. As a result, the productivity of crops increased several folds during the 20th century to feed the increasing world population. However, excess or inappropriate use of nitrogen fertilizers causes environmental problems such as nitrate leaching and contamination in groundwater and rivers and nitrous oxide (N2O) emissions.

This book discusses nitrogen in agriculture, including its usage in crops, the dynamics of soil nitrogen in agricultural fields, and the ecology of and use of nitrogen fertilizers in agriculture to improve nitrogen-use efficiency and reduce ecological problems for sustainable agriculture.

There are four sections in this book: Section 1: "Ecology and Environmental Aspects of Nitrogen in Agriculture," Section 2: "Nitrogen Fertilizers and Nitrogen Management in Agriculture," Section 3: "N Utilization and Metabolism in Crops," and Section 4: "Plant-Microbe Interactions."

The first section includes Chapter 1, "Mitigation of Climate Change by Nitrogen Managements in Agriculture", by Kazuyuki Inubushi and Miwa Yashima; and Chapter 2, "Influence of Heavy Metals on the Nitrogen Metabolism in Plants", by Vitor Nascimento, Glauco Nogueira, Gabriel Monteiro, Waldemar Júnior, Joze Melissa Nunes de Freitas, and Cândido Neto.

The second section includes Chapter 3, "Nitrogen Management in Conservation Agriculture", by Anthony Imoudu Oyeogbe; Chapter 4, "Agronomic Response of Camelina to Nitrogen and Seeding Rate on the Northern Great Plains", by Thandiwe Nleya, Dwarika Bhattarai, and Phillip Alberti; and Chapter 5, "Cover Crop Residue Management for Effective Use of Mineralized Nitrogen in Greenhouse Tomato Production", by Rafael A. Muchanga and Hajime Araki.

The third section includes Chapter 6, "Nitrogen Fixation in Soybean Nodules Affects Seed Protein and Oil Contents: The Suggested Mechanism from the Coordinated Changes of Seed Chemical Compositions and Phosphoenolpyruvate Carboxylase Activity Caused by Different Types of Nitrogen Fertilizer", by Toshio Sugimoto, Naoki Yamamoto, and Takehiro Masumura; Chapter 7, "Modeling of Nitrogen Use Efficiency in Lettuce Culture (*Lactuca sativa*): Isotopic Nitrogen (15 N) and AquaCrop" by Mawhoub Amirouche, Dalila Smadhi, and Lakhdar Zella; Chapter 8, "Nitrogen in Flowers", by Soraya Ruamrungsri, Kanokwan Panjama, Takuji

Ohyama, and Chaiartid Inkham; and Chapter 9, "Nitrogen Storage in Crops: Case Study of Zeins in Maize", by Marija Duvnjak, Kristina Kljak, and Darko Grbeša.

The final section includes Chapter 10, "Conservation of Edible Ectomycorrhizal Mushrooms: Understanding of the ECM Fungi Mediated Carbon and Nitrogen Movement within Forest Ecosystems", by Lu-Min Vaario and Norihisa Matsushita; Chapter 11, "Promotion of Nitrogen Assimilation by Plant Growth-Promoting Rhizobacteria", by Gabriel Monteiro, Glauco Nogueira, Cândido Neto, Vitor Nascimento, and Joze Freitas; and Chapter 12 "Mycorrhizal Fungi and Sustainable Agriculture", by Soibam Helena Devi, Ingudam Bhupenchandra, Soibam Sinyorita, S.K. Chongtham, and E. Lamalakshmi Devi.

We are grateful to all authors who contributed excellent chapters to this book. In addition, we thank external reviewers for their helpful feedback. Finally, we greatly appreciate Author Service Manger Mr. Josip Knapić at IntechOpen for all his kind support.

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Section 1

Ecology and Environmental Aspects of Nitrogen in Agriculture

Chapter 1

Mitigation of Climate Change by Nitrogen Managements in Agriculture

Kazuyuki Inubushi and Miwa Yashima

Abstract

Soil is one of the important sources of nitrous oxide (N_2O), which is generally producing through soil microbial processes, such as nitrification and denitrification. Agricultural soils receive chemical and organic fertilizers to maintain or increase crop yield and soil fertility, but several factors are influencing N_2O emissions, such as types and conditions of soil and fertilizer, and rate, form, and timing of application. Mitigation of N_2O is a challenging topic for future earth by using inhibitors, controlled-release fertilizers, and other amendments, but the cost and side effects should be considered for feasibility.

Keywords: N_2O, nitrification, denitrification, mitigation, soil type

1. Introduction

Global warming is significant and the impact of human activities is no doubt, such as mining of fossil fuels and deforestation, over-grazing, and constant increase of nitrogen fertilizer, resulting in atmospheric concentrations of CO_2, methane (CH_4) and nitrous oxide (N_2O) keep increasing, respectively, as indicated by Intergovernmental Panel on Climate Change (**IPCC**), under the United National Framework Convention on Climate Change (**UNFCCC**) (**Figure 1**, [1]). CH_4 and N_2O are the main Short-Lived Climate Forcers (**SLCPs**) because these participate in air pollution chemistry (ozone production, the oxidizing capacity of the atmosphere) and have very high Global Warming Potential (**GWP**) to compare with CO_2 = 1 as CH_4 GWP = ~28; N_2O GWP = ~298 (100 yr integration on per mole basis).

The Japanese government declared in 2020 that the year 2050 is the target of "Carbon Neutral Society", like other OECD countries. To achieve this target, we should reduce greenhouse gas emissions, not only CO_2 but also CH_4 and N_2O, both strongly related to food production and agriculture sectors.

Soil is one of the important sources of N_2O, which is generally producing through soil microbial processes, such as nitrification and denitrification. Agricultural soils receive chemical and organic fertilizers to maintain or increase crop yield and soil fertility. However, excess amount of chemical N fertilizer application may cause eutrophication and ground water pollution in the hydrosphere. Moreover, many factors are also influencing N_2O emission in the atmosphere, such as types and conditions of soil and fertilizer, and rate, form, and timing of application. Mitigation of N_2O emission to the atmosphere is a challenging topic in

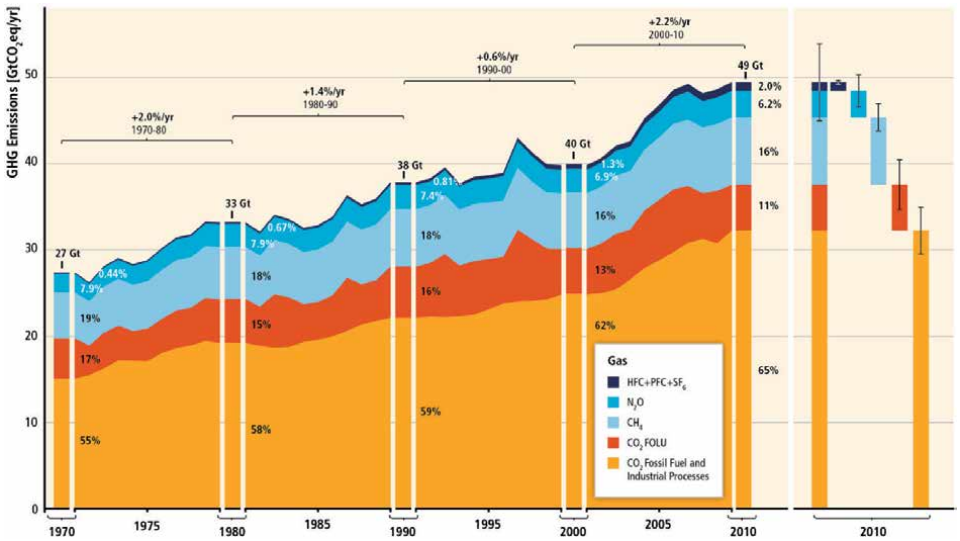

Figure 1.
Total annual anthropogenic GHG emissions by gases 1970–2010 (source: [1]).

sustainable agriculture, such as by using inhibitors, controlled-release fertilizers and other amendments, though the cost and side effects should be considered for feasibility. In this review, processes and influencing factors of N_2O production in the soil is reviewing and some trials for mitigation are introduced.

2. Global N_2O budget and production in soil

Global Carbon Project (GCP) published a comprehensive quantification of global nitrous oxide sources and sinks [2]. This reports details of the global N_2O budget in 21 natural and human sectors between 1980 and 2016 (**Figure 2**), indicating that

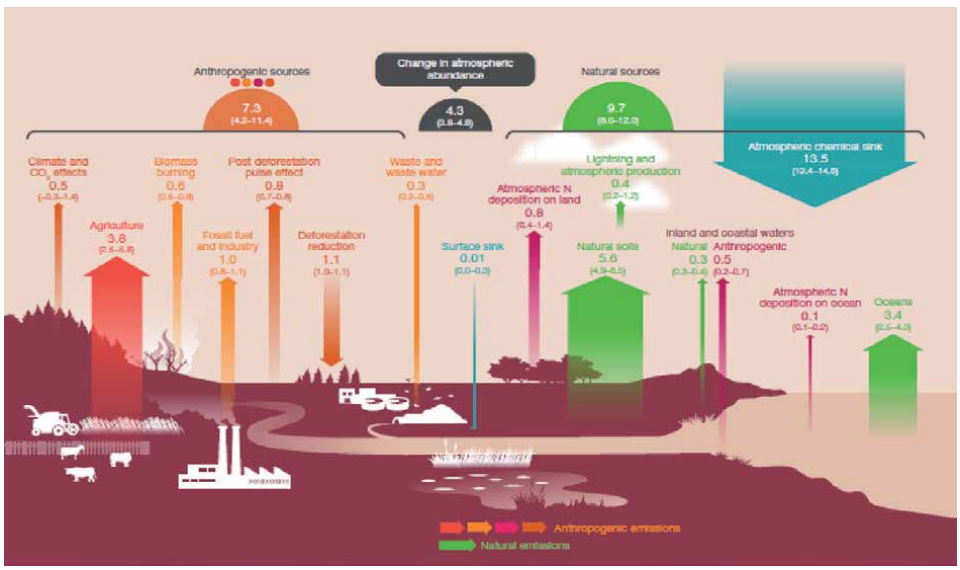

Figure 2.
Global N_2O budget [2].

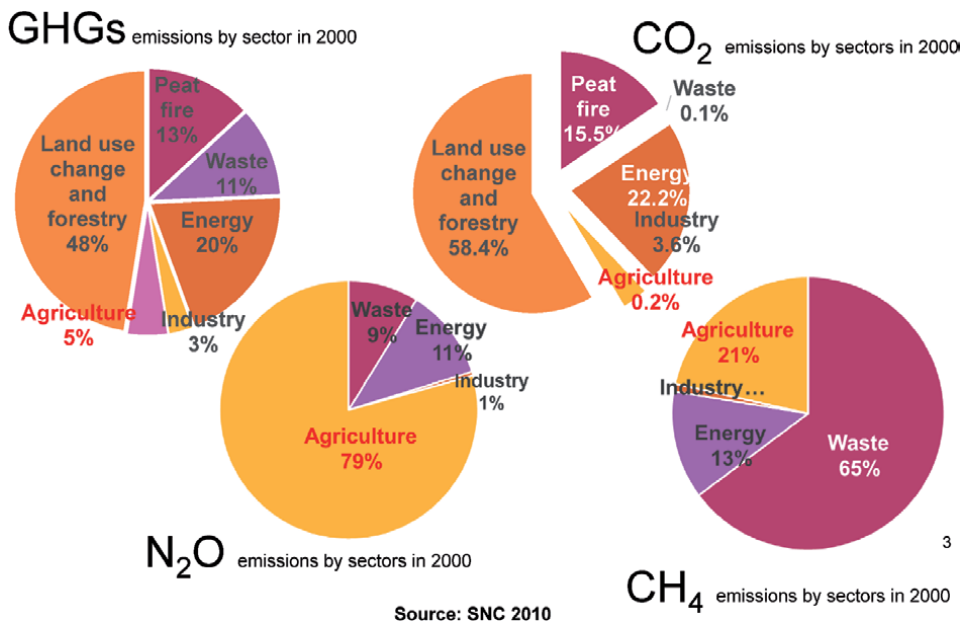

Figure 3.
National contribution of greenhouse gas emission by sector Indonesia source, [3].

natural and anthropogenic sources of N_2O were 57% and 43%, or 9.3 and 7.3 TgN yr^{-1} (1 Tg = 10^{12} g or 1 million ton), respectively and total as 17.0 (minimum 12.2 to maximum 23.5 Tg), while 13.5 Tg sink by atmospheric chemical reactions, resulting 3.5 Tg increase annually. By continental or regional estimates, Africa releases most (3 Tg yr^{-1}) due to large areas with tropical forests where high temperature and soil moisture, followed by Latin America and East Asia, where the agricultural contribution is largest. The annual increase of N_2O emission is more than 1%, and the agricultural sector is largest, especially in Asia, followed by Latin America, Africa, and particularly in East Asia, the input of chemical fertilizer and manure plus direct emission is increasing as more than double in past three decades. National inventory of greenhouse gas emission in developing countries such as Indonesia (**Figure 3**) [3] contributions of agricultural sectors in N_2O and CH_4 are bigger than other sectors.

3. N_2O production and its affecting factors

N_2O is generally producing in the soil through microbial processes, mainly via nitrification and denitrification (**Figure 4**). Nitrification is carried out under aerobic conditions by two groups of autotrophic nitrifiers, namely ammonium oxidizers and nitrite oxidizers, both do not require organic matters, not only in bacteria group but also archaea group. Autotrophic nitrification is the dominant process in aerobic soil (less than 60% water-holding capacity), while heterotrophic nitrification is negligible [4]. N_2O is producing as a byproduct during nitrite oxidation during nitrification. On the other hand, denitrification is carried out under wet and anaerobic conditions, such as in paddy soil and wetland soil, by heterotrophic denitrifiers, not only the bacteria but also fungi, both requires not only nitrate but also N-rich organic matter. N_2O is producing as an intermediate product during denitrification between nitrite and N_2. However, N_2O emission from flooded paddy soil is generally low, probably due to the high solubility of N_2O and complete denitrification to N_2. Chemical denitrification was also negligible [4]. Microbial

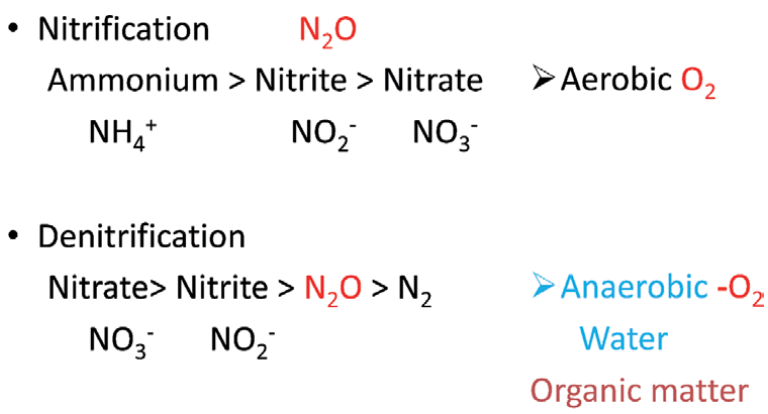

Figure 4.
Main processes of N_2O production in soil.

community structure was investigated also in tropical acid tea soil [5] and peat soil [6, 7]. Anaerobic ammonium oxidation (ANAMMOX) and dissimilatory nitrate reduction to ammonium (DNRA) are also focusing recently to see the possibility to relate N_2O production and contribution in soil N dynamics [8, 9].

Based on above knowledges about N_2O production processes, several mitigation technologies are proposed. To apply such mitigation technologies, it is important to understand factors affecting N_2O production, which are (1) Soil type and amendments such as manure, compost, and biochar, (2) Soil management and mitigation technologies such as controlled-release chemical fertilizers and nitrification inhibitors, and (3) Trade-off effects with other greenhouse gas mitigation such as water management in paddy field to reduce CH_4.

4. Effect of soil types and amendment on N_2O production and plant growth

Generally, soil with a large amount of soil organic matter (SOM) tended to produce more N_2O. However, in a case study using various soils in Japan and Hungary [10], Andosol, typical upland soil in Japan with higher SOM contents, produced less N_2O than Chernozem with lower SOM contents, typical upland soil in Europe, under the same incubation conditions, especially amended with chemical N fertilizer and biochar (**Figure 5**). However sandy soils with fewer SOM contents, N_2O production was small. Biochar is focused on soil C sequestration to build up C stock in soil, but also in Andosol, N_2O tended not to be increased with biochar and N fertilizer. Leafy vegetable (Komatsuna; *Brassica rapa*) growth and yield were also enhanced by amendments of chemical N fertilizer and biochar.

Effects of amendments on N_2O production were studied by many researchers, showing compost and N fertilizer generally increase N_2O production [11–16]. Under field conditions, N_2O emission to the atmosphere was much diversified in space and also soil depth [16, 17], and land-use [18]. Further study is needed for feasible soil and fertilizer management to meet sustainable developments.

5. Soil and fertilizer managements and mitigation technologies

To reduce N_2O emission, controlled-release chemical fertilizers and nitrification inhibitors have been examined [19, 20], and meta-analysis of 113 field experiment

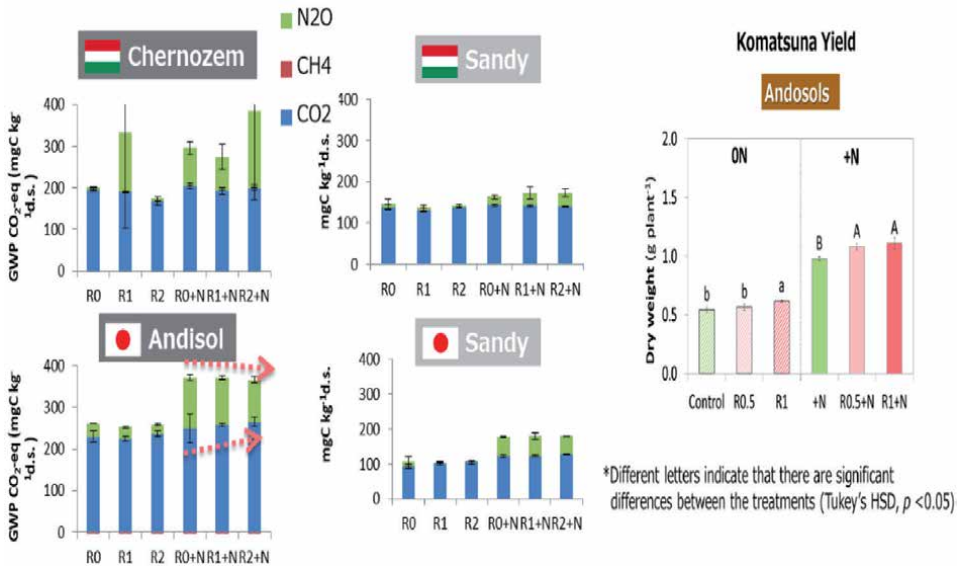

Figure 5.
Effect of soil types and amendment on N_2O production and plant growth (R: Rice husk biochar, 0, 1, 2: Application rate as 0, 1, 2%w/w, respectively, N: Urea).

datasets showed that polymer-coated fertilizers significantly reduced N_2O emissions (mean: −35%, 95% confidential interval: −58% to −14%) and nitrification inhibitors (−38%, −44% to −31%), respectively, depending on soil type and regions [21].

Controlled-release coated urea (CRCU) is a type of polymer-coated fertilizer. CRCU was examined to compare with conventional chemical fertilizer in tropical oil palm plantations over 340–580 days, where vast areas have been converted from rainforest and other plantations. Sakata et al. [22] reported the effect of CRCU compare with conventional fertilizer on N_2O emission and yield (**Figures 6** and 7). In Tungal sandy loam soil, controlled-release nitrogen fertilizer (CRNF; M) showed lower N_2O emission than conventional fertilizer (C), while in Simunjan sandy soil, N_2O was low in both M and C. On the other hand in Tatau peat soil, both M and C emitted a similar amount of N_2O, and much larger than other sites, and even from control (B; without fertilizer) (**Figure** 7). No significant effect on oil palm yield

Figure 6.
Field experimental sites in Sumatra, Indonesia and Sarawak, Malaysia [22], ① Tunggal, ② Simunjan, ③ Tatau.

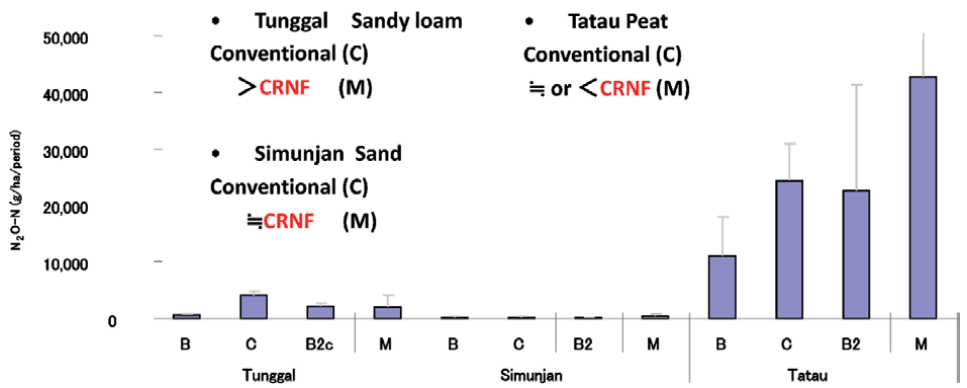

Figure 7.
N_2O emission from field experiments with different soil and fertilizer [22].

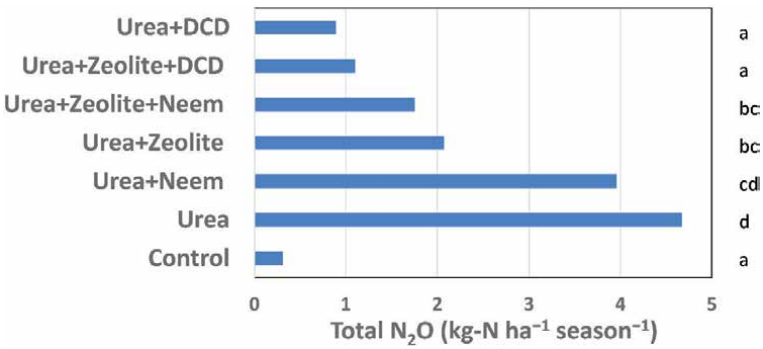

Figure 8.
Nitrification inhibitors on N_2O emission [29].

was observed even N application rate of M was half of C. Tropical peat soil has been pointed out as a significant N_2O emission source, even without fertilizer, strongly influenced by groundwater level [6, 7, 23–27]. Therefore CRCU has a significant impact even under tropical conditions to reduce N_2O in certain mineral soils, but not in organic peat soil. Long-term evaluation and cost–benefit analysis are important with yield evaluation in various soil types under diverse climate conditions.

Another mitigation option, nitrification inhibitors to stop ammonium oxidation have been also examined, typically DCD (Dicyandiamide; [20]) which is biologically and temperature-dependently decomposed [28]. However, it caused contamination in exposed milk powder in New Zealand, so NZ banned DCD from 2013. Nitrapyrin and 3,4-dimethyl pyrazole phosphate (DMPP) are other chemicals of nitrification inhibitors, but less effective. Neem cake is derived from natural compounds, so less expensive, but also less effective to compare with chemical inhibitors [21]. Combined effects of a nitrification inhibitor, including DCD, neem, and clay mineral (zeolite) on N_2O fluxes and corn growth were examined ([29]; **Figure 8**). Another biological nitrification inhibitor is also examined [30].

6. Trade-off with other mitigation

Mitigation options of other greenhouse gases, such as water management in the paddy field to reduce CH_4, have been examined in Japan [31]. The controlling irrigation water level was also examined in Indonesia [32]. Groundwater level

Varietyc	Treatment	CH_4 (kg ha^{-1})	N_2O (kg ha^{-1})	GWP (kg CO_2-eq/ha)	Reduction %
ADT 43	SRI	59.97 a	1.94 a	2077.4	**28.8%**
	MSRI	45.11 a	2.69 a	1929.4	**33.9%**
	CT	99.44 b	1.45 a	2918.1	
CO 51	SRI	51.73 a	2.09 a	1916.1	**27.1%**
	MSRI	50.76 a	1.53 a	1724.9	**34.3%**
	CT	88.86 b	1.36 a	2626.8	

SRI as a type of AWD with reduce seedling numbers, and MSRI as modified SRI with seedling age to compare with control CT [38].

Table 1.
Effects of water management and crop establishments on N_2O emission in field experimental site conducted at Tamil Nadu Rice Research Institute

control by alternate wet and drying (AWD) have established by IRRI and examined in Indonesia, the Philippines, Thailand, Vietnam [33–37] and India [38]. AWD has merit for saving labor and water. However, it may have a trade-off effect to increase N_2O, because of the removal of flooded water to expose anaerobic soil directly to the atmosphere. To examine this trade-off, they measured not only CH_4 but also N_2O emissions in the same field experiments and found that N_2O emission was mostly negligible without losing rice yield although CH_4 was significantly reduced (**Table 1**). Such trade-off should be examined not only for water management but also other soil managements including biochar for soil C sequestration.

7. Conclusions

Nitrogen is one of the most critical elements for food production, local and global environments. Nitrous oxide (N_2O) is an important greenhouse gas emitted from the soil via biological processes in N cycling. N_2O emission keeps increasing to induce global warming, climate change, and stratospheric ozone layer depletion. N_2O production in the soil is related to soil and fertilizer management and is influenced by many factors, such as soil conditions, chemical fertilizer, and organic manures. Mitigation is possible by appropriate soil and fertilizer management (controlled-release fertilizer and nitrification inhibitors), but exceptional soil such as peat soil should be careful. Feasibility is important to harmonize with yield and other factors (cost and economic merits, side effect, etc.). Under the COVID-19 pandemic, the balance of food production, human health, and environmental management become more and more crucial issues. Sustainable development goals become more important view-points than before [39, 40].

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Chapter 2

Influence of Heavy Metals on the Nitrogen Metabolism in Plants

Vitor Nascimento, Glauco Nogueira, Gabriel Monteiro, Waldemar Júnior, Joze Melissa Nunes de Freitas and Cândido Neto

Abstract

As an essential element, Nitrogen is needed in large quantities for being an important component of cellular constituents and for plant metabolism, and its deficiency is one of the most common limitations for plant development. The study of the toxic effects of metal in plants involves a complex system of reactions that can be better determined once having a large attention of the different backgrounds of occurence to determinate how to proceed. The objective of this review is to add scientific knowledge, addressing the main functionalities and characteristics of this relation heavy metals – nitrogen metabolism in plant. Increasing industrialization and urbanization had anthropogenic contribution of heavy metals in biosphere and had largest availability in ecosystems. This toxicity in plants varies with plant species, specific metal, concentration, soil composition, as many heavy metals are considered to be essential for plant growth. Were provided data and reviews regarding the effect of heavy metals on nitrogen metabolism of plants and the responses of plants and the cross-talk of heavy metals and various stressors factors. Is clear to understand the relation between metals amount and the benefit or harm caused on plants, determining then, which mechanism should be activated to protect your physiological system.

Keywords: Plant physiology, human activities, industrialization, plant stress, toxicity

1. Introduction

Nitrogen (N) is an essential nutrient required by all living organisms and often limits primary production in aquatic and terrestrial ecosystems. This element is needed in large quantities, as it is an essential component of proteins, nucleic acids and other cellular constituents. Proteins alone comprise 60% or more of the N of plants and microbial cells [1]. It is also an essential element for plant metabolism and its deficiency is one of the most common limitations for plant development [2]. The preference of plants for the source of N can vary according to the selective pressures and consequent physiological adaptations [3].

The most common and available forms in the soil are the nitrate (NO_3^-) and ammonium (NH_4^+) forms, the first being more abundant and better assimilated by plants [4], as a result of the nitrification process by bacteria. However, depending on soil conditions, the ammoniacal form may be the most abundant due to the

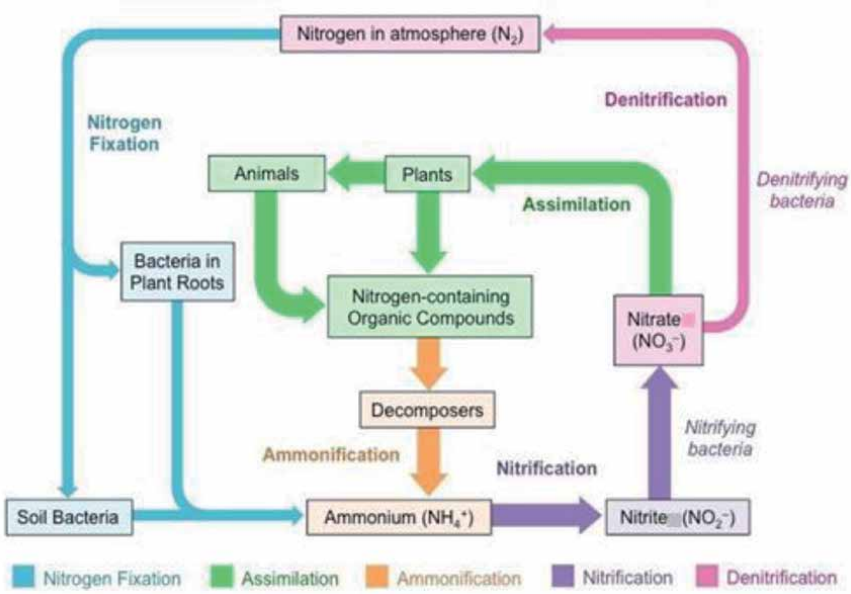

Figure 1.
Nitrogen metabolism in plants. Source: Biology Experts Notes.

inhibition of these organisms [5]. Once in the leaves, it is known that different sources of N can affect plant metabolism differently. The accumulation of NH_4^+ can lead to decreased photosynthesis [6], while the excess of NO_3^- can lead to the formation of reactive species inducing oxidative stress [7].

The metabolism of nitrogen is also closely linked to that of carbon (C), and the photorespiratory process is one of the points of connection between these, in addition to being a metabolic route that naturally produces a reactive oxygen species (**Figure 1**).

The study of the toxic effects of metal in plants involves a complex system of reactions that can be better determined once having a large attention of the different backgrounds and regions of occurence to determinate how to proceed. Therefore, the objective of this review is to add scientific knowledge, addressing the main functionalities and characteristics of this relation heavy metals – nitrogen metabolism in plant.

2. Heavy metals

According to Lenntech [8], heavy metals were significant environmental pollutants because their toxicity is a problem of increasing significance for ecological, evolutionary, environmental and nutritional reasons. Including copper (Cu), manganese (Mn), lead (Pb), cadmium (Cd), nickel (Ni), cobalt (Co), iron (Fe) and others, they are group of metals and metalloids with atomic density greater than 4 g/cm^3, or 5 times or more, greater than water [9]. Environmentally it is defined as total circumstances surrounding an organism or group of organisms especially, the combination of external physical conditions that influence and affect the growth, survival and development of the organisms [10].

Heavy metals are most found in rock formations with a dispersed form. Increasing industrialization and urbanization had anthropogenic contribution of heavy metals in biosphere and had largest availability in soil and aquatic ecosystems

and to a relatively smaller portion in atmosphere as vapors or particulates. This toxicity in plants varies with plant species, specific metal, concentration, chemical form, pH and soil composition, as many heavy metals are considered to be essential for plant growth. According to Mildvan [11], some of these heavy metals like Cu and Zn could serve as activators of enzyme reactions and cofactor, exhibiting metallic properties such as ductility, malleability, cation stability, conductivity and ligand specificity were characterized by relatively high density and high relative atomic weight with an atomic number greater than 20, as said by Raskin et al. [12].

In Brazil, the reference levels for investigating the levels of heavy metal and other chemical substances in soils and groundwater are defined in CONAMA Resolution No. 420 [13], which facilitates the assessment of contamination and the creation of indicators that control and take care of the areas exposed to metals and the living beings in it. All forms of life can be affected by the presence of heavy metals depending on the dose and chemical composition [14]. Another key factor to be considered is the degree of exposure that is directly related to the amount of bioavailability in the environment, as the free ions of the metal can be linked to organic matter, reducing bioavailability [15].

3. Heavy metals in plants

According to Becher et al. [16], responses of plants to environmental stresses involve defenses, as well as stress-inducible reactions. Stimulation of important tolerance routes, such as enhancement of antioxidant enzymes activity, osmolyte accumulation, induction of membrane-localized transporters. Therefore, plants have developed mechanisms for homeostasis of ions that enable them to cope with a certain limited excess of heavy metals. Plants in the course of evolution demonstrate tolerance or even an adaptation to various biotic and abiotic stress factors.

According to Hall and Williams [17] chaperons, chelators and specific transmembrane transporters have evolved. In plants non-adapted to given environmental conditions, that are challenged with excess of ions, an enhanced biosynthesis of complexing substances such as metallothioneins, phytochelatins and/or organic acids can occur (**Figure 2**) [18, 19].

Nitrogen assimilation is an important plant metabolic process, which not only controls development plant growth but also plays an important role in plant survival under stress conditions. As said by Burger and Jackson [20], NO_3^- and NH_4^+ are major nitrogen sources and are required during different metabolic processes. Nitrate is converted into NH_4^+ via a process constituted of two steps; during the first step, NO_3^- is converted into nitrite with the action of the enzyme nitrate reductase, and in the second step nitrite is converted into NH_4^+ with the action of nitrite reductase.

Pérez-Tienda et al. [21] reported that nitrate reductase is located in the cytoplasm, while nitrite reductase is located in chloroplast and uses energy and some reductants such as NADH or NADPH from respiration or photosynthesis process to carry out such NO_3^- to NH_4^+ conversion reaction. The first step of this reaction occurs in the cytosol, while the second step occurs in the plastid. Following NH_4^+ production, it has to be incorporated in carbon skeleton, and this process occurs primarily via GOGAT cycle.

Moreover, as related by Mevel and Prieur [22], is possible to visualize two isoforms of GS, GOGAT and their localization has been found in a tissue-specific manner, e.g., in roots, GS1 and NADHGOGAT are primarily involved in the assimilation of nitrogen, in leaves the GSII and GOGAT are predominantly involved in the nitrogen assimilation.

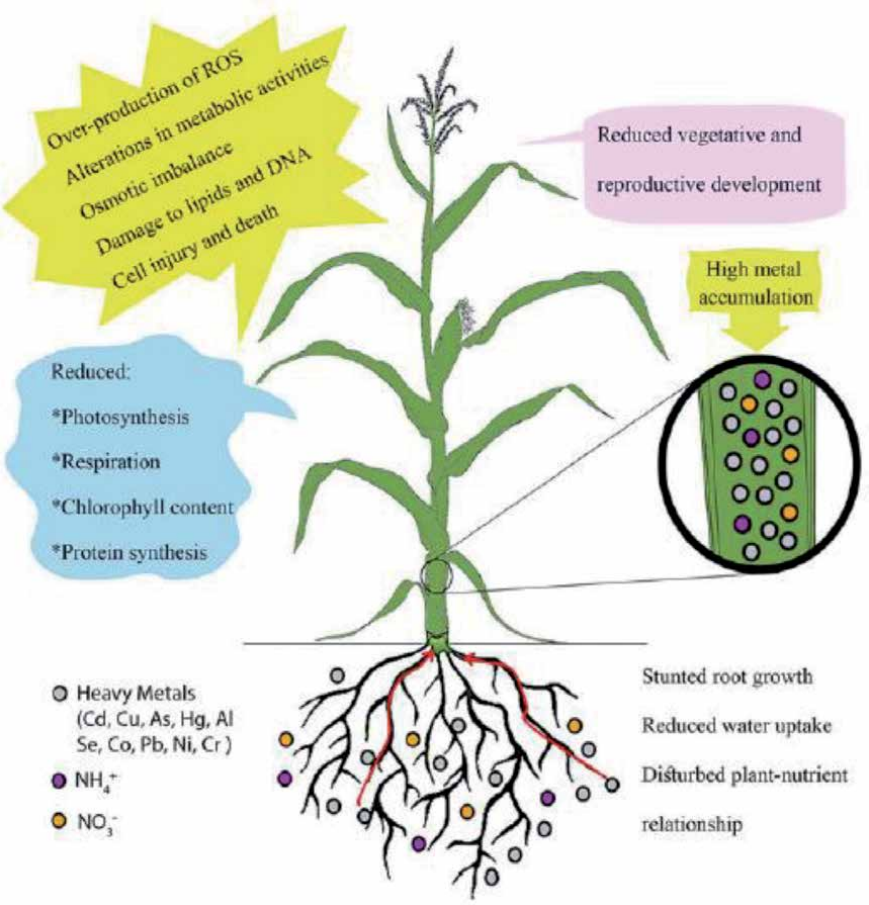

Figure 2.
Morphophysiological responses of plants to metal toxicity in soil.

Metal toxicity significantly reduces nitrogen assimilation process. However, the level of reduction depends on the sensitivity and localization of enzymes to heavy metal toxicity. Moreover, concentration, mobility and duration of heavy metal ions in growth medium further aggravate alterations in the process of nitrogen assimilation.

The exposure to metals at higher concentrations could result in severe damage to various metabolic activities leading consequently to the death of plants. The exposure of excess levels of metals to plants inhibits physiologically active enzymes [23], inactivates photosystems [24], and can possibly destroy the mineral metabolism. Janas et al. [25] have analyzed the impact of Cu on lipid peroxidation, growth, localization in lentil seedlings and phenolic compound accumulation. According to Xie et al. [26] and Chen [27] previous experiments have reported that dissolved soil organic substances have significant effects on heavy metal transformations by increasing the solubility of metal, plant uptake and root growth.

4. The effects of some heavy metals on plants

4.1 Cu

According to Burkhead *et al*. [28], due to the ability to cycle between the reduced Cu and oxidized Cu states, it is involved in biological processes such as

photosynthesis, respiration, oxygen superoxide scavenging, ethylene sensing, lignification and cell wall metabolism. As an essential element, Cu can also be highly toxic [29]. Cu in a free form catalyzes reactions that generate hydroxyl radicals causing damage to lipids, proteins and DNA [30]. According to Bernal *et al.* [31], copper also has been reported to interfere with iron homeostasis. A highly reduction of plant biomass, inhibition of root growth, chlorosis, and necrosis are the most reported symptoms of a excess od Cu due to increased production of reactive oxygen species and harmful interactions at the cellular level.

4.2 Zn

The roles that zinc plays in cellular processes is a good example of the diverse biological utility of metal ions. Zn is involved in protein, lipid metabolism, carbohydrate and nucleic acid. Moreover, Zn is critical to the control of biological processes regulated by proteins containing DNA-binding Zn-finger motifs and gene transcription [32]. The precise cause of Zn toxicity is unknown, but the metal may bind to inappropriate intracellular ligands, or compete with other metal ions for transporter proteins or enzyme active sites. In order to play these diverse roles in cells, and because it cannot passively diffuse across cell membranes, Zn must be transported into the intracellular compartments of the cell where it is required for these Zn-dependent processes.

4.3 Ni

Ni is essential to several metabolic phenomena and is extremely toxic to plants when present at excessive levels in nutrient solutions or in the soil to which plants are exposed. According to Rahman et al. [33], the general signs associated with Ni toxicity in plants, include: reduced shoot and root growth, poor development of the branching system [34], deformation of various plant parts [35], abnormal flower shape [36, 37], decreased biomass production [33], leaf spotting [38], mitotic root tip disturbances [36], inhibition of germination [39], Fe deficiency leading to chlorosis [40, 41], and foliar necrosis [42].

4.4 Cd

According to Chen et al. [43], among all the heavy metals, cadmium (Cd) is considered to have high toxicity to humans and all other living organisms as it has no known biological functions in aquatic or terrestrial organisms. Through its effects on various biochemical and physiological processes in plants, Cd could inhibit plant growth and cause cell death above critical levels [44, 45]. Studies by Hassan et al. [46] reported that cadmium-induced growth reduction might be explained on the basis of inhibition of carbon fixation due to a decrease in photosynthetic rate and chlorophyll content.

5. Conclusion

In the presented chapter, we have provided data and reviews regarding the effect of heavy metals on nitrogen metabolism of plants and the responses of plants and the cross-talk of heavy metals and various stressors factors. Moreover, is clear to understand the relation between metals amount and the benefit or harm caused on plants, determining then, which mechanism should be activated to protect your physiological system. Additionally, we briefly show physiologically how this process occurs.

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Section 2

Nitrogen Fertilizers and Nitrogen Management in Agriculture

Chapter 3

Nitrogen Management in Conservation Agriculture

Anthony Imoudu Oyeogbe

Abstract

Transitioning to conservation 'sustainable' agriculture (CA) from the conventional 'industrial' agriculture often increase nitrogen (N) limitation, particularly in the first few years. Inadequate N availability is associated with the permanent crop residues on the soil surface. The soil available N for crop uptake is immobilized by microbial sources of organic residues mineralization. The increase in N immobilization contributes to yield declines, and thus, researchers are advocating for the inclusion of N management as the fourth principle in CA. The challenge for CA under optimized N fertilization is how to reduce environmentally-damaging greenhouse gases (GHG) emissions from yield-related productivity. This paper focuses on efficient N management under CA system. Here, we showed the impacts of adaptive N management on crop yields increase, soil health enhancement, and greenhouse gases mitigation. We conclude that efficient N management using innovative technologies and good agronomic practice can scale-up the adoption of CA. An adaptive N management in CA can maintain environmental benefits while contributing to improved soil health and crop productivity. Moreover, the implementation of adaptive N management must be tailored to crop and soil types and location-specific.

Keywords: N immobilization, adaptive N fertilization, crop N demands, soil N test value, sensor-N guidance

1. Introduction

Conservation agriculture (CA) is a resource-efficient system that is capable of increased soil quality, crop productivity, and environmental sustainability [1]. CA system provides multiple ecosystem services and promotes agrobiodiversity ([2], Montpellier [3]). It is characterized and quantified by three principles practised simultaneously, namely; zero/minimum tillage; permanent soil cover; and diversified crop rotation:

- Continuous zero or minimum soil tillage: direct seeding or planting into undisturbed or untilled soil, to maintain or improve soil organic matter content, soil structure, and soil health. The disturbance area must be less than 15 cm or 25% of the cropped area, in addition to no interrupting tillage.

- Permanent organic matter soil cover with cover crops or crop residues: this shields the soil surface, conserves nutrients and water, promotes soil biological activity, and contributes to weed management. Soil cover should be preferably 100%, however, surface soil cover of 30% is seen as adequate.

- Diversified crop rotation: both annuals and perennials as inter-and sequential-cropping, contributing to dietary diversity, human and livestock nutrition, and enhanced cropping system resilience. Mono-cropping is allowed provided no productivity limitation is envisaged in the cropping area.

The synergy of CA principles contributes to on-farm resource use efficiency, sufficiency and sustainability [4]. However, N immobilization under CA-based cropping systems is a major trade-off between resource use efficiency and sustainability. In promoting CA as a productivity-enhancing, resource-saving and eco-friendly paradigm of sustainable intensification, there is the need to address the challenges of increased limitation of soil available N [5, 6]. The inclusion of adaptive N management in CA can contribute to increased crop yields during the early (first three) years of transition from the conventional production system to CA.

2. Conservation agriculture: sustainable agriculture and food security

Feeding the projected 9+ billion people come 2050 call for the implementation of sustainable production systems globally [7]. Conventional agriculture disrupts agroecosystem sustainability, and are a major source (19–29%) of anthropogenic greenhouse gas emissions [8]. Thus, the quest for sustainable crop production intensification has dominated both the scientific and policy thinking space in the last two decades with regards to food security [5]. CA is a paradigm of sustainable intensification with numerous agricultural and environmental benefits (Montpellier [3]). It promotes on-farm biodiversity and ensures ecosystem sustainability [2, 9].

Currently, CA is practised on 155 million hectares of land [10], equivalent to 9% of global arable land [11]. Research studies on CA have highlighted the numerous agricultural and environmental benefits, which includes increased crop yields [12, 13], soil carbon sequestration [14, 15], microbial diversity [16, 17], soil-water retention capacity [18, 19], GHG emissions mitigation [20, 21], early planting time, labour, and energy savings [22, 23], and dietary diversity for human and livestock nutrition [1].

Nevertheless, the multiple benefits of CA have not provided the impetus for robust implementation across scales. Several on-farm research under CA management has reported a reduction in crop yields, particularly in the early phase of transition [11, 24–27]. The decrease in crop yield is ascribed to the increased N immobilization by organic residues, which limits soil available N uptake for crop growth. Researchers have advocated the need for the inclusion of N management [6, 28]. Tailoring N management in CA-based cropping systems can improve the soil organic matter efficiency while contributing to crop yield increase.

3. Nitrogen immobilization: a tradeoff in conservation agriculture

N immobilization is one of the major tradeoffs in CA, which is associated with the permanent organic residues soil cover. Increased N immobilization affect crop yields, particularly in the early stages (1–3 years) of CA implementation [29]. Other trade-offs in CA includes soil compaction, incompatible machinery, and technical know-how. These trade-offs in CA have affected widespread adoption [24]. Thus, the need for a soil-based approach in managing N fertilizers [30], including locally-adapted N management can contribute to yield increase in CA [6, 28].

The significance of CA is the improvement in soil quality, crop productivity, and environmental sustainability [9]. CA practices applied together are of critical importance to soil processes and ecosystem functioning. More specifically, the synergies of minimal soil disturbance, permanent soil cover and crop diversification create an optimal soil environment that stimulates the organic matter efficiency. Increased soil organic matter influences the microbial communities, which are responsible for improving the soil and crop productive capacity. However, N availability in CA is negatively affected by the permanent crop residues on the soil surface. The diverse microbial communities in soil utilize the available soil N for residue-C decomposition, which is detrimental to the crop N uptake in a short time.

Based on a global data set and across a broad range of crops, Lundy et al. [25]; Pittelkow et al. [11, 26] and Rusinamhodzi et al. [31] reported the impact of N fertilization in CA. These authors showed that adequate N fertilization can offset yield declines in CA systems, particularly in tropical regions. Furthermore, they reported that the effects of implementing CA with and without N fertilizer, residue management, and crop rotation in various crops and climates showed yield declines under CA by 12% without inorganic N fertilizer and 4% with N fertilizer addition. For instance, the addition of inorganic N fertilizer (80–120 kg N ha^{-1}) reduced yield by 4% under CA. Also, the inclusion of legumes in CA-based cropping systems produced comparable yield to that of conventional tillage without N addition.

4. Nitrogen management and availability in conservation agriculture

Dynamics of N availability is the net amount of inorganic and organic inputs in soil undergoing decomposition, mineralization and immobilization [32]. Also, the quantity and quality of organic residues influence the N availability [33, 34]. The mineralization of organic residues increases with N fertilization [35], and this offsets the temporary immobilization of available N [34, 36]. Adequate N fertilization during the transition from conventional to CA would contribute to the rapid mineralization of organic residues, which in turn minimizes microbial N immobilization and increases N availability for crop uptake [37, 38]. Therefore, ensuring adequate N fertilization is an immediate strategy of alleviating N limitations in residue-laden soils under CA. However, increasing inorganic N fertilization might hasten organic residues N mineralization, which is associated with the potent greenhouse nitrous oxide (N_2O) emissions [39].

The appropriateness of N fertilizer application is a recommended management practice in minimizing crop yield declines in CA [11, 13, 25, 26, 35]. Increasing N fertilizer rate in CA is more important in the tropics than the temperate region [25]. For instance, decreases in crop yields were observed at low N fertilization in the first 2 years of adoption under tropical conditions compared to the temperate. However, the addition of N (75–100 kg N ha^{-1} $yr.^{-1}$) fertilizer improved yields by up to 12% under tropical environment [25, 26]. In the Indo-Gangetic Plains, Oyeogbe et al. [4, 13] showed that optimizing N fertilizer dose in maize and wheat to 180 and 150 kg N ha^{-1}, respectively, increased the grain yield by 20 and 14%. Also in northwest India, wheat grain yields under precision N management increased by 14% compared to farmers fertilization practice [21]. In Germany, adjusting the N input from 65 to 105 kg N ha^{-1} in maize produced significant yield increases up to 16% under conservation tillage system [23].

Adaptive N management using good agronomic practices and novel technologies can optimize N availability in CA. Oyeogbe et al. [13] and Sapkota et al. [21] demonstrated that N fertilizer management by soil N test assessment and optical sensor (GreenSeeker™) technology increased the grain yields of maize and wheat

compared to farmers practice under CA of the Indo-Gangetic Plains. Yadvinder-Singh et al. [35] reported that split N fertilizer applications following the optical sensor guidance improved the yields of wheat under CA. In-season N fertilization guided by the optical sensor ensures that adequate N is available for organic residues mineralization and crop uptake [35, 40, 41].

Also, organic amendments can influence N availability in a CA-based system. To reduce soil N immobilization in cereal-based CA cropping systems, Flower et al. [42] included high biomass oat cover crop to reduce soil N immobilization. Pittelkow et al. [26] and Lundy et al. [25] reported that crop yields response with inorganic N additions were similar to that of conventional tillage system. Combining organic and inorganic N fertilizers can contribute to a more efficient soil available N under long-term CA system [43]. In the Indo-Gangetic Plains, brown manuring is becoming an effective organic N strategy under CA [12]. Oyeogbe et al. [4, 13] showed that the inclusion of brown manuring had a positive effect on yields of maize and wheat by supplying additional N.

5. Nitrogen fertilizer and nitrous oxide emissions in conservation agriculture

Agricultural soils are the largest source of N_2O emissions, and N fertilizer use is a major contributor to N_2O emissions [44]. N_2O is mostly produced by microbial transformations of N in soils and is often enhanced where available N exceeds crop demand [45]. Under the CA system, N_2O emissions are influenced by increased organic residues mineralization. Moreover, optimized N fertilizer in CA would contribute to larger N_2O emissions. Thus, the challenge for CA is how to effectively manage the permanent organic residues and optimized N fertilization, and reduce environmentally-damaging GHG emissions from yield-related productivity. CA is an eco-friendly 'greener' production system, which is capable of alleviating the GHG emissions compared to conventional 'industrial' production system [46]. It emphasizes on the efficient use of fertilizer, pesticides, and farm machinery are important strategies to mitigate GHG emissions while improving crop productivity [23, 47]. However, there are negating views about the positive impacts of CA practices on GHG emissions. Several research findings reported increased N_2O emissions from the organic residues decomposition under CA [20, 48, 49]. Retaining crop residues on the soil surface is susceptible to increased microbial N transformations and associated N_2O emissions.

Several factors such as high temperature and N fertilization can influence larger emissions of GHG in CA. High temperature and N fertilizer application increase the decomposition and mineralization of organic residues [49, 50]. However, organic N mineralization and associated N_2O emissions decrease in CA due to the absence of soil tillage [51]. Ito et al. [52] indicated that tillage exerted stronger effects on nematode community structure than organic residue management. And thus, it can be argued that the mineralization of organic residues is lower in CA due to less contact between the organic residues and soil organisms compared to conventional tilled system associated with greater N mineralization [49]. Del Grosso et al. [53] simulated the N_2O emission rates from conventional tilled and no-till soil, larger emissions rate were found in the conventional system compared to CA soil. Oyeogbe et al. [4] demonstrated that tailoring N fertilizer application to crop demands can reduce N_2O emissions under CA. Adaptive N-rate (i.e. 155 and 133 kg ha^{-1} for maize and wheat, respectively) influenced yield gains of about 20 and 14%, respectively, while reducing N_2O emissions in the first two years of implementation [4, 13]. Furthermore, Oyeogbe et al. [13] demonstrated that N_2O emissions based on

the global warming potential (conversion to CO_2-eq) was decarbonized through increased soil carbon sequestration efficiency under adaptive N fertilizer management in CA. Therefore, efficient N fertilization in CA can improve crop productivity, enhance nutrient use efficiency [35], reduce N leaching losses [54, 55], and deactivate N_2O emissions [4, 21].

6. Conclusion

Increased N limitation in CA contributes to crop yield declines, particularly in the first few years of implementation. In promoting CA both as a productivity-enhancing and resource-saving paradigm, there is a need to tailor N availability to crop demands. Adaptive N management in CA can alleviate N limitation of microbial origin and contribute to yield increase, soil quality and environmental sustainability. More importantly, adaptive N management in CA should align with the crop and soil types in diverse agroecological conditions. Therefore, integrating good agronomic practices and innovative technologies in CA such as N management could lead to wide-spread adoption.

Conflict of interest

The author declares no conflict of interest.

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Chapter 4

Agronomic Response of Camelina to Nitrogen and Seeding Rate on the Northern Great Plains

Thandiwe Nleya, Dwarika Bhattarai and Phillip Alberti

Abstract

Camelina (*Camelina sativa* L. Crantz,) a new oilseed crop in the Brassicaceae family has favorable agronomic traits and multiple food and industrial uses. Appropriate production practices for optimal camelina yield in temperate climates of North America are lacking. This study investigated the response of camelina seed yield and quality, and agronomic traits to applied N (5 levels, 0, 28, 56, 84, 140 kg ha^{-1}) and four seeding rates (4.5, 9, 13, 17.5 kg ha^{-1}). Separate experiments were conducted at four environments (site-years) for N and three environments for seeding rate in South Dakota. In three of the four environments, the highest N rate increased seed yield by 30 to 60% compared to the control. The increase in seed yield with increasing N rate was linear in a high yielding environment and quadratic in a low yielding environment. Increasing seeding rate increased plant stands but had inconsistent impacts on seed yield depending on location and year. Seed oil concentration ranged from 149 to 350 g kg^{-1}, was inversely related to N rate but was not influenced by seeding rate.

Keywords: *Camelina sativa* L., camelina, N fertilizer, seeding rate

1. Introduction

Camelina (*Camelina sativa* L. Crantz,) commonly known as camelina or false flax, is an annual herbaceous oilseed crop commonly grown in the temperate region of Europe and North America [1]. Although the actual origin of this crop is uncertain, most studies report that the crop likely originated in southeastern Europe and southwestern Asia [2, 3]. Camelina was introduced as a weed in North America; however, at present, it is widely grown as an oilseed crop that can perform well in diverse climate, requires low nutrient and is highly resistant to diseases and pests [2, 4]. Camelina seed oil has diverse uses including edible products, biodiesel feedstock [4, 5], bio-jet fuel [4, 6] and other chemical derivatives [7, 8]. Seed oil content is reported to range from 26.7% to 46.0% [9, 10]. Camelina oil mainly consists of poly- and mono-saturated fatty acids. The most important ones are linolenic (C 18:3, 25–42.52%), linoleic (C 18:2, 12.34–21.3%), oleic (c 18:1, 11.89–20.51%) and eicosenic acid (C 20:1, 12,54–18.30%) [10]. Other notable fatty acids include palmitic (C 16:0), stearic (C 18:0), eicosanoic (C 20:0), and erucic (C 22:1) [8]. Because the oil is high in omega-3 (linolenic) and omega-6 (linoleic) acids and low in saturated fatty acids, the crop is considered a high quality edible oil. The oil also contains vitamin E

which acts as an antioxidant and also increases the stability and the shelf life of camelina oil compared to other omega-3 oils [11]. Camelina oil has a high smoke point (246°C) and therefore can withstand high-heating cooking methods like frying. However, overheating the oil can reduce beneficial compounds such as antioxidants and can impact the overall taste of the oil so it is not recommended that camelina oil be heated for prolonged periods of time. The erucic acid (C 22:1) content of camelina oil is variable depending on the genotypes and can range 2.11 to 4.30% [10] higher than the maximum allowed in food grade rapeseed oil (2%) [11]. High content of erucic acid in edible oils is of concern as excessive consumption of erucic acid has often been linked to heart diseases. However, there is significant genetic variation for erucic acid and camelina breeding lines with lower erusic acid have been identified [11]. Camelina meal can be used as animal feed including cattle, swine and poultry [12–14] and has been reported as a potential aquaculture feed [15]. The increasing demand of high quality biofuel derived from polyunsaturated fatty acids, [16], has prompted interest for the development of sound agronomic practices for camelina production as an industrial oilseed in Northern Great Plains (NGP) of the US. Thus, there is a need to identify the optimal management practices for camelina including seeding rate, and nitrogen (N) fertilization for achieving yield and seed quality goals in both humid temperate and semi-arid production zones of the NGP.

Nitrogen is an important component required for physiological functions of all plants; it has a crucial role in photosynthesis and is a component of protein and enzymes. Many studies have suggested that camelina has a low nitrogen requirement compared to other oilseed crops like canola and sunflower. Research conducted in Kansas, US reported that N fertilizer application affected plant height, plant stand, protein content of camelina seeds, and biomass and seed yield [9]. The optimum N rate was 50 kg ha^{-1} producing about 760 kg ha^{-1} seed yield suggesting camelina has a low N requirement. This was supported by other research results conducted in US [17–19]. More recently, in a study conducted in Minnesota, US, Johnson et al. [17] reported that a rate of 34 kg ha^{-1} N was sufficient to achieve economically viable camelina seed and oil yields. Mohammed et al. [18] reported that application of 60 kg ha^{-1} N produced maximum seed yield and oil yield while Wysocki et al. [19] reported optimum N rates ranging from 0 to 90 kg ha^{-1} depending on annual precipitation and available N. On the other hand, Solis et al. [20] reported that under conditions of high productivity, camelina maximum seed yield is achieved at N rate of 185 to 300 kg ha^{-1} N. These findings were supported by Malhi et al. [21] who reported that camelina responded to high N rates (170 kg ha^{-1}) similar to *Brassica juncea* and Jiang and Caldwell [22] who concluded that camelina responded positively to increased N rates up to 200 kg ha^{-1} but that seed yield response to applied N depended on genotype. Previous research seem to agree however, that seed oil content decreases as N application rate increases [18, 20, 22]. The above research shows that the response of camelina to increasing N rate varies considerable depending on environmental conditions.

Stand establishment varies depending on tillage practices, environmental conditions and variety and therefore higher seeding rates are often recommended to avoid stand losses due to poor seedbed conditions. In camelina as with most agronomic crops, uniform crop emergence and plant development are crucial to achieving optimum yields and using the appropriate seeding rate is very important to achieve this goal. Previous studies on impact of seeding rate on camelina yield often suggest that yield is not reduced by low plant populations due to growth plasticity of camelina plants [23–25]. Optimum seeding rates for camelina range from 4 to 6 kg ha^{-1} [8]. In a study conducted in Canada optimum seeding rates ranged 400 to 600 seeds m^{-2} [25], which is similar with the recommendation suggested by Berti, et al., [8]. Gesch et al. [23] recommended a seeding rate of no less than 3 kg ha^{-1} for

good stand establishment of spring camelina when seeded using a drill. Although these low seeding rates would be enough for optimum yield, higher seeding rates are often recommended to compensate for low stand establishment due to poor seedbed preparation, lack of soil moisture and poor competition of camelina with weeds. This means further evaluations under different environmental conditions are warranted.

The overall goal of this study is to develop management strategies for camelina production practices at two growing different agro-zones in South Dakota. The specific objectives were to determine the effects of N fertilization rate and seeding rate on growth, seed yield and seed oil content of camelina.

2. Material and methods

2.1 Study site

The study was conducted at two locations, near Brookings (44° 18' 40.8863" N, 96° 47' 54.1957" W) and near Pierre (44° 22' 5.9362" N, 100° 21' 3.4794" W) in South Dakota in 2015 and 2016 for N fertilization study and 2016 and 2017 for the seeding rate study. The Brookings study was conducted on a Brandt silty clay loam (fine-silty, mixed, superactive, frigid Calcic Hapludolls) while the Pierre study was conducted on a Dorna silty loam soil (coarse-silty over clayey, superactive, mesic Fluventic Haplustolls). The previous crop at the Brookings location was winter wheat (*Triticum aestivum* L.) in all three years. At the Pierre location, the previous crops were teff [*Eragrotis tef (Zucc.)Trotter*] in 2015 and corn (*Zea mays* L.) in 2016 and 2017. Soil analysis details for each location in each year are shown on **Table 1**. Soils at each location were sampled in the spring of each year at planting time. Four soil cores were sampled diagonally across each field using a tee-handled push probe to a depth of 0–15 cm. At the Brookings location, the study was managed using conventional tillage while at Pierre the study was under no-tillage system.

2.2 Nitrogen fertilization study

The experimental design was a randomized complete block design with treatments replicated four times. Treatments included five different N fertilizer rates: 0, 28, 56, 84, and 140 kg N ha^{-1}, and one camelina variety (S-40). Nitrogen fertilizer in the form of urea (46% N), was broadcast manually on each plot soon after planting using an automatic hand-held spreader to ensure even application. In 2015, plots were planted on 3 April at Brookings and 16 April at Pierre. In 2016 the planting dates were 26 April at Brookings and 14 April at Pierre. Planting was accomplished

Location	Year	Texture class	pH	Soluble salts (mmho/cm)	Organic matter (%)	Nitrogen-NO3 (ppm)	Olsen-P (ppm)	K (ppm)
Brookings	2015	Medium	5.7	0.2	4.8	12.0	9.0	171.0
Brookings	2016	Medium	5.6	0.1	4.8	12.0	16.0	349.0
Brookings	2017	Medium	5.6	0.1	4.7	10.0	10.0	141.0
Pierre	2015	Medium	6.1	0.2	2.9	10.8	8.9	208.0
Pierre	2016	Medium	6.1	0.2	3.0	18.1	21.7	626.0

Table 1.
Site description and soil characteristics at the 0–15 cm depth for the two South Dakota locations where studies were conducted in 2015 and 2016.

using a seven-row Hege 500® (Wintersteiger- Austria) at Brookings; seeding at Pierre was done using a Light Duty Grain Drill® (Almaco- Iowa). Individual plot size was 1.6 x 9.1 m (14.6 m^2) at Brookings and 1.6 x 8.2 m (13.1 m^2) at Pierre. Each plot had seven rows, 22 cm apart. The seeding rate was 9 kg ha^{-1} at both locations.

Number of days to maturity were recorded when 50% of pods on the main stem of plants within a plot had turned yellow. Plant height was determined by measuring height of five random plants within each plot from soil line to the top of the plant and averaging the height. Yield was determined by harvesting whole plots using a Kincaid 8XP® crop research combine (Kincaid Equipment and Manufacturing- Haven, KS) with the assistance of the H2 High Capacity GrainGage® (Juniper Systems Inc.- Logan, UT). In 2015, camelina was harvested on 12 August at Brookings and 14 August at Pierre. In 2016, the study was harvested on 9 August at Brookings and 6 August at Pierre. Seed from each plot was dried to a constant weight, cleaned and sieved before weighing to determine seed yield.

2.3 Seeding rate study

The experimental design was a randomized complete block (RCBD) design with treatments replicated four times. Treatments included four different seeding rates (4.5, 9, 13, and 17.5 kg ha^{-1}) and two camelina cultivars ('SO-40 and SO-50) arranged in a factorial design to give a total of eight treatments. In 2016, the planting dates were April 26 at Brookings and April 15 at Pierre. In 2017, the planting dates were April 24 at Brookings. Planting was accomplished using a seven-row Hege 500® (Wintersteiger- Austria) at Brookings; seeding at Pierre was done using a Light Duty Grain Drill® (Almaco- Iowa). For the Brookings location, individual plot size was 1.62 x 9.14 meters (14.86 m^2) and 1.62 x 8.23 meters (13.37 m^2) at Pierre. Each plot had seven rows, 22 cm apart.

In 2016, 56 kg ha^{-1} N fertilizer in the form of urea (46% N) was broadcast manually using an automatic hand-held spreader to ensure even application ~4 weeks after planting. In 2017, 112 kg ha^{-1} N and 22 kg ha^{-1} S in the form of urea (46% N) and ammonium sulfate (21% N and 24% S) mixture was applied in a split application to ensure continuous supply of N. The first application occurred at planting and the second application occurred around the bolting stage. The fertilizer was broadcast manually using an automatic hand-held spreader to ensure even application.

Four weeks after seeding, plant stands were assessed by counting the number of plants in a 4 ft^2 and converted to plants m^{-2}. Days to flowering (50% of flowers open within each plot) and days to maturity (50% of plant with pods turned yellow within each plot) were also determined for each plot. At physiological maturity, average plant height was determined by measuring height of five random plants within each plot from soil line to the top of the plant. Lodging notes were taken and rated on a scale from 1 to 9 (1 = no lodging, 9 = completely lodged). Shattering notes were taken based on percent of pods shattered at the time of harvest within each plot. In 2016 only, random 10 plant samples were obtained from all plots and both locations, the number of seeds per 15 pods and number of pods per 10 plant determined. From these values, the average number of seeds pod^{-1} and pods $plant^{-1}$ were determined.

Once camelina had appropriately dried down, it was harvested using a Kincaid 8XP® crop research combine (Kincaid Equipment and Manufacturing- Haven, KS) with the assistance of the H2 High Capacity GrainGage® (Juniper Systems Inc.- Logan, UT). In 2016, the camelina was harvested on August 9 at Brookings and August 5 at Pierre. In 2017, the camelina was harvested on August 21 at Brookings.

2.4 Oil analysis

For both the N fertilizer and the seeding rate study, once the plots were harvested the seed was cleaned using a sieve and a blower to remove unwanted plant material; cleaned seed was collected and total seed yield (kg) was determined. Sub-samples of the harvested seed were collected and placed into individual manila envelopes and stored in a cold room (~10° C) for oil content determination. Two replications for all treatments were sent to SGS Mid-West Seed Services, Inc. (Brookings, SD, USA) for oil content analysis using a hexane solvent extraction. The results of this analysis were used to calibrate the "minispec mq" (Bruker- Billerica, Massachusetts) NMR instrument for further camelina oil analysis. The remainder of the samples from both years and locations were then analyzed using the "minispec mq."

2.5 Weed management

For both experiments, weeds were managed with pre-plant application at all locations of Prowl H_20 (Pendimethalin, BASF, Research Triangle, NC) herbicide. The herbicide was applied at a rate of 2.8 L ha^{-1} and incorporated 5 cm deep via two-pass incorporation. Herbicide application was at approximately two weeks before planting for both locations and all years. Once the crop had emerged, weeds were managed by manually removing weeds from within each plot, as necessary.

2.6 Statistical analysis

Data collected from each year were analyzed using PROC MIXED in SAS (Version 9.4, SAS Institute, Cary, NC) to determine the impact of N fertilizer and seeding rate on agronomic traits, seed and oil yield. The fixed effects in the model were N fertilizer rates in the first experiment and seeding rate and varieties in the second experiment while replication was considered random. The LINES option of LSMEANS statement was used to compare the differences among treatments at 95% confidence level. Different models (linear plateau, quadratic and quadratic plateau) were fit to seed yield, and seed oil concentration data to examine the response to N fertilizer rate. The choice of the best model was based on model significance (significantly different from zero based on t-test at $P = 0.05$), and coefficient of determination (R^2) [26, 27]. A Shapiro–Wilk test was used to test for normality.

3. Results and discussion

3.1 Climatic information

Temperature and rainfall data were collected at weather stations located within each farm throughout the crop growing period in both years (**Tables 2** and **3**). The climate data shows that the Pierre location was drier (especially during June and July) and hotter than the Brookings location. Overall, the 2015 growing season had rainfall and temperature conditions closer to the 30-year average at both locations. The trials were planted early to late April and the crop reached the flowering stage 52–56 days after planting meaning flowering and seed filling occurred in June and July. In 2016, both Brookings and Pierre had lower than average rainfall in June and July, coinciding with bolting, flowering, and seed filling periods, a critical time for seed development and quality. In particular, the Pierre location experienced higher temperatures and lower precipitation for a longer duration during this critical growth stage resulting in lower yields at this location.

	April	May	June	July	August	Total
	Rainfall (mm)					
Brookings 2015	5.1 (−49.0)	137.2 (+62.3)	43.2 (−65.8)	111.8 (+28.7)	71.1 (−6.9)	368.3 (−30.7)
Brookings 2016	48.3 (−5.8)	60.2 (−14.7)	66 (−43.0)	124.5 (+41.4)	142.2 (+64.2)	441.2 (+42.2)
Brookings 2017	40.7 (−13.5)	88.9 (+14.0)	5.1 (−103.9)	160.0 (76.9)	157.5 (+79.5)	542.1 (+53.0)
Pierre 2015	12.4 (−33.6)	157.0 (+77.0)	96.2 (+6.4)	58.2 (−7.4)	63.2 (+17.2)	387 (+59.6)
Pierre 2016	99.6 (+53.6)	30.5 (−49.5)	70.9 (−18.9)	28.5 (−37.1)	54.4 (+8.4)	283.9 (−43.5)

Table 2.
Monthly total rainfall throughout the crop growing period for camelina grown at Brookings and Pierre, SD in 2015, 2016 and 2017 (numbers in parentheses indicate deviations from 1981 to 2010 average).

3.2 N fertilizer effects

The N rate effects on plant height were significant at both locations in 2016 but were not significant in 2015 at both locations (**Tables 4** and **5**). At both locations in 2016, plant height increased with increasing N rate with the tallest plants recorded in the highest N rate of 140 kg ha^{-1}. These results agree with previous research on camelina where plant height increased with increasing N rate was inconsistent among environments [20]. In their study and in the current study, higher rates of N increased plant lodging. The influence of N rate on plant height and therefore lodging should be taken into consideration in environments with potential lodging problems such as areas with high wind speeds and high rainfall.

The number of days to maturity was recorded only in 2015 and was significantly influenced by nitrogen rate at both locations (**Table 4**). Days to maturity increased in response to N rate with a rate of 140 kg N ha^{-1} resulting in the longest time to maturity while plants in the control treatment took the shortest time to reach maturity (**Table 4**). These results confirm findings from previous studies suggesting that increased N fertilization rates can result in delayed crop maturity, due to prolonged periods of vegetative growth [28, 29]. Prolonged vegetative growth in the NGP would delay flowering and seed setting, the two most important growth stages determining yield potential, to later in the season (late-June–July) when high temperature and drought stress often occur. Under such environmental conditions, earlier planting dates and promoting earlier and shortened duration of flowering to avoid unfavorable conditions have been shown to increase yield in *Brassica* species [30, 31].

Seed yield was significantly influenced by nitrogen rate at Brookings in 2015 and at both locations in 2016 (**Tables 4** and **5**). At Brookings in 2015, N rates of 28 to 140 kg ha^{-1} yielded the same and significantly greater than the control. In 2016, on the other hand, the highest N rate of 140 kg ha^{-1} yielded greater than all other N rates and the control at the Brookings location. At a drier environment in Pierre, N rate did not impact seed yield in 2015 but in 2016, seed yield increased in response to N rate, with greatest seed yields occurring at the N rate of 140 kg N ha^{-1} rate (817 kg ha^{-1}) with this value statistically different from the yield obtained at all other N rates. The control treatment yielded significantly lower (416 kg ha^{-1}) than all other N rates. The effects of N fertilizer on seed yield of camelina have been reported by other researchers [20–22]. Jiang and Caldwell [22] reported that seed yield responded positively

	April		May		June		July		August	
Location	**Max.**	**Avg.**	**Max.**	**Avg.**	**Max.**	**Avg.**	**Max.**	**Avg.**	**Max.**	**Avg.**
	Temperature ^{0}C									
Brookings 2015	15.8 (+3.0)	8.7 (+2.0)	18.6 (−0.8)	13.0 (−0.3)	25.1 (+0.1)	19.6 (+0.7)	27.3 (−0.5)	21.7 (+0.6)	25.1 (−1.6)	19.5 (−0.5)
Brookings 2016	13.8 (+1.0)	7.9 (+1.2)	20.8 (+1.4)	14.6 (+1.3)	27.3 (+2.3)	21.3 (+2.4)	27.1 (−0.7)	21.6 (+0.5)	26.3 (−0.4)	20.9 (+0.9)
Brookings 2017	13.2 (+0.5)	7.2 (+0.5)	19.4 (0.0)	13.2 (0.0)	26.1 (+1.1)	19.4 (+0.5)	28.9 (+1.1)	22.8 (+1.7)	23.3 (−3.4)	18.9 (−1.1)
Pierre 2015	17.7 (+2.1)	9.8 (+1.5)	19.3 (−1.8)	12.9 (−1.5)	27.1 (+0.4)	20.6 (+0.6)	30.9 (−0.8)	23.4 (−0.5)	30.0 (−0.6)	22.6 (−0.7)
Pierre 2016	15.8 (+0.2)	8.9 (+0.6)	22 (+0.9)	14.4 (0)	30.1 (+4.4)	22.2 (+2.2)	32.3 (+0.6)	24.4 (+0.5)	30.2 (+0.4)	22.7 (+0.6)

Table 3.
Maximum and average temperature throughout the crop growing period for camelina grown at Brookings and Pierre, SD in 2015, 2016 and 2017 (numbers in parentheses indicate deviations from 1981 to 2010 average).

	Height	Maturity	Seed yield	Oil conc	Oil yield	Lodging	Shatter
N Rate (kg ha^{-1})	(cm)	(days)	kg ha^{-1}	g kg^{-1}	kg ha^{-1}	(1–9)	(%)
Brookings 2015							
0	73.2	109^{b}	1116^{b}	327^{a}	373	1.25	2.25
28	73.6	112^{a}	1360^{a}	258^{bc}	353	2.25	1.75
56	74.5	111^{a}	1397^{a}	275^{b}	388	2.75	1.25
84	78.6	113^{a}	1340^{a}	268^{b}	361	4.00	1.75
140	76.7	113^{a}	1455^{a}	226^{c}	332	4.75	1.00
Pierre, 2015							
0	79.2	110^{b}	1186	228^{a}	268^{a}	7.30	3.00
28	76.5	108^{b}	1353	188^{b}	254^{a}	8.00	3.00
56	84.8	111^{b}	1396	185^{b}	258^{a}	8.00	2.67
84	81.8	115^{ab}	1348	177^{b}	238^{ab}	9.00	2.33
140	76.2	116^{a}	1393	149^{c}	207^{b}	8.00	2.00

Within each column and location, means followed by the same letter are not significantly different at P< 0.05.

Table 4.
Nitrogen (N) fertilization rate effects on plant height, days to maturity, seed yield, oil concentration, oil yield, lodging and pod shatter in camelina grown at two locations in SD in 2015.

	Height	Seed yield	Oil concentration	Oil yield
N Rate (kg ha^{-1})	(cm)	kg ha^{-1}	g kg^{-1}	kg ha^{-1}
Brookings 2016				
0	68.7^{e}	988^{b}	350^{a}	345^{b}
28	74.7^{d}	1215^{b}	324^{a}	392^{ab}
56	77.7^{c}	1112^{b}	293^{b}	317^{b}
84	81.1^{b}	1197^{b}	321^{ab}	386^{ab}
140	88.2^{a}	1579^{a}	286^{b}	451^{a}
Pierre, 2016				
0	58.0^{d}	416^{d}	271^{a}	91^{b}
28	67.4^{c}	657^{c}	204^{a}	134^{a}
56	70.4^{bc}	723^{bc}	193^{ab}	139^{a}
84	71.7^{b}	770^{b}	176^{b}	135^{a}
140	75.4^{a}	817^{a}	138^{c}	113^{ab}

Within each column and location, means followed by the same letter are not significantly different at P< 0.05.

Table 5.
Nitrogen (N) fertilization rate effects on plant height, seed yield, oil concentration, and oil yield in camelina grown at two locations in SD in 2016.

to applied N to 200 kg ha^{-1} N although the response was different depending on genotype. Similarly, Mahli et al. [21] reported that maximum seed yield of camelina was achieved at an N rate of 170 kg ha^{-1} supporting the notion that camelina has a high demand for N. However, studies conducted in the US suggest that camelina has a lower N requirement. Wysocki et al., [19] reported that camelina seed yield in the Pacific Northwest was maximized between 44 and 88 kg ha^{-1} depending on location.

In a study conducted in Montana, Mohammed et al., [18] reported that application of 60 kg ha^{-1} N produced maximum agronomic seed and oil yield. In the current study, regression analysis showed that seed yield had a linear relationship with N rate at a higher yielding environment at Brookings while the relationship was quadratic at the lower yielding environment at Pierre (**Figure 1**). Other researchers have also shown similar results indicating different response of camelina to N rate depending on genotypes and environmental conditions. Wysocki et al. [19] reported camelina seed yield response to N rate varied widely from one location to the other depending on annual precipitation and soil available N. They reported linear increase in camelina seed yield with increase in N rate at locations with high precipitation and while locations with lower precipitation showed no response. Research conducted in Canada reported both linear and quadratic response depending on camelina genotype [22].

Seed oil concentration was significantly influenced by N rate at both locations and in both years (**Tables 4** and **5**). Seed oil concentration decreased at a rate of 0.40 to 0.60 g kg^{-1} for 1 kg ha^{-1} increase in N fertilizer rate at Brookings and at a

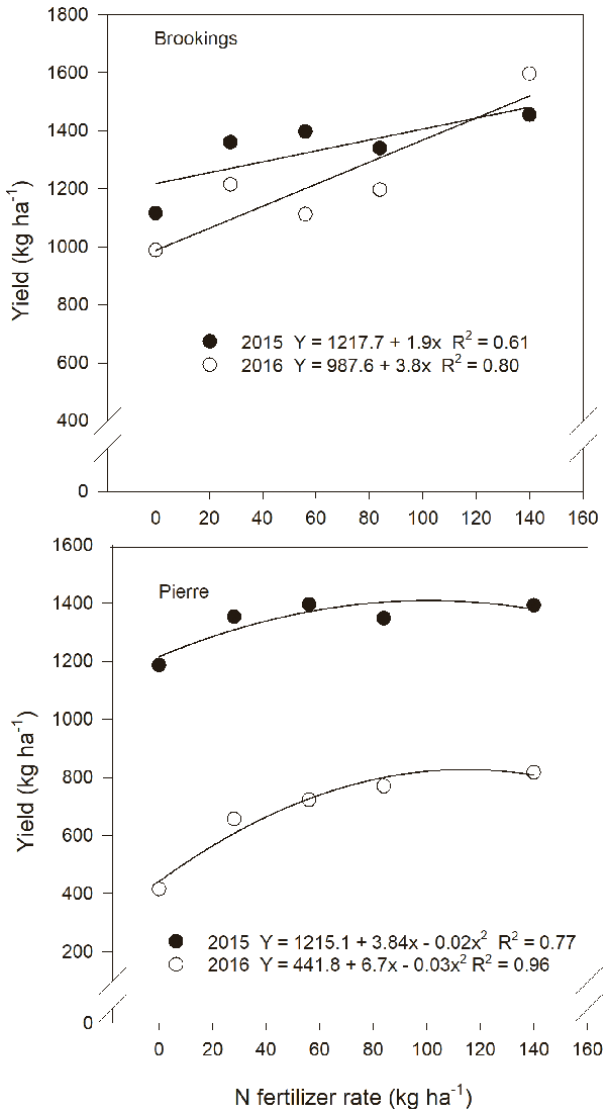

Figure 1.
Camelina seed yield as a function of nitrogen fertilization rate at Brookings and Pierre, SD in 2015 and 2016.

higher rate of 0.50 to 0.80 g kg^{-1} for 1 kg ha^{-1} increase in N fertilizer rate at Pierre (**Figure 2**). These findings agree with earlier findings which show seed oil concentration decreasing linearly in response to increasing N rate [18, 20, 21]. In both years and both locations, the control treatment had the greatest oil concentration while the lowest oil concentration was observed in the highest N rate of 140 kg N ha^{-1} (**Tables 4** and **5**) likely due to the dilution effect [18]. In addition, increased N availability to the plant results in protein formation at the expense of fatty acid synthesis due to competition during carbohydrate metabolism [32]. Location impacted oil concentration with seed from the wetter environment in Brookings showing greater oil concentrations than at Pierre and decreasing less with increase in N fertilizer rate. The low oil concentration at Pierre is most likely due to overall lower precipitation and higher temperature throughout the growing season. Accelerated growth

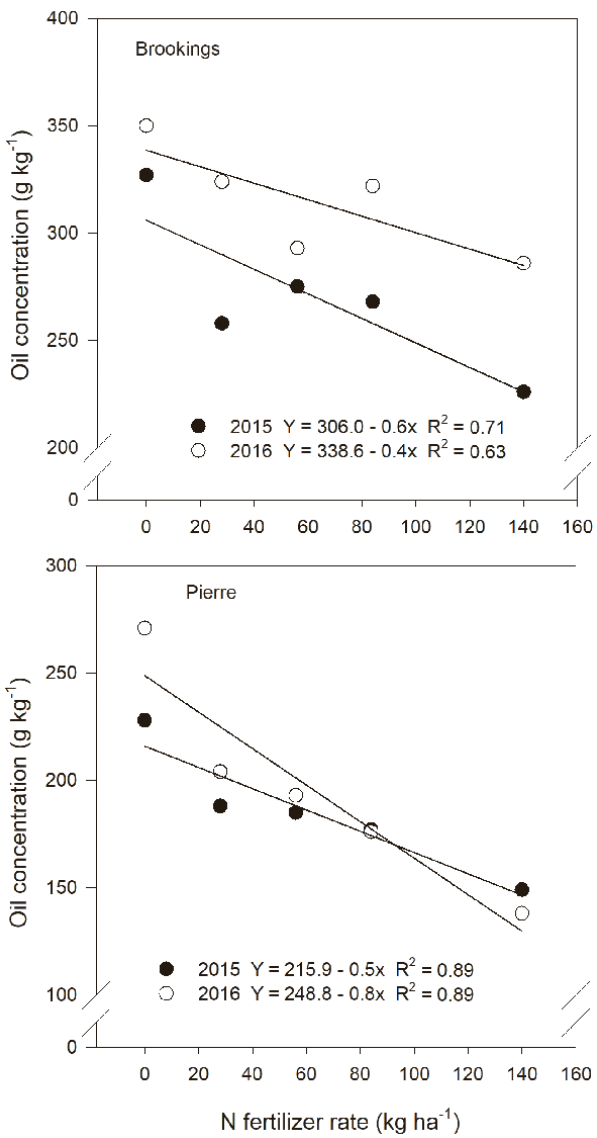

Figure 2.
Camelina seed oil concentration as a function of nitrogen fertilization rate at Brookings and Pierre, SD in 2015 and 2016.

and short growing seasons in semi-arid environments negatively impacts seed maturity and oil accumulation resulting in low seed oil concentrations [33].

Seed oil yield was significantly influenced by N rate at Pierre in 2015 and at both locations in 2016 (**Tables 4** and **5**). At Pierre in 2015, the highest N rate of 140 kg ha^{-1} had the lowest oil yield (207 kg ha^{-1}) while the greatest oil yield (268 kg ha-1) was recorded in the control treatment though this value was similar to oil yields obtained at N rates of 28 to 84 kg ha^{-1} (**Table 4**). The results were reversed in 2016 at Pierre, with the control treatment having significantly lower oil yield compared to all other N rates except the highest N rate of 140 kg ha^{-1} (**Table 5**). At Brookings on the other hand, the highest N rate of 140 kg ha^{-1} had the greatest oil yield (451 kg ha^{-1}) with this values significantly greater than the oil yield for the control treatment (345 kg ha^{-1}) but similar to intermediate N rates (**Table 5**). Oil yield was influenced by location, as the Brookings location produced greater average oil yields than the Pierre location, which resulted from greater seed oil concentration at Brookings in both years (**Tables 4** and **5**). Higher temperatures during seed filling have a negative effect on seed oil content [34, 35] explaining the lower oil yields at Pierre where temperatures were extremely high during the seed filling period.

3.3 Seeding rate effects

Plant population density increased with seeding rate at Brookings in 2016 and 2017 and at Pierre in 2016. At Brookings in 2016 and 2017, plant stand counts significantly increased with every seeding rate increase (**Tables 6** and **7**). At Pierre,

	Plant stand	Height	Pods	Seeds	Seed yield	Oil conc	Oil yield	Shatter
	plant m^{-2}	cm	plant^{1}	pod^{-1}	kg ha^{-1}	g kg^{-1}	kg ha^{-1}	%
Brookings 2016								
Seeding Rate (kg ha^{-1})								
4.5	394^{d}	84.7	77^{a}	13	1293^{a}	296	384	31.2^{a}
9	475^{c}	81.6	65^{b}	13	1160^{a}	301	345	29.4^{a}
13	638^{b}	83.2	63^{b}	11	1362^{a}	277	383	23.7^{b}
17.5	820^{a}	87.0	35^{c}	11	928^{b}	273	253	23.7^{b}
Varieties								
SO-40	584	83.7	61	12	1138	283	323	27.2
SO-50	580	84.5	59	11	1233	291	359	26.9
Pierre 2016								
Seeding Rate (kg ha^{-1})								
4.5	192^{c}	74.4^{a}	71	10	567^{b}	217	123	3.7
9	283^{b}	73.3^{a}	69	11	647^{ab}	203	131	6.2
13	339^{ab}	70.5^{b}	67	12	764^{a}	215	179	8.3
17.5	365^{a}	66.9^{c}	71	12	594^{b}	199	120	8.1
Varieties								
SO-40	296	71.3	55^{b}	11	607	192^{b}	117	5.7
SO-50	294	71.2	70^{a}	11	678	225^{a}	160	7.5

Within each column and location, means followed by the same letter are not significantly different at P< 0.05.

Table 6.
Seeding rate and camelina variety effects on plant height, pods per plant, seeds per pod, seed yield, oil concentration, oil yield and pod shatter in camelina grown at two locations in SD in 2016.

	Plant Stand	Height	Yield	Shatter
	plant m^{-2}	cm	kg ha^{-1}	%
Seeding Rate (kg ha^{-1})				
4.5	155[d]	68.3	1214	32.5[a]
9	193[c]	68.1	1249	31.2[a]
13	221[b]	66.7	1190	27.5[b]
17.5	293[a]	69.5	1265	26.9[b]
Varieties				
SO-40	216	68.3	1126[b]	30
SO-50	215	68.1	1332[a]	29.1

Within each column and location, means followed by the same letter are not significantly different at P< 0.05.

Table 7.
Seeding rate and camelina variety effects on plant height, seed yield, and pod shatter in camelina grown at Brookings, SD in 2017.

the lower two seeding rates of 4.5 and 9 kg ha^{-1} had similar plant stands with stands increasing significantly with each increase in seeding rate for the higher two seeding rates (**Table 6**). These findings agree with other reports [23, 25]. In 2016 average plant stand counts was greater in Brookings (582 plants m^{-2}) than in Pierre (295 plants m^{-2}). Plant stand counts were lowest in Brookings in 2017 (216 plant m^{-2}). The variation in stand counts between locations and years suggest emergence rates likely depend on environmental conditions and seed bed preparation which were inconsistent among environments. For example, at Brookings the study was conducted under conventional tillage making for a more favorable seedbed whereas in Pierre no till practices exposed seeds to a poorer seedbed.

Plant height was only significantly influenced by seeding rate at Pierre in 2016 likely due to lodging at the highest seeding rate of 17.5 kg ha^{-1}. Number of pods per plant and seeds per pod were measured at both locations only in 2016. At Brookings, number of pods per plant decreased with increasing seeding rate with the highest seeding rate of 17.5 kg ha^{-1} having a significantly lower number pods (35 pods per plant) compared to the lower three seeding rates. The lowest seeding rate of 4.5 kg ha^{-1} had the greatest number of pods (77 pods per plant). Similar to the current findings, Urbaniak et al. [25] reported a decrease in pods per plant with increasing seeding rate. The lack of change in pod number as seeding rate increased at Pierre is likely due to poor stand establishment resulting in lower plant populations at all seeding rates.

Seeding rate had a significant impact on seed yield at Brookings and Pierre in 2016 but seed yield was not affected by seeding rate at Brookings in 2017. At the Brookings location, the lower three seeding rates (4.5, 9 and 13 kg ha^{-1}) yielded the same and significantly greater than the highest seeding rate of 17.5 kg ha^{-1}. At a drier environment in Pierre, on the other hand, the greatest seed yield was obtained at a seeding rate of 13 kg ha^{-1} with this yield similar to the yield obtained with a seeding rate of 9 kg ha^{-1} but significantly greater than the yield obtained at the lowest seeding of 4.5 kg ha^{-1} and the highest seeding rate of 17.5 kg ha^{-1}. Other researchers have also reported inconsistent results on camelina seed yield response to seeding rate. Gesch et al. [23] reported no response to seeding rate and attributed the lack of response to plasticity of camelina and therefore the crop's ability to compensate for lower plant densities. While the current results agree with the above observation, our results further suggest this ability to compensate for lower plant densities is likely influenced by environmental conditions. We observed higher yield

compensation ability of camelina at a higher yielding environment at Brookings compared to a harsher lower yielding environment at Pierre. Urbaniak et al. [25] reported that seeding rate strongly affected plant population but that seed yield response to seeding rate was weak. McVay and Khan [24] reported that a stand count reduction of 90% at the rosette stage only reduced seed yield by 19% supporting the above assertion that seed yield response to seeding rate is weak.

Seed oil concentration and oil yield were not influenced by seeding rate. This is similar to previous reports [23, 24]. We observed that lower plant populations due to lower seeding rate had a higher rates of pod shatter compared to higher seeding rates (**Tables 6** and 7). This suggests that increasing seeding rate can result in slight decreases in pod shatter. This association is difficult to explain since earlier reports [24] suggest that stand reduction at rosette stage of camelina delays plant maturity. Therefore, increasing seeding rate would be expected to reduce days maturity, thus increasing the period where mature pods are exposed to hot and dry conditions which promote shattering. However, plants in thin stands are more exposed the elements of wind thus causing more pod shatter. Pod shatter at Brookings in 2016 was slightly higher than in 2017 due to prolonged periods of late-season rains that delayed dry-down and harvest time resulting in increased prevalence of pod shatter.

The two camelina varieties used in the study performed the same for most measured traits except for number of pods per plant and seed oil concentration at Pierre in 2016 and seed yield at Brookings in 2017. The variety SO-50 outperformed SO-40 in all the three traits that were different between the two varieties.

4. Conclusion

These results show that although considered a low-input crop, camelina can respond positively to high N rates in high yielding environments as indicated by a linear response to N rate observed at a high yielding environment in Brookings. This suggests that camelina has a potential for incorporation into both low-input as well as high-input cropping systems of the NGP. However, camelina yields were much lower in a dry year in the lower yielding environment suggesting the crop is unlikely to be economically viable in such environments. High N rates had a negative impact on seed oil concentration. The seeding rate study showed that camelina had a weak response to seeding rates supporting the theory that the crop has a great capacity to compensate for yield at low plant densities. However, we also observed the yield compensation capacity is influenced by environmental conditions. Poor stand establishment under no-till or a dry seedbed can reduce plant densities limiting the plasticity of camelina plants. This suggests that higher seeding rates may be necessary to help compensate for reduced stand establishment in drier environments under no-till.

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Conflict of interest

The authors declared no conflict of interest.

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Chapter 5

Cover Crop Residue Management for Effective Use of Mineralized Nitrogen in Greenhouse Tomato Production

Rafael A. Muchanga and Hajime Araki

Abstract

Adequate residue management may enhance the benefits of cover crops on greenhouse tomato (*Solanum lycopersicum* L.) productivity, soil N pool, N cycling, and environmental quality. Regardless of management, cover crops may maintain or increase soil N storage at 10 cm depth compared with bare fallow. Cover crops may also enhance microbial biomass N, as a result, soil N availability may increase with cover crops, except rye (*Secale cereale* L.), more so with hairy vetch (*Vicia villosa* R.; HV) incorporation than HV mulch and the biculture of HV and rye. Residual inorganic N at surface soil may increase with cover crops, more so with HV and rye monocultures than the biculture. Tomato yield may increase more with the biculture than either HV incorporation or HV mulch because of an efficient residue-N use by tomatoes. The biculture may change the N release pattern from both cover crops: rye of the biculture may release more N than the monoculture, while HV may release a similar or more N in the late than in the early period of tomato growth. With adequate seeding HV/rye ratio (2/1), biculture may maintain or increase soil N storage, increase N cycling and tomato yield, and improve environmental quality.

Keywords: soil and environmental quality, N dynamics, hairy vetch, rye, tomato yield

1. Introduction

Nitrogen (N) is the most important nutrient required by most crops, and if adequately used in cropping systems can contribute to increasing food production over the long-term, which may help sustain the growing world population. Nitrogen fertilizers have been used in amounts that often exceed the crop N requirements over the years worldwide. Nitrogen fertilizer rates in vegetable production systems worldwide can be as high as 200 to 900 kg N ha^{-1} [1, 2]. However, high N fertilization rates may result in increased soil residual inorganic N [3] that can be lost from the soil–plant system through volatilization to the atmosphere [4] or leaching to groundwater [5]. Cover cropping is an improved management practice that can help reduce N leaching to groundwater through soil N uptake and/or by reducing N fertilizer inputs into cropping systems. However, the efficiency of cover

crops in reducing N leaching varies with plant species. Nonlegumes are about three times more efficient in reducing N leaching than legumes [5] because of greater above- and belowground biomass production [3, 6]. Also, nonlegumes can establish root systems and produce dry matter under cool conditions better than legumes. On the other hand, the ability of legume cover crops in reducing N leaching is limited by the fact that legumes meet some or all of their N requirements from symbiotic N_2 fixation [5].

The sustainability of the cropping systems depends on the maintenance or improvement of soil carbon (C) and N levels. Cover crops can maintain or improve soil organic C and N levels by adding large amounts of residues that provide C and N to the soil [7, 8]. An increase in soil C and N levels may result in a range of ancillary benefits to the growing plants such as improved microbial biomass and activity and soil structure [9, 10], crop growth and yield [10–12]. Cover cropping may also help mitigate greenhouse gas emissions by sequestering atmospheric C to the soil and through improving N use efficiency by crops [13, 14]. As with N leaching, the cover crops' ability to influence soil C and N levels, crop N uptake, N use efficiency, and crop yields vary with plant species. Because of greater biomass production, nonlegumes may be more effective in improving soil C pool than legumes, while legumes with higher N concentration may be more effective in improving soil N pool and crop yields [12]. Nitrogen use efficiency from plant residues by crops including tomatoes (*Solanum lycopersicum* L.) is often less than 50% [3, 15], and part of unrecovered N amount is prone to be lost from the soil–plant system; this low N recovery is, in part, related to the nature of plant residues added to the soil. Nonlegumes decompose and release N slowly to the growing crop because of high C/N ratio, whereas legume plant residues decompose rapidly after application in the soil, releasing high N amounts in the early growth stages when the crop requires low N amounts. Thus, there is a need to adopt plant residue management practices that help increased N use efficiency by crops thereby improving N cycling, environmental quality, and crop yields. This chapter discusses cover crop use in greenhouse tomato production systems and residue management practices that optimize N use efficiency and tomato yield, reduce or limit residual N accumulation while maintaining or improving soil N storage.

2. Greenhouse tomato production systems and cover crops

Tomato is one of the most widely grown and eaten vegetables worldwide, and the second most important after potato (*Tuberosum solanum* L.) [16]. In Japan, tomato is the most important vegetable crop, and the fresh-market tomato industry is larger than that of processing tomatoes. Fresh-market tomatoes are mainly grown in greenhouses, especially plastic high tunnels, throughout the year, mainly in warm regions. In these systems, farmers cultivate tomatoes continuously and often apply high amounts of synthetic N fertilizers (>200 kg N ha^{-1}) to maximize net returns, as a result, salt accumulation and fruit injury became serious problems [17]. The increased concerns about environmental problems and the consumer's preference for vegetables produced with fewer chemicals have become the driving forces for the development of alternative and sustainable fresh-market tomato production systems. The use of cover crops, especially legumes such as hairy vetch (*Vicia villosa* R.; HV), was seen as a promising practice that could help reduce N fertilizer inputs, while maintain or improving tomato yield and soil organic matter. Hairy vetch and rye (*Secale cereale* L.) cover crops were evaluated in the field and Wagner pots under plastic high tunnel conditions in northern Japan and the results are discussed below.

2.1 Hairy vetch and rye C and N accumulation and residue decomposition

Hairy vetch is a winter-hardy cover crop that can produce large biomass and accumulate about 92.1 to 187 kg N ha^{-1} in the open-field systems and 98.1 to 301 kg N ha^{-1} in greenhouse systems (**Table 1).** Because of a C/N ratio < 25 (**Table 1**) [23], HV residues breakdown rapidly after incorporation in the soil, releasing about 40% of its total N within the first 4 weeks after incorporation of residues in the soil [24]. This rapid N release increases the likelihood of N loss from the soil–plant system when used as an N source for crop production, especially in the open-field systems [3, 25].

Rye is the coldest tolerant and the easiest to establish, the most productive and the earliest to head among temperate region nonlegume cover crops [26]. It can produce as much as 6.75 Mg ha^{-1} of aboveground biomass in the open-field systems (**Table 1**). Because of the C/N > 25 (**Table 1**) [23], rye residues breakdown slowly after application in the soil resulting in a lack of synchrony between residue-N release and crop N demand, which leads to low residue-N recovery and crop yields. Our results from a litterbag experiment under the plastic high tunnel conditions showed that only 16.5% of buried dry weight rye residues decomposed during the first 4 weeks after the initiation of the experiment, whereas, during the same period, HV showed decomposition of 59.8% of buried dry weight residues (**Figure 1**). A more significant rye decomposition was observed after the first 4 weeks, whereas by the end of the experiment, 12 weeks after burying, the percentage of the initial dry weight of rye residues that remained in the soil was 24.5%, higher than 1.2% of HV (**Figure 1**). Despite greater biomass production, rye adds fewer N amounts to the soil compared with HV [6, 11] because of low N accumulation. In some cases, the application of rye residues (C/N ratio > 25) depletes soil N availability and decreases crop yields because of N immobilization by soil microbes [11, 25].

Alternatively, the biculture of HV and rye, with an intermediate C/N ratio between HV and rye (**Table 1**), may show a moderate decomposition speed that may result in an increased soil N availability and residue-N recovery by tomatoes. This assumption is supported by the results from a litterbag assay under the open-field conditions of Chinta et al. [27], who reported, with few exceptions, an intermediate decomposition level of residues of the biculture of HV and rye between pure HV (higher) and rye (lower) residues. Also, because of greater seeding rates, biculture of HV and rye may accumulate greater biomass and add more C to the soil than HV, and more N than rye monoculture [6, 11], thereby influencing soil C and N dynamics more significantly.

2.2 Effects of cover crop residue management on tomato yield, N uptake, and cover crop N recovery

Because of high N accumulation and low C/N ratio, HV adds more N to the soil and enhances N uptake and the yield of the subsequent crop better than nonlegumes or bare fallow [8, 11]. Cover crop treatments showed a 13.9 to 32.7% greater marketable yield than the bare treatment in 2017 (**Table 2**). The biculture of HV and rye (HV + RYE) showed the highest marketable yield. Similarly, the cover crop treatments showed 31.5 to 68.3% greater shoot biomass and N uptake compared with the bare treatment. Greater marketable yield with the biculture than with HV incorporation (HVI), although a similar total N uptake and residue-N recovered, may be explained by more efficient use of cover crop N by tomatoes throughout the growing period. Biculture with a moderate decomposition speed (C/N = 17.6 [22]) may have released more N during the period of high N demand, while HVI with fast

Cover crop	DW Biomass (Mg ha^{-1})	C applied (kg ha^{-1})	N applied (kg ha^{-1})	C/N ratio	Examination period	Reference
Open-field systems						
Hairy vetch	4.97	2107	159	13.3	1989	Clark et al. [11]
	4.80	2112	187	11.4	1996–1997	Sainju et al. [12]
	4.23	1688	136	12.4	2000–2002	Sainju et al. [6]
	2.19	1049	92.1	11.4	1999	Horimoto et al. [18]
	—	1482	123	10	1992–1993	Kuo et al. [8]
Rye	6.75	3737	74.0	50.5	1989	Clark et al. [11]
	—	1630	47.5	35.0	1992–1993	Kuo et al. [8]
	6.37	2954	107	27.7	1996–1997	Sainju et al. [12]
	4.05	1795	41.6	43.1	2000–2002	Sainju et al. [6]
Hairy vetch/rye	6.63	2822	193	14.6	2000–2002	Sainju et al. [6]
	7.96	3956	168	23.6	1989	Clark et al. [11]
Greenhouse systems						
Hairy vetch	4.71	2050	203	10.1	2007–2012	Araki [19]
	6.52	2693	281	9.60	2017	Muchanga et al. [20]
	4.80	2037	193	10.6	2016–2017	Muchanga et al. [21]
	6.78	3018	301	10.0	2017	Muchanga et al. [22]
	2.46[z]	1003	98.1	10.2	2018	Muchanga et al. [22]
Rye	6.19	2639	43.1	61.3	2018	Muchanga et al. [22]
Hairy vetch/rye	8.02	3465	197	17.6	2017	Muchanga et al. [22]
	6.55	2777	117	23.7	2018	Muchanga et al. [22]

[z]Low biomass resulted from a late planting of hairy vetch (planted on 25 April 2018) due to the low survival rate of the previous hairy vetch planting (planted on 21 September 2017) caused by a high snowpack (>1 m) and a long period of snow cover.
—Aboveground biomass was not reported by the authors.

Table 1.
The dry weight (DW) biomass, carbon (C) and nitrogen (N) accumulation, and C/N ratio of cover crops grown in the open-field and greenhouse systems.

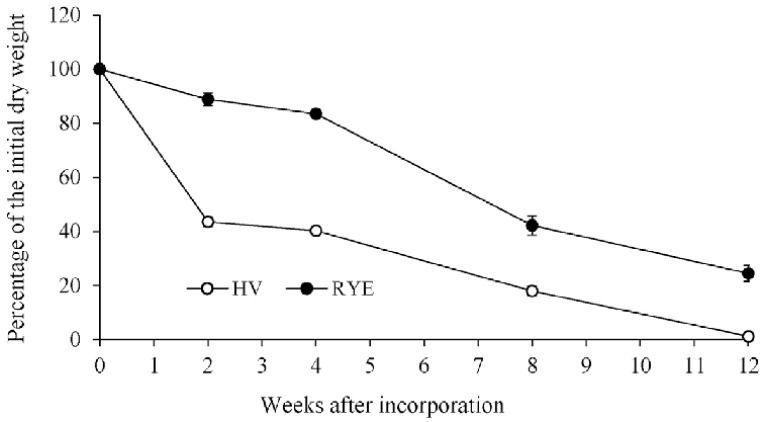

Figure 1.
Decomposition of 5 g dry weight of hairy vetch (HV) and RYE (RYE) residues buried at 10 cm soil depth in tomato plots during 12 weeks under the plastic high tunnel in 2018. Vertical bars represent standard errors (n = 3); only shown when larger than the symbols.

Treatments[z]	Marketable yield		Dry weight shoot biomass		Total N uptake[y]		Recovered cover crop N[x] Total		Percentage of N input
	Mg ha^{-1}		Mg ha^{-1}		kg N ha^{-1}		kg N ha^{-1}		
2017									
BARE	101	c[w]	4.89	c	186	c	—		—
HVI	116	b	7.10	a	313	a	127	a	42.0
HVM	115	b	6.43	b	267	b	81	b	27.0
HV+RYE	134	a	7.47	a	312	a	126	a	63.9
2018									
BARE	60.1	b	4.37	ab	184	b	—		—
HVI	84.3	a	5.96	a	233	a	49.0	a	50.7
HVM	64.8	b	4.98	ab	195	b	11.0	b	11.4
RYE	49.8	c	3.64	b	153	c	-31	—	-71.8
HV+RYE	60.7	b	4.81	ab	188	b	4.0	c	3.42

[z]BARE, no cover crop but fertilized with 150 kg N ha^{-1}; HVI, hairy vetch incorporation; HVM, hairy vetch mulch; RYE, rye monoculture; HV+RYE, biculture of hairy vetch and rye. All cover crop treatments received the controlled-release N fertilizer at a rate of 150 kg N ha^{-1}.
[y]Nitrogen uptake from the soil and cover crops = shoot N content × shoot dry weight biomass + fruit N content × fruit total dry weight.
[x]Calculated as the ratio of total N recovered from cover crops (N uptake in cover crop – N uptake in BARE) to the cover crop N input. Cover crop N input in HVI, HVM, and HV+RYE was 302, 300, and 197 kg N ha^{-1} in 2017, and 99.3, 96.8, and 117 kg N ha^{-1} in 2018, respectively. Rye residues (RYE) added to the soil 43.1 kg N ha^{-1} in 2018.
[w]Means followed by the same letters in each column and year are not significantly different at 5% by Tukey's honestly significant difference test.

Table 2.
Effects of cover crop residue management on tomato marketable yield, shoot biomass, N uptake and recovery in 2017 and 2018 (Muchanga et al. [22]).

decomposition speed (C/N = 10.2), may have released more N in the early period of tomato growth [24], a period of low N demand. This assumption is supported by a lower growth index (GI = plant length × stem diameter × number of expanded leaves [28]) in the early period of tomato growth (5 and 7 weeks after transplanting; WAT) with the biculture than with HVI (**Table 3**). However, the rate of increase of GI from 7 to 9 WAT was higher with the biculture (82.9%) than with HVI (68.9%), which resulted in a similar GI of the biculture to HVI at 9 WAT. The results of Sugihara et al. [24] who found more HV-derived N concentration in the 1st and 2nd tomato fruit clusters than in upper fruit clusters, also supported the assumption that HV incorporation released more N in the early period of tomato growth, which favors more shoot biomass accumulation rather than fruit set and enlargement.

As opposed to 2017, the marketable yield, shoot biomass, and total N uptake increased with HVI only, compared with the bare treatment, in 2018. The biculture and HV mulch (HVM) showed similar marketable yield, shoot biomass, and total N uptake to the bare treatment, whereas rye treatment (RYE) showed adverse effects on tomato yield, shoot biomass, and total N uptake due to N immobilization (**Table 2**). The adverse effect of rye residues resulted from their C/N ratio > 25 [23]. Soil microbes require N for their growth and if residues cannot meet their N requirements, microbes immobilize soil inorganic N, resulting in soil inorganic N depletion. Sainju et al. [12] reported 27.2 and 28.9% lower tomato yield and plant biomass, respectively, in rye than in bare plots without N fertilization in 1997. Likewise, Clark et al. [11] reported a decrease in corn grain yield by 32.7% in rye plots compared with no cover crop plots. The increased effectiveness of the biculture on marketable yield observed in 2017 was not repeated in 2018. Biculture showed no effect on shoot biomass, tomato yield, and N uptake possibly because of low residue-N recovery in 2018. Tomatoes utilized only 3.42% of the biculture N amount applied (N applied by residues was 117 kg N ha^{-1} [22]) (**Table 2**). This ineffectiveness of the biculture may be explained by the higher C/N ratio of residues in 2018 (23.7) than in 2017 (17.6) [22]. This result highlights the importance of the C/N ratio in controlling the plant residue N release, so the decision on seeding rates HV/rye should be based on the expected C/N ratio of biculture residues. Greater tomato yield with HV than with bare fallow was reported by several researchers [10, 12]. Araki et al. [17] reported greater tomato yield and plant growth

Treatments[z]	Growth index[y]						
	WAT						
	3	5		7		9	
BARE	4031	12,474	b[x]	29,185	c	46,074	c
HVI	4344	15,801	a	34,578	a	58,406	a
HVM	4112	12,634	b	32,577	b	55,906	b
HV + RYE	3967	13,316	b	32,173	b	58,846	a
Significance	NS						

[z]BARE, no cover crop but fertilized with 150 kg N ha^{-1}; HVI, hairy vetch incorporation; HVM, hairy vetch mulch; HV+RYE, biculture of hairy vetch and rye. All cover crop treatments received the controlled-release N fertilizer at a rate of 150 kg N ha^{-1}.
[y]GI = Plant length (cm) × stem diameter (mm) × number of expanded leaves. WAT, weeks after transplanting.
[x]Means followed by the same letters in each column are not significantly different at 5% by Tukey's honestly significant difference test.
NS, not significant.

Table 3.
Tomato growth index (GI) as influenced by cover crop residue management in 2017.

with HV mulch than with bare fallow. Likewise, Muchanga et al. [21] reported greater marketable and total yields and shoot biomass with HV incorporation than with the bare fallow.

2.3 Effects of cover crop residue management on soil N pool

2.3.1 Soil microbial biomass N and N availability

The quantity, quality, and management of cover crop residues may influence N dynamics and storage in the soil, which affects crop yield and environmental quality. Regardless of residue management (residue placement or the mixing of legumes and nonlegumes residues), the addition of cover crops residues to the soil increased significantly soil microbial biomass N (MBN) levels at 0–10 cm bulk soil by 25.4 to 121% at 4 and 8 WAT in 2017, and by 26.8 to 187% at 2 and 8 WAT in 2018, compared with the bare treatment (no cover crop but fertilized with 150 kg N ha^{-1}) (**Figure 2**). In both years, HV incorporation showed the highest increment of MBN, whereas, despite a similar N input [22], HV mulch showed the least increment of MBN. This fact points out that the placement of residues in the soil may determine the cover crop N mineralization and contribution to the growing crop. The increase in MBN with cover crops than with no cover crop resulted from greater C and N inputs, especially N because it is the most limiting nutrient for microbial growth [29, 30]. However, with high levels of soil inorganic N, even residues with low N content, such as rye residues, may increase MBN levels in the early period after residue application because if plant residues do not satisfy microbes N requirements, microbes obtain N from the soil [29].

Soil N availability (soil inorganic N during the crop growing period) varied significantly ($P < 0.05$) with treatments at 0–10 cm depth at 2, 8, and 16 WAT in 2017 and 2018 (**Figure 3**). Averaged across sampling dates, soil N availability followed these orders: HVI > HV + RYE > BARE > HVM in 2017, and HVI = HVM > HV + RYE > BARE > RYE in 2018. The decrease in soil N availability in HVM in 2017 may be the result of slow N mineralization of mulch residues compared with incorporated residues [31], and higher N uptake compared with the bare treatment (**Table 2**). On the other hand, the decrease in soil N availability in RYE in 2018 resulted from N immobilization due to the C/N ratio > 25 [23]. Therefore, even in greenhouse production systems where water is applied regularly and the soil temperature is relatively higher compared to that of the open-field systems, cover crops such as rye, with a high C/N ratio, may have little or no N contribution to the growing crop, so biculture of HV and rye may be a better management option. It's noteworthy that the effectiveness of biculture in increasing MBN and soil N availability diminished from 2017 to 2018 as a result of the increase in the C/N ratio of residues. Therefore, the use of adequate seeding HV/rye rates is crucial to maximizing residue-N utilization by the growing crop.

2.3.2 Soil total N

Increasing or maintaining soil N levels is vital for sustaining soil quality, crop growth and yield [8]. Soil N storage may vary with cover crop species due to differences in biomass production and N accumulation [32], and residue management. In 2017, HV incorporation increased significantly soil total N (STN) by 11.3% at 0–10 cm depth and 8.14% at 10–30 cm depth, compared with the bare treatment (**Table 4**). The biculture showed a 1.76% increase in STN compared with bare treatment at 10–30 cm depth only. In contrast, HVM showed no or negative effect on STN in 2017. In 2018, all cover crop treatments increased STN by 10.1% to 12.6%

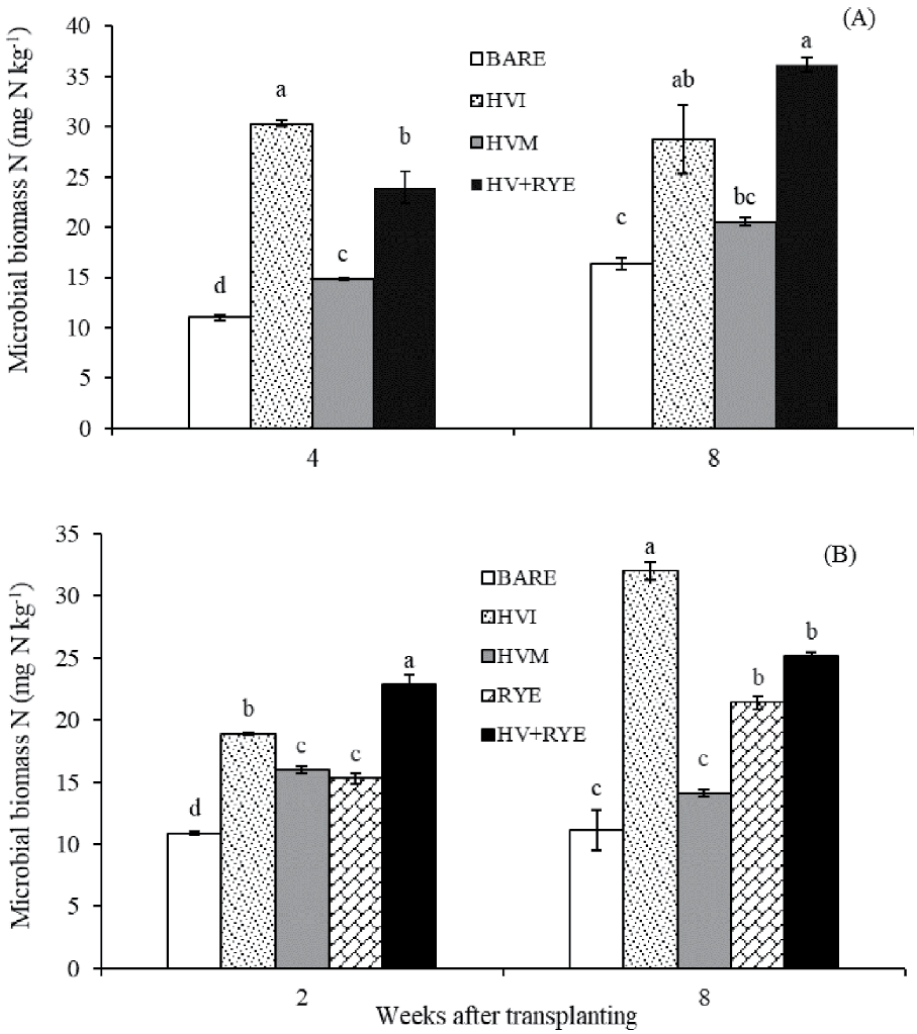

Figure 2.
Effects of cover crop residue management on microbial biomass nitrogen at surface 10 cm bulk soil depth during the period of tomato cultivation in (A) 2017 and (B) 2018. Vertical bars represent standard errors (n = 3). Means followed by the same letters on each sampling date and year are not significantly different at 5% by Tukey's honestly significant difference test (Muchanga et al. [22]).

at 0–10 cm depth only, compared with no cover crop treatment. Greater STN with cover crops than without cover crops observed mostly in 2018 agreed with the results of Kuo et al. [8] and Sainju et al. [12] and may be the result of greater C and N inputs by cover crop residues. In both years, HVI was the most effective treatment in increasing STN. The positive effects of HVI on STN shown in **Table 4** agreed with the results of our previous study [21] where the effects of HV (incorporation) were compared to those of livestock compost. In that study, HV incorporation showed 7.29% greater STN stock than the bare treatment (no HV and compost) and a 17.3% increase of STN stock compared with the baseline stock (initial STN stock measured before any treatment application). However, HV was not as effective as the livestock compost in building up STN stock.

The cover crops and bare treatments shown in the **Table 4** were fertilized with the controlled-release N fertilizer at a rate of 150 kg N ha^{-1} to sustain crop growth when the cover crop N supply ceases or reduces. This N fertilizer follows a sigmoidal pattern (S-type), releasing 80% of its total N slowly for 70-day after a lag period

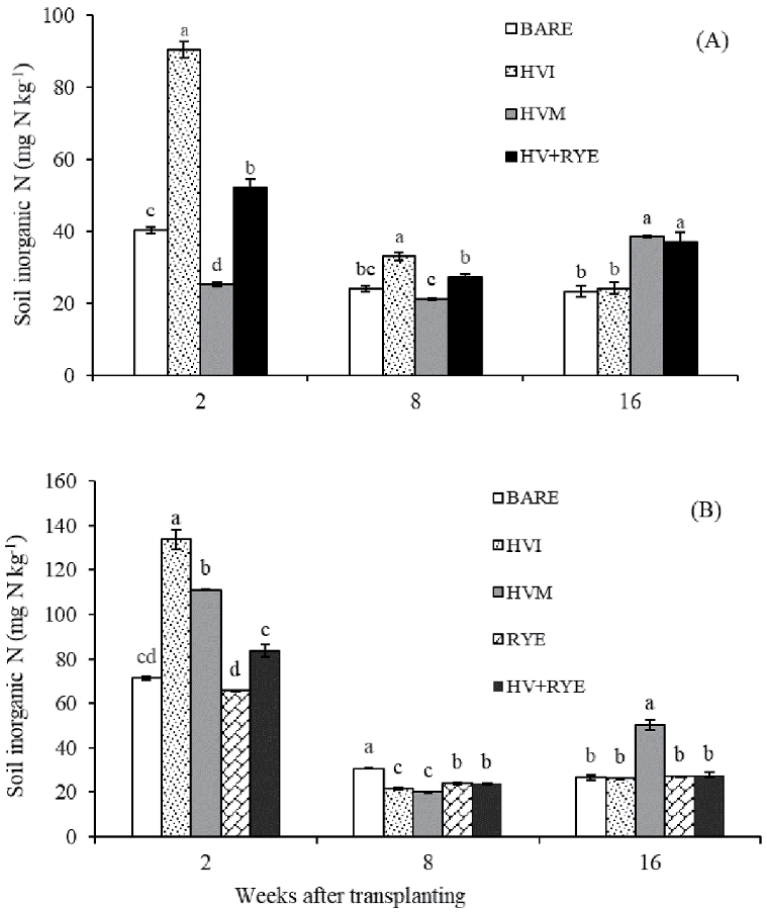

Figure 3.
Effects of cover crop residue management on soil inorganic nitrogen ($NO_3^- - N + NH_4^+ - N$) at surface 10 cm soil depth during the period of tomato cultivation in (A) 2017 and (B) 2018. Vertical bars represent standard errors (n = 3). Means followed by the same letters on each sampling date and year are not significantly different at 5% by Tukey's honestly significant difference test (Muchanga et al. [22]).

of 30-day in the soil at 25° C (LPS100, 41%N; JCAM AGRI, Tokyo, Japan). Because of its N release pattern, it was included in our experiments for developing low N input systems. The N fertilizer effects on soil C and N were examined in 2016 under plastic high tunnel conditions (**Table 5**). The controlled-release N fertilizer showed no effect on soil organic C (SOC) and STN at 0–10 cm depth, but HV × fertilizer interaction was significant for SOC. The N fertilizer enhanced the positive effects of HV mulch on SOC, whereas it diminished the effectiveness of HV incorporation in increasing SOC (**Table 5**). As opposed to fast-release N fertilizers that were reported to decrease SOC and STN [33, 34], this controlled-release N fertilizer may be used safely in greenhouse tomato production systems, preferably applying N amounts ≤ 150 kg N ha^{-1}.

2.3.3 Soil residual N

Soil residual inorganic N may represent 50 to 80% of total fertilizer N applied to crops, more so in vegetable than field crop systems because of higher N inputs and lower N recovery of vegetable crops including tomatoes [35]. Thus, the N loss potential from the soil–plant system through volatilization and/or leaching is higher in vegetable systems. Because temperate region soils are negatively charged

Treatments[z]	STN				Change[y]	
	0–10 cm		10–30 cm		0–10 cm	10–30 cm
	Mg N ha^{-1}				%	
2017						
BARE	3.10	b[x]	7.37	bc	—	—
HVI	3.45	a	7.97	a	11.3	8.14
HVM	3.20	b	7.25	c	—	–1.63
HV + RYE	3.22	b	7.50	b	—	1.76
2018						
BARE	2.47	b	5.94		—	—
HVI	2.78	a	5.99		12.6	—
HVM	2.73	a	5.92		10.5	—
RYE	2.75	a	5.92		11.3	—
HV + RYE	2.72	a	5.80		10.1	—
Significance			NS			

[z]BARE, no cover crop but fertilized with 150 kg N ha^{-1}; HVI, hairy vetch incorporation; HVM, hairy vetch mulch; RYE, rye monoculture; HV+RYE, biculture of hairy vetch and rye. All cover crop treatments received the controlled-release N fertilizer at a rate of 150 kg N ha^{-1}.
[y]Change (%) = (STN in cover crop – STN in BARE)/STN in BARE × 100
[x]Means followed by the same letters in each column and year are not significantly different at 5% by Tukey's honestly significant difference test. NS, not significant.

Table 4.
Soil total nitrogen (STN) at surface 0–30 cm depth as influenced by cover crop residue management in 2017 and 2018 (Muchanga et al. [22]).

Treatments[z]	Soil N				Soil organic C	
	Inorganic		Total			
	mg N kg^{-1}		g kg^{-1}			
BARE-NF	15.8	b[y]	2.39	b	29.2	b
BARE-F	19.9	b	2.18	b	29.5	b
HVI-NF	15.5	b	2.67	a	32.3	a
HVI-F	43.6	a	2.59	a	30.4	ab
HVM-NF	21.6	b	2.52	a	30.8	ab
HVM-F	50.1	a	2.68	a	31.8	a
Hairy vetch (HV)	***		*		***	
Fertilizer (F)	***		NS		NS	
HV × F	**		NS		***	

[z]BARE-NF, no cover crop and no N fertilization; BARE-F, no cover crop with N fertilization; HVI-NF, hairy vetch incorporation without N fertilization; HVI-F, hairy vetch incorporation with N fertilization; HVM-NF, hairy vetch mulch without N fertilization; HVM-F, hairy vetch mulch with N fertilization. Controlled-release N fertilizer applied at a rate of 150 kg N ha^{-1}.
*[y]Means followed by the same letters in each column are not significantly different at 5% by Tukey's honestly significant difference test. NS, *, **, ***Not significant or significant at $P < 0.05$, 0.01, and 0.001, respectively.*

Table 5.
Effects of hairy vetch residue management and nitrogen fertilization on soil carbon (C) and nitrogen (N) at 0–10 cm depth in 2016.

retaining most of NH_4^+ −N [32], NO_3^- −N leaching is more important, especially after winter when a large amount of snow melts. The use of cover crops may reduce N fertilization rates and thereby reducing N leaching potential. Moreover, the management of cover crop residues may affect the decomposition speed of the residues thereby influencing crop N uptake and residual inorganic N. Generally, the decomposition speed of residues that are incorporated in the soil is faster than that of residues that are placed on the soil surface [29, 31], therefore the net N release of surface-placed residues is delayed [36].

The effects of cover crop residue management on soil residual N (soil inorganic N levels after tomato harvest) in tomato production in a Gleysol were assessed in 2017 and 2018 in northern Japan. Because of greater N input and slow or fast decomposition speed, HVM and HVI showed 25.2 to 386% greater NO_3^- −N and soil inorganic N (SIN) at 0–10 cm depth in 2017 (**Table 6**). On the other hand, despite a higher N input (biculture residues added 117 kg N ha^{-1} to the soil [22]), the biculture showed NO_3^- −N and SIN levels similar to those of the bare treatment. Least NO_3^- −N and SIN levels with the biculture may be the result of higher residue-N recovery by tomatoes (**Table 2**) possibly due to a moderate decomposition speed of residues.

As opposed to 2017, NO_3^- −N, NH_4^+ − N, and SIN levels increased by 54.2 to 218% with all cover crop treatments compared with the bare treatment in 2018. In both years, HVM showed the highest NO_3^- −N and SIN levels suggesting its use in greenhouse tomato production systems may represent a high risk of N leaching to groundwater after winter. As opposed to 2017, the fact the biculture increased NO_3^- −N and SIN levels in 2018 may be explained by the increase of its C/N ratio to 23.7, which resulted in low N recovery due to slow residue decomposition [37].

Treatments[z]	NO_3^-−N		NH_4^+ − N		SIN		Percentage of SIN-derived from cover crop N input[y]
	kg N ha^{-1}						%
2017							
BARE	4.59	c[x]	23.0	b	27.6	c	—
HVI	9.14	b	25.4	b	34.5	b	2.30
HVM	22.3	a	32.0	a	54.3	a	8.92
HV + RYE	4.11	c	22.8	b	26.9	c	−0.91
2018							
BARE	20.8	d	5.7	c	26.4	d	—
HVI	35.7	c	11.4	a	47.1	c	20.8
HVM	73.9	a	10.2	ab	84.1	a	59.5
RYE	47.5	b	12.7	a	60.2	b	78.3
HV + RYE	32.0	c	10.0	ab	42.0	c	13.3

[z]BARE, no cover crop but fertilized with 150 kg N ha^{-1}; HVI, hairy vetch incorporation; HVM, hairy vetch mulch; RYE, rye monoculture; HV+RYE, biculture of hairy vetch and rye. All cover crop treatments received the controlled-release N fertilizer at a rate of 150 kg N ha^{-1}.
[y]%SINdfCCNinput = (SIN in cover crop – SIN in BARE)/Cover crop N input ×100. Cover crop N input in HVI, HVM, and HV+RYE was 302, 300, and 197 kg N ha^{-1} in 2017, and 99.3, 96.8, and 117 kg N ha^{-1} in 2018, respectively. Rye N input was 43.1 kg N ha^{-1}.
[x]Means followed by the same letters in each column and year are not significantly different at 5% by Tukey's honestly significant difference test.

Table 6.
Soil inorganic nitrogen (SIN; NO_3^-−N + NH_4^+ − N) after tomato harvest at surface 10 cm depth as influenced by cover crop residue management in 2017 and 2018 (Muchanga et al. [22]).

In 2017, the proportion of the seeding rates of HV/rye was 2/1 (20 kg ha^{-1} HV/10 kg ha^{-1} rye), while in 2018 was 1/1 (50 kg ha^{-1} HV/50 kg ha^{-1} rye). The reasons for using different seeding rates among the years were explained by Muchanga et al. [22]. Thus, seeding rates of HV/rye in a proportion of 2/1 (in kg ha^{-1}) that may lead to a C/N ratio of about 17.6 are recommended. The percentage of SIN-derived from cover crop N input (%SINdfCCN$_{input}$) was lower in 2017 than in 2018 (**Table 6**), suggesting that more residue-N applied in 2017 was utilized by plants than in 2018. Hairy vetch mulch and rye showed higher %SINdfCCN$_{input}$ than other treatments in 2018, suggesting that more residue-N was released in the late period, more so from rye than HV mulch.

The data from **Table 5** suggests that the increase in residual SIN in HVM and HVI plots is more related to the addition of the controlled-release N fertilizer, so the rate of 150 kg N ha^{-1} added to HVI and HVM plots may be excessive. A further study determining a better N fertilization rate for HVI and HVM that leads to a high yield, increased soil N storage, and least residual SIN is needed.

2.4 Rye-derived nitrogen dynamics in the soil-tomato system in a greenhouse using ^{15}N tracer technique

Since neither HV nor rye can simultaneously provide N and enhance tomato yield, increase soil N storage, and reduce N leaching through residual N uptake, the use of the biculture of HV and rye in tomato production is seen as a promising practice that can provide most of the benefits [32]. The positive effects of the biculture on tomato yield and residue-N recovery observed in 2017 discussed in the previous section suggest that residue management may improve the N contribution of cover crops to tomato production, and more importantly, the N release pattern of HV and rye may change when applied together in the soil. While dynamics of N derived from HV in the soil−tomato system have been studied [24], studies on dynamics of N derived from rye in the soil−crop system are limited, possibly due to detrimental effects of rye on crop yields. A ^{15}N tracer examination was conducted in Wagner pots in a plastic high tunnel in northern Japan to understand the reasons for the high effectiveness of the biculture in increasing residue-N recovery and tomato yield by examining the uptake and recovery, and retention in the soil of N derived from rye residues applied as a monoculture and biculture with HV. The major findings are discussed below.

2.4.1 Rye-derived nitrogen accumulation in tomato, uptake and recovery

Because of higher rye-derived N input, tomatoes (shoot + fruit) in the rye treatment (N input was 1943 mg N/plant [38]) accumulated more rye-derived N than those in the biculture treatment (N input was 972 mg N/plant) on all sampling dates (**Figure 4A**). Rye-derived N accumulation in both treatments increased from 2 to 8 WAT and then decreased afterward. This decline of rye-derived N accumulation indicates the cessation of N uptake (determined as the positive difference between rye-derived N accumulation of a given week and the preceding week). The cessation of N uptake by tomato shoot and fruit or the decline in N accumulation after 8 WAT may be the result of N partitioning to roots, a process that occurs when soil inorganic N is least [39, 40]. Rye-derived N uptake by tomatoes was 70.2% and 75.5% greater with rye than with the biculture at 0–2 and 4–8 WAT, respectively (**Table 7**). Rye is often regarded as a delayed-N release, so the fact that rye treatment released a high amount of N at 0–2 WAT may be explained by a C/N ratio < 25 (C/N ratio = 17.4) [23], which is lower than a normal range (25.3 to 66.9) reported by several researchers [6, 8, 10, 11]. This lower C/N ratio resulted from N

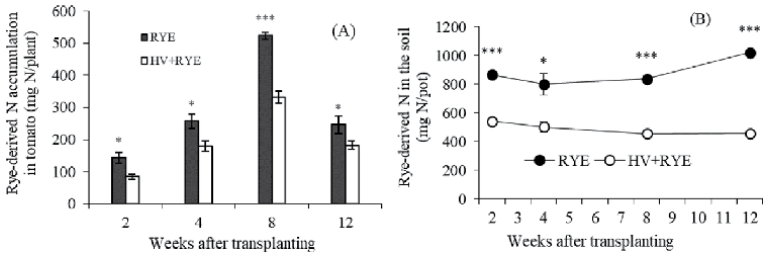

Figure 4.
*Influence of residue management on rye-derived nitrogen accumulation in tomato shoot and fruit (a) and retained in the soil (B). Vertical bars represent standard errors (n = 3); only shown when larger than the symbols. *, ***significant at* $P < 0.05$, *and 0.001, respectively (Muchanga et al. [38]).*

		Rye-derived N							
Treatments[z]	**Input in the soil (mg N/pot)**	**Uptake[y] (mg N/plant)**				**Recovery[x] (%)**			
		WAT			**Total**	**WAT**			**Total**
		0–2	2–4	4–8		0–2	2–4	4–8	
RYE	1943	144	114	265	523	7.39	5.87	13.7	26.9
HV + RYE	972	84.3	94.9	151	331	8.67	9.77	15.6	34.0
t-test	***	**	NS	***	***	**	NS	***	***

[z]RYE, 15N-labeled rye residues; HV+RYE, biculture of hairy vetch and 15N-labeled rye residues.
[y]Determined as the positive difference of rye-derived N accumulation (Figure 4A) between a given week and the preceding week.
[x]Rye-derived N recovery (%) = rye-derived N uptake/ rye-derived N input × 100.
*NS, **, *** Not significant or significant at P < 0.01, and 0.001, respectively.*

Table 7.
Rye-derived nitrogen input in the pot, and its uptake and recovery by tomatoes (shoot + fruit) as influenced by residue management in 2018 (Muchanga et al. [38]).

fertilization with $^{15}NH_4Cl$ and soil N uptake (rye grew in soil with the initial NO_3^-—N concentration of 65.2 mg N kg^{-1} [38]).

As opposed to rye-derived N uptake, the biculture showed a total rye-derived N recovery of 34%, higher than 26.9% of rye treatment (**Table 7**). This result suggests that biculture of HV and rye may be an effective management practice to increase the N contribution from rye to tomato growth and fruit production. Consequently, if more N from residues is used by tomatoes, less supplemental N fertilizer may be needed, and the environmental problems associated with high N fertilization rates may be avoided. The biculture may provide many benefits such as enhancing tomato yield and residue-N recovery better than HV and rye monocultures and reduce residual N better than HV [41]. Several researchers [3, 25] have reported increased residual nitrate levels and leaching to groundwater with HV than with no cover crop, more so when high N fertilization amounts were added.

Although the N contribution from rye to tomato growth and fruit production increased when applied with HV to the soil, HV in the biculture may play a major role by contributing more N than rye in the early (0–4 WAT) and late periods (4–8 WAT) of tomato cultivation (**Table 8**). Hairy vetch and rye N contributions from 0 to 8 WAT represented 25.3% and 17.3% of the total N uptake (1914 mg N/plant [38]), respectively. Sugihara et al. [24] reported N recovery by tomatoes (shoot + fruit) of 40.3% by 4 WAT and an additional 15% at 4–10 WAT (HV-derived N uptake ceased at 10 WAT). In this study, HV contributed 9.65% more N to shoot growth and fruit production at 4–8 WAT than at 0–4 WAT, suggesting that the biculture may change the N release pattern from both cover crops: rye may release

Component of the biculture	N uptake from cover crops (mg N/plant)			Percentage of total N uptake[z]
	WAT		Total	
	0–4	4–8		
Hairy vetch	231	253	484	25.3
Rye	179	151	331	17.3
t − test	*	**	***	—

[z]*Calculated as the ratio of the total N uptake from hairy vetch or rye to the total N uptake of the biculture (1914 mg N/plant [38]).*
**, **, ***Significant at $P < 0.05$, 0.01, and 0.001, respectively.*

Table 8.
Nitrogen uptake by tomatoes (shoot + fruit) from each cover crop of the biculture (Muchanga et al. [35]).

more N when mixed with HV, in turn, HV may release a similar or more N amount after 4 weeks following the transplanting than before that period.

2.4.2 Retention in the soil of nitrogen derived from rye

Residue management and the quantity of residues applied to the soil [7, 8] may influence the amount of N retained by the soil. Rye-derived N retained in the soil was markedly higher with rye monoculture than with the biculture on all sampling dates (**Figure 4B**). The rye treatment showed an increasing trend of rye-derived N levels in the soil from 2 to 12 WAT, more so at 8 to 12 WAT. In contrast, the biculture showed a decreasing trend of rye-derived N levels in the soil from 2 to 12 WAT. Greater rye-derived N retention with rye than with the biculture treatment may be the result of higher rye-derived N input in rye treatment [(1943 mg N/plant) (**Table 7**)] than in biculture (972 mg N/plant), and also lower N mineralization rate of rye residues in RYE treatment than in HV + RYE treatment. The decreasing trend of rye-derived N in the soil with biculture suggests that slow decomposition of residues may be advantageous over fast decomposition in building up STN.

By 12 WAT, the amount of rye-derived N retained in the soil represented 52.5% and 47.0% of the total rye-derived N input in rye monoculture and biculture, respectively. The ability of the soil to retain plant-derived N is stronger than the ability of different loss mechanisms to remove it [15]. Thus, the lower rye-derived N recovery observed in both treatments resulted from increased soil N retention. Rye-derived N retained in the roots and/or lost (difference of rye-derived N input and rye-derived N uptake and retained in the soil) was estimated at 20.6% and 19.0% in rye monoculture and biculture treatments, respectively.

3. Conclusion

Adequate cover crop residue management may help increase N contribution from residues to tomato production, thereby enhancing tomato yield, reduce N fertilization rates, and maintain or improve soil and environmental quality. Regardless of residue management cover crops may maintain or increase soil N storage at surface 10 cm soil depth. Residual soil inorganic N at surface 10 cm soil depth, subject to leaching losses after tomato harvest, may increase with cover crops, more so with hairy vetch (incorporation and mulch) and rye monocultures than the biculture of hairy vetch and rye because of rapid or delayed N release.

With adequate seeding hairy vetch/rye ratio (2/1), the biculture may be a better management practice to increase tomato yield and residue-N recovery, and maintain or increase soil N storage at surface 30 cm depth with no or least residual N. Biculture may promote efficient use of residue-N by tomatoes by releasing high amount of N in both the early and late periods of tomato growth. Biculture may change the N release pattern of both hairy vetch and rye residues: hairy vetch may release a similar or more N in the late (reproductive growth stage) than in the early period (vegetative growth stage) of tomato growth, while rye may release more N when applied with than without hairy vetch.

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Section 3

N Utilization and Metabolism in Crops

Chapter 6

Nitrogen Fixation in Soybean Nodules Affects Seed Protein and Oil Contents: The Suggested Mechanism from the Coordinated Changes of Seed Chemical Compositions and Phosphoenolpyruvate Carboxylase Activity Caused by Different Types of Nitrogen Fertilizer

Toshio Sugimoto, Naoki Yamamoto and Takehiro Masumura

Abstract

The contents of seed storage compounds, protein and oil, determine the best use of soybean seeds, namely materials for food processing and oil production. Genetic and environmental factors could affect the chemical compositions of soybean seeds. However, the mechanisms of how the accumulation of these primary seed compounds is regulated are mostly unclear. In this chapter, we describe the different effects of nodulation on the protein and oil contents in soybean seeds and the crucial role of phospho*enol*pyruvate carboxylase (PEPC) in the protein accumulation of soybean seeds. Based on our previous studies on soybean seeds, we introduce five manners deduced; (1) protein accumulation is independent of oil accumulation, (2) nitrogen fixation results in decreasing oil amount per seed and decreased seed oil content, (3) a high pseudo negative correlation between protein and oil contents in seeds is likely to be observed under less nitrogen supply from the soil, (4) nitrogen absorbed from soil during the late growth stage promote seed production, (5) plant-type PEPC, ex. Gmppc2 in soybean could play a role in amino acid biosynthesis for storage protein accumulation in seeds during the late maturation period.

Keywords: carbon metabolism in immature seeds, Gmppc2, plant-type, principal component analysis, role of PEPC, seed yield, slow-release N fertilizer

1. Introduction

Soybean seeds contain about 40% protein, 20% oil, 35% carbohydrate, and 5% minerals on a weight basis [1]. Contents of these compounds vary among cultivars

(CVs) and environments of plant growth. Seeds having higher protein content are preferable for food material, and which is called food bean. On the other hand, those having higher oil content are for vegetable oil production, and which is called oil bean. Germplasm stock of USDA exhibits a protein concentration from no less than 35% to more than 50% with an oil concentration of 7% to 28% [2]. Contents of these storage compounds were negatively correlated with each other, implying competition of the synthesis of these storage compounds during the seed maturation period. For the production of soybean seeds to have better quality, it was necessary to clarify the mechanism of how these storage compounds contents were genetically controlled and affected by environmental conditions.

One of the characteristics of soybean plants is N_2 fixation ability concerted with symbiotic microorganisms located in nodules on roots. Soybean plants supply photosynthate, sucrose, to nodules as the nutrient. Symbiotic microorganisms in nodules utilize sucrose for the source of energy required for N_2 fixation and the carbon (C) skeleton of nitrogenous compounds, ureides (allantoin and allantoic acid) [3]. Ureides are suitable forms for the transportation of nitrogen (N) in the plant body. Soybean plants supply less amount of sucrose to nodules when the soil offers enough amount of N (as a form of nitrate) [4]. It is well known that a high concentration of soil nitrate depresses the formation of nodules and N_2 fixation [3].

We did experiments to clarify two possible factors to affect seed protein content, the supply of N to seeds and C metabolism in immature soybean seeds. Firstly, we describe that N_2 fixation in nodules affects seed protein content among soybean plants having different N_2 fixation activity grown on soils with different types of coated urea slow-release N fertilizers (CUSLNFs) in Section 2. Secondly, we describe the role of a CO_2 fixing enzyme, phospho*enol*pyruvate carboxylase (PEPC), on the protein accumulation in maturing seeds in Section 3. Here we introduce our results in the experiments on plants cultivated in the field of the Faculty of Agriculture, Kobe University, where the soil was granite-based udorthents.

2. Association of nodulation and N_2 fixation activity with the accumulation of protein and oil in soybean seeds

2.1 Soybean plants with different nodulation status reveals the effect of nodulation and N_2 fixation activity on the seed protein and oil contents

2.1.1 Estimation of N derived from nitrogen fixation (Ndfa), N derived from N fertilizer (Ndff) and N derived from soil (Ndfs) in seeds by monitoring $\delta^{15}N$

We used the most popular cultivar in Japan, Enrei, and its two near-isogenic lines (NIL) to evaluate the effect of nodule's N_2 fixation activity on seed protein and oil contents. One of the NILs [5, 6], En1282, was the no nodulating isoline [7], and another NIL, En-b0–1, was the hyper nodulating isoline [8]. As high soil nitrate levels inhibit the nodule formation and N_2 fixation activity of soybean plants [3], we applied different types of coated urea slow-release nitrogen fertilizers (CUSLNFs) having different lifespan to inhibit the N_2 fixation activity of plants for a certain period. The used CUSLNFs continuously emit N contained in them for 0 to 30 days for M5, 60 to 120 days for MS9, and 0 to 100 days for M15, respectively. We designated four types of experimental plots with different expected soil N levels (H, high or L, low) during the early and late periods of plant growth: the plots 'L-L', 'H-L', 'L-H', and 'H-H' were with no CUSLNF, M5, MS9, and M15, respectively. N_2 fixation of Enrei and En-b0–1 were expected to be suppressed during their working period. **Table 1** shows $\delta^{15}N$ values of matured seeds of the three NILs grown on the

Genotype	N Treatment			
	L-L	H-L	L-H	H-H
En1282	3.36	2.57	1.22	1.07
	(0.60)	(0.51)	(0.66)	(0.14)
Enrei	−0.20	2.12	0.33	1.88
	(0.36)	(1.36)	(0.14)	(0.36)
En-b0–1	−0.61	1.46	1.06	1.69
	(0.22)	(0.02)	(0.09)	(0.22)

The $\sigma^{15}N$ value of an urea coated slow-released nitrogen fertilizer (Meister 15) was −1.41.
$\sigma^{15}N$ values: Averages of duplicated samples. Parentheses indicate the SD (n = 2).

Table 1.
The $\sigma^{15}N$ values of mature seeds from plants of three genotypes of soybean.

four types of plots. We discriminated N derived from N_2 fixation, soil and CUSLNFs using the $\delta^{15}N$ values of seeds based on the authorized method [9]. Namely, ratios of seed N from the three origins in the NILs were estimated by the equations described in the footnotes of **Tables 2** and **3**. Since the En1282 plants utilized the mineral N that was available in the experimental fields, the $\delta^{15}N$ value of En1282 seeds of the L-L plot (3.36) was of the N supplied from the soil and the basal compound fertilizer. We distinguished the CULSNF-N from the N from the soil and compound fertilizer by using the $\delta^{15}N$ values of En1282 seeds of H-L, L-H, and H-H N plots then estimated the ratios of CULSNF-N as 16.6%, 44.8%, and 48.0%, respectively (**Table 2**). In the case of En-b0–1 plants grown on the L-L plot, the N from both the soil and the compound fertilizer was assumed to be negligible as %Ndfa of the plants was estimated to 118.1%. We presumed that the $\delta^{15}N$ value (−0.61) of En-b0–1seeds of L-L N plot was of the N from N_2 fixation. The contribution rates of the N_2 fixation in Enrei grown on the L-L, H-L, L-H, and H-H plots were 89.9%, 14.2%, 48.9%, and − 47.9%, respectively, and those of En-b0–1 were 100%, 35.0%, 8.9%, and − 36.5%, respectively (**Table 3**). The contribution rates of fixed-N ratio in the seeds from the plants of these nodulating genotypes grown on the H-H plot were negative values because these $\delta^{15}N$ values were higher than those of the En1282 plants. The contribution to the total N from the N_2 fixation in both Enrei and En-b0–1 was assumed to be zero. The number of nodules in a nodulating soybean

N	Ratio, %		Amount, gN/plant		
Treat.	Soil + BF	CUSLNF	Soil + BF	CUSLNF	Sum
L-L	100	0	0.63	0	0.63
H-L[§]	83.4	16.6	1.50	0.30	1.80
L-H[§]	55.2	44.8	1.20	0.98	2.18
H-H	52.0	48.0	1.32	1.22	2.53

The values were calculated by the equation described below under the assumption that the two types of CUSLNF, M-5 and MS-9, had the same $\sigma^{15}N$ values with that of Meister 15. The ratios of these N origins of seeds from plants of En1282 grown on the other N plots were estimated by using these $\sigma^{15}N$ values.
$\sigma^{15}N(En1282_{i–ii}) = (3.36) \times X_{i–ii}/100 + (-1.41) \times Y_{i–ii}/100$ ($X_{i–ii} + Y_{i–ii} = 100$), where X indicates the percentage of the sum of soil N and compound fertilizer N against the total N, Y represents the percentage of CUSLNF-N against the total N, and i–ii is the estimated N levels of the early and late periods of plant growth (H or L levels of N).

Table 2.
The ratios and amounts of N originated from the two origins, soil + compound fertilizer and CUSLNFs, in the total N in seeds from plants of non-nodulating genotype, En1282.

N	Genotype	Ratio, %	Amount		gN/plant			
Treat.		Soil + BF	SLNF	N_2 fix.	Soil + BF	SLNF	N_2 fix.	Sum
L-L	Enrei	10.1	0	89.9	0.28	0	2.48	2.75
	En-b0–1[†]	0	0	100	0	0	0.53	0.53
H-L[§]	Enrei	71.6	14.2	14.2	1.21	0.24	0.24	1.70
	En-b0–1	54.2	10.8	35.0	0.40	0.08	0.26	0.73
L-H[§]	Enrei	28.2	22.9	48.9	0.97	0.79	1.68	3.43
	En-b0–1	50.3	40.8	8.9	0.53	0.43	0.09	1.06
H-H	Enrei[‡]	76.9	71.0	−47.9	1.67	1.54	−1.04	2.17
		(52.0)	(48.0)	(0)	(1.13)	(1.04)	(0)	(2.17)
	En-b0–1[‡]	71.0	65.5	−36.5	0.95	0.87	−0.49	1.33
		(52.0)	(48.0)	(0)	(0.64)	(0.69)	(0)	(1.33)

[†]En-b0–1 plants grown on L-L soil; it was assumed that N was only from N_2 fixation.
[‡]The numbers of each fraction of nitrogen origins in parentheses were calculated by the equation described in the text of Ref [5] in the cases in which N_2 fixation did not work.
[§]The values were calculated by the equation described in the text of Ref. [5] under the assumption that the two types of CUSLNF had the same $\sigma^{15}N$ values with that of Meister 15.

Table 3.
The ratios and amounts of N originated from the three origins, soil + compound fertilizer, CUSLNFs, and N_2 fixation, in total N in seeds from plants of the two nodulating genotypes, Enrei and En-b0–1.

line T202 per plant during the seed maturation stages was affected by the type of applied CUSLNF (**Figure 1**) [10]. The same effects of CUSLNF was observed in Enrei (data not shown). Pot experiments under the conditions corresponded to the L-L and H-H plots showed that N_2 fixation activities of Enrei and En-b0–1 worked on L-L but did not work well on the H-H plot, respectively, judging from the changes in ureides' concentrations in xylem saps [6]. Thus, nodulation in Enrei was almost inhibited in the H-H plot supported the estimation that the amount of N from N_2 fixation was very low in this experiment. We also observed that the number

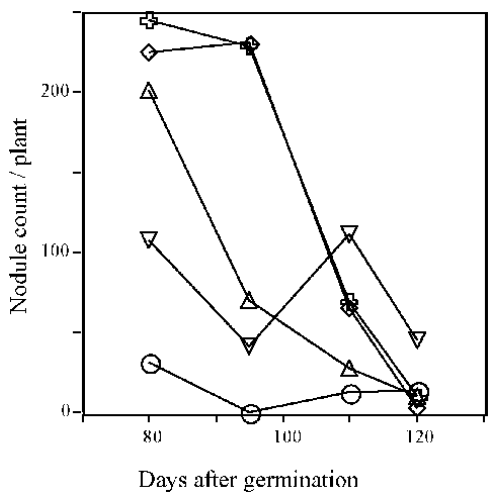

Figure 1.
Effects of N fertilizations on the changes in nodule counts per plant during seed maturation of cv T202. Symbols indicate soil type (fertilizers applied before planting) as follows circle.M-15; triangle, M-5; inverted triangle, MS-9; rhombus, urea; cross, no fertilizer.

of nodules in T202 grown in the H-L plot was rapidly decreased during the seed maturation period (**Figure 1**) [10]. The number of nodules of H-L was less than half of that of L-L at 95 days after germination, and we speculated that nodules in Enrei of the H-L plot did not develop well at the early stage of vegetative growth, resulted in much less N stored in nodules.

2.1.2 Coated urea slow-release N fertilizers differently affected seed chemical compositions and seed yields in the three NILs

Seed yields per plant of the 3 NILs are shown in **Figure 2**. Differences in seed yields of En1282 certified that fertilizers affected seed yield as expected and that the seed yields were proportional to the amount of N excreted from fertilizers. Seed yield of Enrei grown on the L-L plot was almost the same with that grown on the H-H plot, implying that the amount of N_2 fixation-derived N in seeds of the L-L plot was almost same with the amounts of fertilizer-derived N in seeds of the H-H plot. Seed yields of En-b0–1 were proportional to the amount of N applied from fertilizers, and this suggested N_2 fixation activity in En-b0–1 did not work well on this growth condition. This result is inconsistent with the low value of $\delta^{15}N$ value in seeds of the L-L plot and high ureides concentration in xylem saps from plants grown on the L-L condition in pot experiment.

The estimated amounts of N from the two origins (the soil with the compound fertilizer and the SLNF) in En1282, grown with the 4 N treatments, are listed in **Table 2**. Those from the three origins (the soil, the compound fertilizer, and N_2 fixation) in the two nodulating genotypes grown with the 4 of N conditions are listed in **Table 3**. In En1282, the amounts of N/plant from both the soil and compound fertilizer were similar among H-L, L-H, and H-H, and the estimated

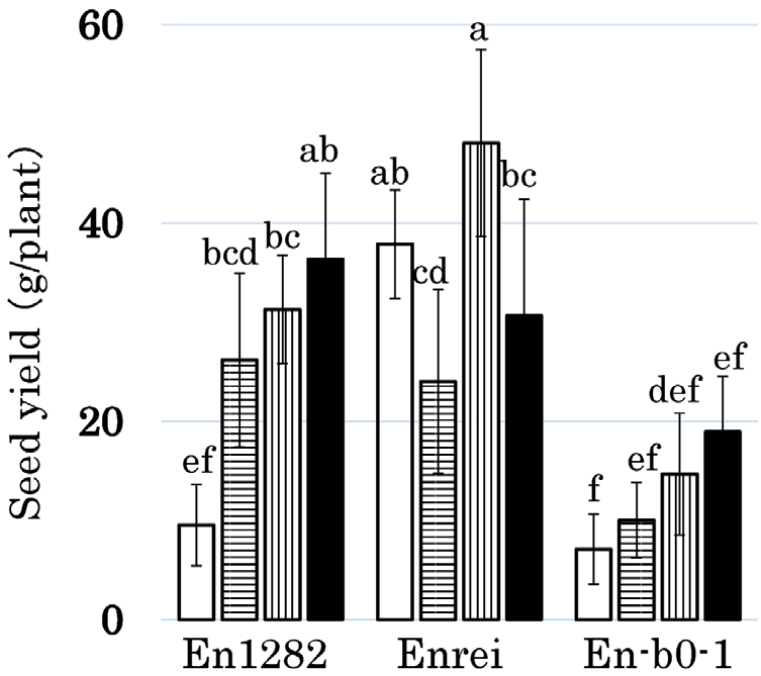

Figure 2.
Effects of the N fertilizations on the seed yields of three genotypes of soybean. Symbols indicate the types of N treatment where plants were grown: white, L-L; horizontal stripe, H-L; vertical stripe, L-H; black, H-H. Different letters indicate significant differences between genotypes and N treatments.

amounts of N/plant from the CUSLNF were 0.30, 0.98, and 1.22 g for the plants grown on H-L, L-H, and H-H, respectively. In Enrei, the amount of N/plant from N_2 fixation of L-H was 1.68 g, which was two-thirds of that on L-L. In contrast, the amount of N/plant from N_2 fixation in En-b0–1of L-H was 0.09 g/plant, which was one-fifth of the amount from the plants grown on L-L.

In the Enrei and En-b0–1 plants grown on H-H, the amount of N from N_2 fixation was negligible, and the amount of N from the CUSLNF was the highest among the N amounts from the 4 of N conditions.

For Enrei, the N supply from the soil had no apparent effect on the seed yield or seed weight (SW). It means that N derived from N_2 fixation compensates for the low supply of soil N for plant growth. Among the Enrei plants grown on the 4 types of N conditions, we observed a lower yield, a smaller SW, and a smaller number of seeds on H-L (**Figure 2**). Our observation that the $\delta^{15}N$ values of mature seeds of H-L was the highest among the Enrei plants grown on the 4 types of N conditions (**Table 1**), which implies that the ratio of N from N_2 fixation to the total N of the plants grown on H-L was less than that of the Enrei plants grown in the other conditions. Enrei plants develop nodules during the early stage of plant growth, and soil nitrate inhibited nodule growth of soybean plants [11]. The $\delta^{15}N$ value of seeds from plants grown on L-H (where changes in the amount of N released from the CUSLNF was opposite to the changes in the plants grown on H-L) was slightly higher than that of the plants grown on L-L. Considering this observation and the result that En1282 assimilated N well in the late growth stage (**Table 2**), the importance of N assimilation at the late plant growth was suggested. Moreira *et al* reported that foliar N application at the pod formation period increased seed yield under a certain environmental condition [12]. The work of Takahashi *et al* (1991) showed that the effectiveness of N fertilization at the late period of plant growth of soybean by the deep placement of N fertilizers that improved seed yields [13].

The inter-relationships among the seed protein content, seed oil content, and SW are illustrated for the three genotypes described in 2.1.1. (**Figures 3** and **4**). The protein content was proportional to the SW in En1282 and Enrei (**Figure 3A**). There was no such a relationship in En-b0–1. The protein content inversely correlated with the seed oil content in En-b0–1 (**Figure 3C**). The seed oil content uncorrelated with the SW and the protein content in En1282 and Enrei (**Figure 3B,C**). En-b0–1 seeds on L-L showed the highest protein content and the lowest oil content among the three genotypes (**Figure 3C**). The results of En1282 of L-L was opposite to those (**Figure 3C**).

The effects of the N treatments on the interrelationship between protein and oil contents in seeds differed among the three genotypes (**Figure 4**). The seed protein contents and seed oil contents of En1282 of L-H and H-H were higher and lower than those of L-L and H-L, respectively. The reverse was true for En-b0–1 on the same types of N conditions. The seed oil contents in Enrei of the 4 of N conditions were almost the same. The seed protein content of Enrei of L-L was the highest among the 4 N conditions, and the protein and oil contents in seeds of the three genotypes grown on H-H were almost the same.

We calculated the amounts of seed protein and seed oil by multiplying the contents of protein or oil by the SW (**Figure 5**). In En1282, the amount of oil per seed was proportional to the amount of protein per seed irrespective of the N treatment types (the r of the coefficient line was 0.998). The relationship between the amounts of oil and protein per seed seemed to be dependent on the N treatments in the two nodulating genotypes. The trends of the oil and protein amounts in Enrei and En-b0–1 seeds of L-H and H-H were similar to those of H-L and L-L. In other word, higher N_2 fixation activity of Enrei plants decreased amount of oil accumulation in seeds.

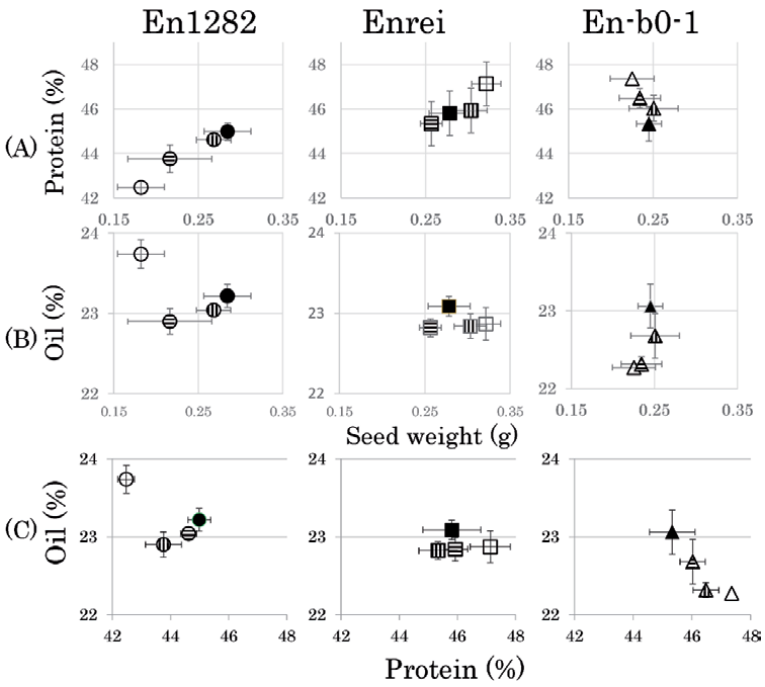

Figure 3.
Effects of N fertilizations on the contents of protein and oil in seeds from plants of three genotypes of soybean. (A) Relationships between protein content and seed weight. (B) Relationships between oil content and seed weight. (C) Relationships between oil and protein content. Shapes of symbols, circles, squares and triangles, indicate the CVs, En1282, Enrei and En-b0–1, respectively. Patterns inside symbols indicate types of soil where plants were grown as follows: white, L-L; vertical stripe, H-L; horizontal stripe, L-H; black, H-H. Correlation coefficients between protein content and seed weight from plants of En1282 and Enrei were 0.982 and 0.907, respectively. Significance levels were 0.02 and 0.10 for En1282 and Enrei, respectively. Correlation coefficient between protein content and oil content from plants of En-b0–1 was 0.924, and it's significance level was 0.10.

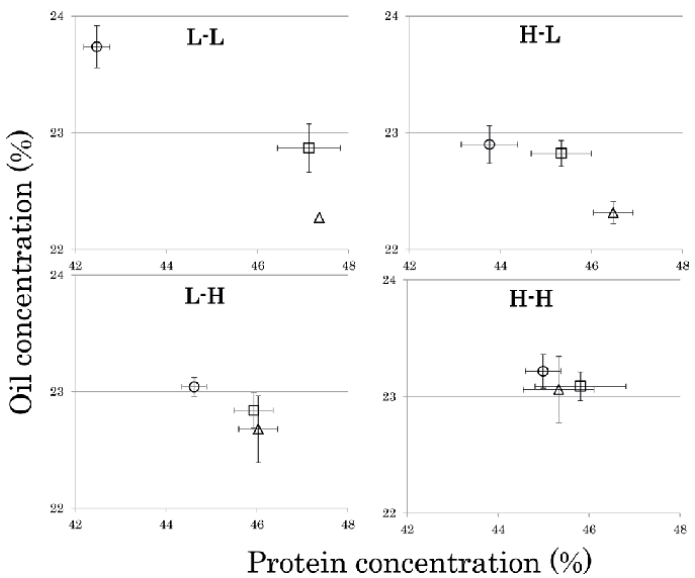

Figure 4.
Interrelationships between the protein content and oil content in seeds from plants of the three soybean genotypes. The four panels indicate the N plots as follows. Left-upper panel: L-L, right-upper panel: H-L, left-lower panel: L-H, right-lower panel: H-H. shapes of symbols indicate the same genotype as those described in the ***Fig. 2*** *legend.*

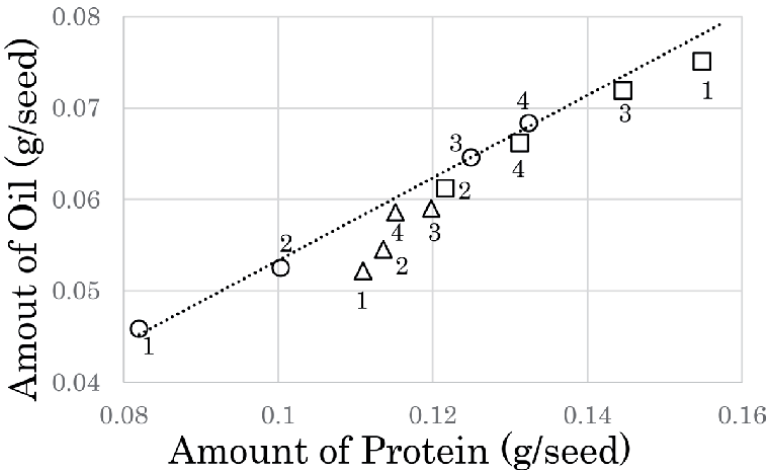

Figure 5.
Effects of the N fertilizations on the amounts of protein and oil per seed from plants of the three soybean genotypes. Shapes of symbols indicate the same genotype as those described in the ***Fig. 1*** *legend. Numbers beside marks indicate types of N plots where plants were grown as follows; 1:L-L, 2:H-L, 3:L-H, and 4: H-H. the* dotted line *indicates the coefficient line between the amount of oil per seed and the amount of protein per seed from En1282 plants (p = 0.01).*

2.2 Effect of different types of N fertilizers on seed chemical composition suggested independently regulated accumulation of protein and oil in soybean seeds

T202 plants were grown on 4 types of N fertilization conditions, where soil N levels were changed in different manners [14]. The 4 N fertilization types were (1) no N fertilizer, (2) urea, (3) M-5, and (4) M-15. The N fertilization condition (1), (3), and (4) were similar to L-L, H-L, and H-H described in 2.1, respectively. Plants of T201, a non-nodulating NIL of T202, were also grown on the 2 types of N fertilization conditions (3) and (4). Firstly, we investigated on the relationship between seed protein content and seed oil content per individual plant basis (**Figure 6**). T202 and T201 exhibited very similar protein and oil contents under the N condition (4), being due to less N_2 fixation in T202. T202 under the N condition (2) exhibited similar protein contents and slightly less oil contents with those of T202 and T201 of (4). This would be due to the different characteristics of M-15 and urea as N fertilizers. T202 and T201 of (1) and (3), both of which offer low nitrogenous conditions at the seed maturation stages, exhibited negative correlations between protein contents and oil contents in each condition, implying these correlations are dependent on the availability of N from soil.

Next, we investigated on the relationship between amounts of protein and oil per seed under the 4 nitrogenous conditions (**Figure 7**). Notably, observed relationships between amounts of protein and oil were exactly opposite to those of seed protein and seed oil contents of individual plants. Namely, each plant samples of (2) and (4) showed positive correlation relationships between amounts of protein and oil (**Figure 7**). In addition, a weaker positive correlation between amounts of protein and oil per seed was observed in T202 grown on the N condition (3). T201 of the N condition (4) exhibited a positive correlation between amounts of protein and oil per seed; it was similar to that of T202 grown on the same N condition. T202 of the N condition (1) did not show any correlation between amounts of protein and oil per seed, being contrast to that of protein and oil contents among individual plants. This could be due to the low variation of the amounts of protein and oil per seed in these samples. Hence, these results suggested that the observed negative

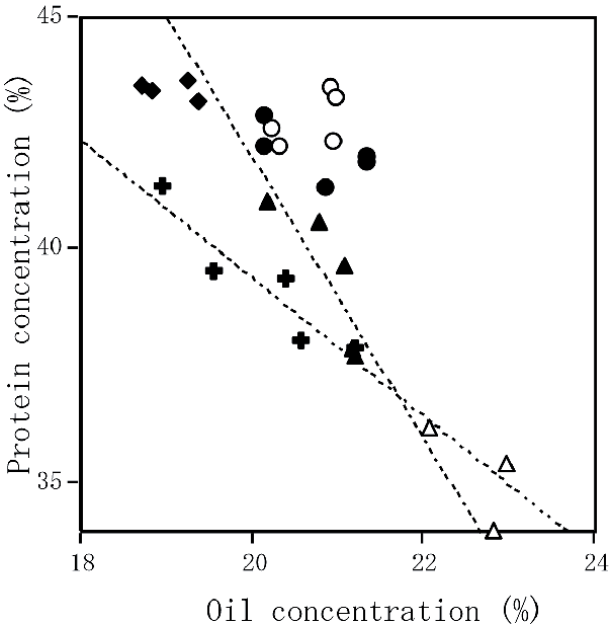

Figure 6.
Effects of soil N level and nodulation on concentrations of protein and oil in soybean seeds. Symbols indicate soil type as follows: circle, M-15; triangle, M-5; rhombus, urea; cross, no fertilizer. Solid and open symbols indicate nodulated cv, T202 and non-nodulated cv T201, respectively. Equations of correlation lines were as follows: line a, Y = -3.01X + 102.19 (r^2 = 0.709) for seeds from T202 on M-5 soil and line b, Y = -1.42X + 68.93 (r^2 = 0.864) for those from L soil, respectively. Y and X denote protein and oil concentrations, respectively.

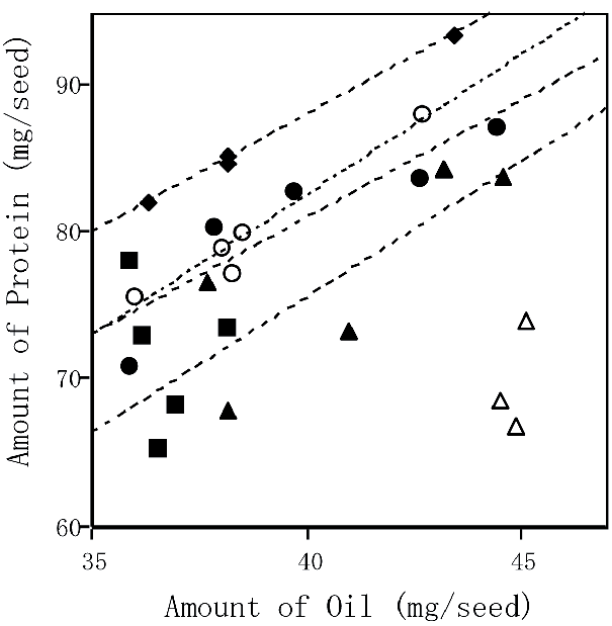

Figure 7.
Effects of soil N level and nodulation on amounts of protein and oil per seed in soybean seeds. Symbols indicate soil type as follows: Circle, M-15; triangle, M-5; rhombus, urea; cross, no fertilizer. Equations and coefficients of correlation lines were as follows: Line a, Y = 1.59X + 17.34 (r^2 = 0.817) for seeds from cv T202 on M-15 soil; line b, Y = 1.84X + 1.1.78 (r^2 = 0.632) for those on M-5 soil; line c, Y = 1.62X + 23.39 (r^2 = 0.997) for those on urea-soil, and line d, Y = 1.91X +6.21 (r^2 = 0.946) for those of cv T201 on M-15 soil, respectively. Y and X denote seed contents of protein and oil, respectively.

correlations of protein and oil contents in the N condition (1) was caused by different seed protein amounts per seed, which were made by receiving insufficient N to immature seeds. The observation that T201 of the N condition (3) (where offers low N supply during seed maturation) exhibited different protein contents and similar oil contents among individual plants implied that insufficient N supply did not affect oil accumulation in seeds.

The regression lines between amounts of protein and oil per seed in T202 of (2), (3), and (4) had similar slopes. This result implied that carbon transported into immature seeds was distributed to the biosynthesis of protein and oil in the same ratio among these plants. In addition, the Y-intercepts of the regression line of T202 under the N conditions (2) and (3) were larger and smaller than those of (4), respectively. Since the amount of N supplied to immature seeds from vegetative tissues would be increased in the order of (1), (3), (4), and (2), the differed Y-intercepts implicate another mechanism which is independent from carbon partitioning for the biosynthesis of amino acids and fatty acids. As mentioned in 2.3, seed oil accumulation ceased before seed maturation, but seed protein accumulation continued till seed maturation. The difference in the Y-intercept may be due to the different amount of accumulated proteins after the oil accumulation has ceased.

2.3 Inhibited N_2 fixation by N application at the flowering stage did not promote the protein accumulation but did the oil accumulation and dehydration in soybean seeds

2.3.1 Ammonium sulfate and NaCl applications at the flowering stage differently affects the protein and oil contents in soybean seeds

Soybean plants were grown on soil, which allows developing nodules well, and high amount of ammonium sulfate (AS) was applied (10 g of solid AS was spread around a plant) to T202 at the flowering stage [14]. We observed that the AS application did not change the protein content per seed but increased the oil content although seed protein content in AS-applied T202 individuals was lower than that in control (**Table 4**) [11]. The ureide concentrations in xylem saps from the NaCl and AS applied plants were lower than untreated ones in the same extent with each other till 25 days after the applications, which implied that plants of both treatments were suppressed their N_2 fixation activities to the same level as each other (**Figure 8**). Oil contents per seed of both treatments were more than those of the no treatment (**Table 4**). On the other hand, protein contents per seed from the AS treated plants were comparable to those from the no treated plants, which was quite different from those from the NaCl treated plants, of which protein content was

	Concentration, mg/g DM		Content, mg/seed		Seed weight
	Protein	Oil	Protein	Oil	g/seed
No	411	230	69.0	38.6	0.168 (0.022)
AS	391	239	69.6	42.5	0.178 (0.020)
NaCl	388	239	65.6	40.4	0.169 (0.020)

In the seed weight column, the standard deviations of seed weight from 70 grains are indicated in parentheses. Mature seeds were harvested 72 days after application. Protein and oil contents per seed were calculated by multiplying their concentrations by seed weight, respectively.

Table 4.
Effects of application of ammonium sulfate (AS) and NaCl at flowering stage on seed storage composition of soybean plants (cv T202).

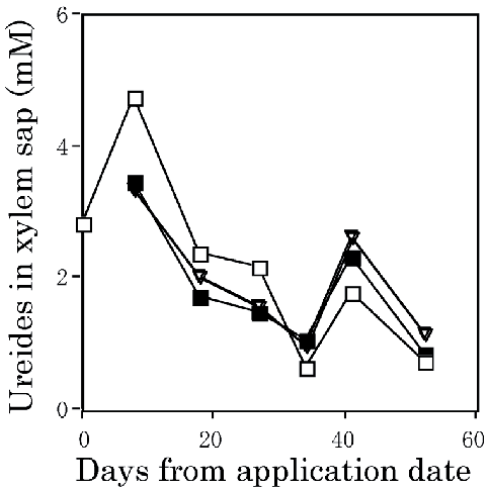

Figure 8.
Effects of application of ammonium sulfate and NaCl at the flowering stage on concentration of ureides in xylem saps from nodulated soybean plants (cv T202).Symbols indicate chemicals applied as follows: Open square, no treatment; solid square, ammonium sulfate; and rectangular triangle, NaCl, respectively. Values of ureides in the xylem saps were given as means of those from two plants from each treatment groups.

lower than those from the no treated plants (**Table 4**). These results suggested that applied AS compensated the decrease in the amount of N from N_2 fixation. That suppression of N_2 fixation activity caused an increase in the amount of oil in seeds.

2.3.2 Different effects of ammonium sulfate application at the flowering stage on the accumulation of protein and oil in soybean seeds

Application of AS at flowering stage to Enrei and Tamahomare decreased protein and increased oil contents in matured seeds of both CVs [15]. Averaged protein and oil contents in seeds from the AS dressed plants were 2% lower and higher than those from the undressed plants, respectively, in Enrei. Accumulation profiles of protein and oil per seed during their ripening period were quite different from each other (**Figures 9** and **10**). The regression curve of the increase in the amount of protein per seed during seed maturation was almost identical between plants that received N at the flowering stage and those that did not. Contrary to this, the regression curves of oil content per seed showed seeds from N applied plants accumulate oil faster than those of control plants did. Seeds of both N treatments stopped in increasing oil content at the late period of seed maturation. N applied plants showed a faster seed dehydration rate than the control plants on the results of changes in the water contents of seeds during seed maturation (**Figure 11**). Matured seeds from N applied plants had less protein content than those from N unapplied ones. An increase in seed weight was higher in N applied plants than those in the control plants. These results implied that a high amount of N application at the flowering stage suppressed N_2 fixation activities of nodules, causing a decrease in sucrose consumption by nodules. The dehydration rate of seeds from N applied plants was faster than those from N unapplied plants. The fact implied that the accumulation rate of storage compounds, protein and oil, in seeds from N applied plants was faster than those from N unapplied plants. In other words, a higher amount of C (sucrose) was supposed to supply to maturing seeds in the case of N applied plants than in the case of N unapplied ones. Sudden suppression of N_2 fixation of nodules by ammonium sulfate application to plants was supposed to cause the decreased consumption of sucrose in nodules and the increase in the amount of sucrose imported into maturing seeds. Thus seeds increased the oil content resultantly.

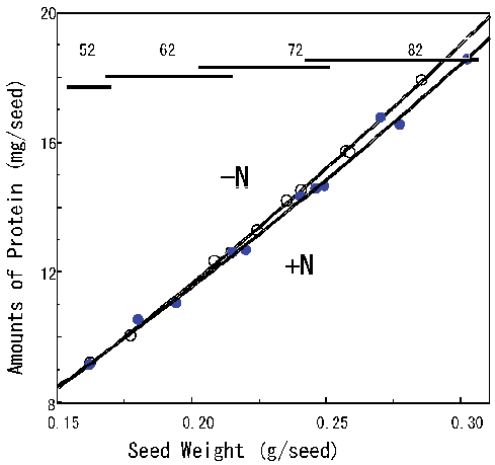

Figure 9.
Effects of N application at the flowering stage on the increase of protein content in soybean seeds during maturation. Open and solid symbols show the protein content per seed from the N undressed and dressed plants, respectively. Symbols indicated the days from the dressed day as follows: Circle, 52; square, 62; triangle, 72; inverted triangle, 82, respectively. Regression curves for values of the each treatment are shown. Equations for the curves are $Y = 56.90X^2 + 45.40X + 0.35$ *(*$r^2 = 0.999$*) and* $Y = 47.96X^2 + 44.90X + 0.69$ *(*$r^2 = 0.995$*) for the protein contents of undressed and dressed plants, respectively. Y and X means amount of proteinous N (mg) in a seed and seed dry weight (g), respectively.*

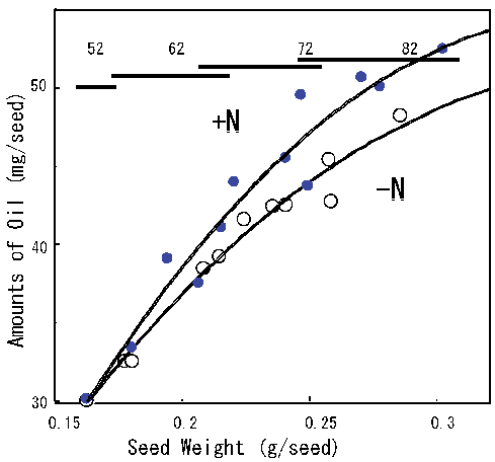

Figure 10.
Effects of N application at the flowering stage on the increase of oil content in soybean seeds during maturation. Symbols were the same as those in ***Figure 8****. Regression curves for values of the each treatment are shown. Equations for the curves are* $Y = -0.495X^2 + 0.366X - 0.0163$ *(*$r^2 = 0.977$*) and* $Y = -0.657X^2 + .467X - 0.0284$ *(*$r^2 = 0.941$*) for the oil contents of undressed and dressed plants, respectively. Y and X means amount of oil (mg) in a seed and seed dry weight (g), respectively.*

2.4 Different effects of CUSLNFs on seed protein concentrations of plants producing high and low protein content seeds – Accumulated proteins in nodules may be a factor to affect seed protein content

Plants of 13 CVs producing low-, medium- and high- protein content seeds were grown on similar conditions to the L-L, H-L, and H-H plots described in 2.1.1, and the protein and oil contents of harvested seeds were compared (**Figure 12**) [10].

Values subtracted seed protein contents from plants grown on H-H soil from those grown on L-L soil were compared among 13 CVs based on the protein concentrations

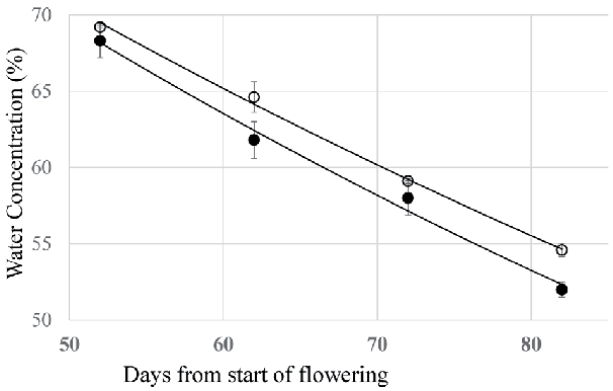

Figure 11.
Effects of N application at the flowering stage on the changes in water content of soybean seeds during maturation. Open and solid symbols show the water contents from the N undressed and dressed plants, respectively.

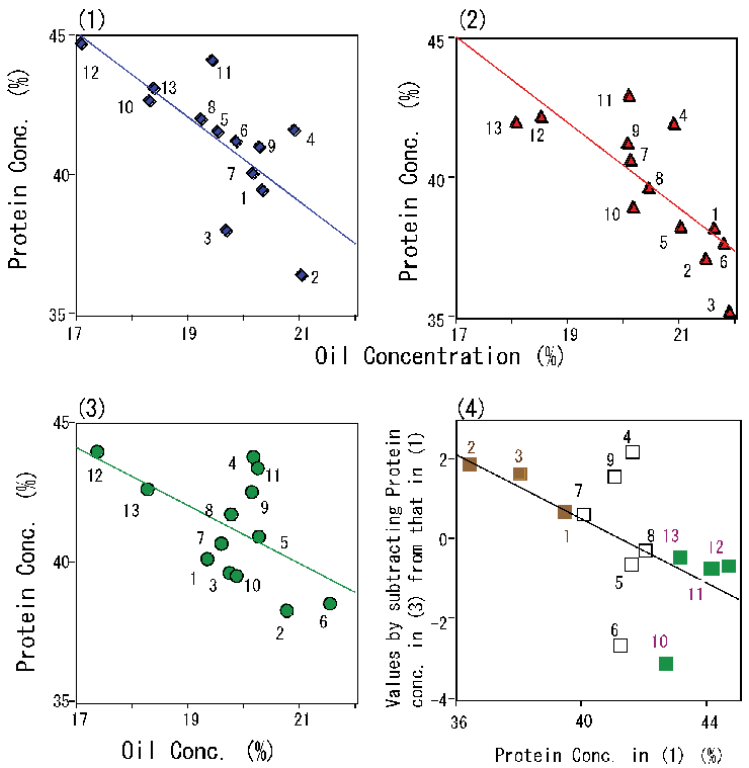

Figure 12.
Different effects of CUSLNFs on the contents of protein and oil in mature seeds of 13 cultivars of soybean. Left-upper panel, (1) interrelationships between the protein content and oil content in seeds from plants grown on L-L soil. Right-upper panel, (2) interrelationships between the protein content and oil content in seeds from plants grown on H-L soil. Left-lower panel, (3) interrelationships between the protein content and oil content in seeds from plants. Right-lower panel, (4) interrelationship between the value of protein content of seeds from plants grown on H-H soil (3) subtracted from that on L-L (1) soil and protein content of seeds from plants grown on L-L soil (1). Numbers in the figure indicate the cultiver names as follows: 1, Akishirome; 2 Ginrei; 3, Tamahomare; 4, Ohtsuru; 5, Kyu-kei 273; 6, Tamamasari; 7, Nishimusume; 8, Asagoao; 9, Mizukuguriokute; 10. Toyoshirome; 11, Fukuyutaka; 12, Miyagiaosho; and 13, Bunjyocha. Cvs of which number 1 to 3, 4 to 9 and 10 to 13 were classified to those producing low (less than 40%), medium (40 to 42%) and high (more than 42%) protein content seeds, respectively, based on the results shown in panel (1). Colors of symbols in panel (4) indicate the class of seed protein content of CV as follows: brown, low; white, medium; green, high. The X-axis and Y-axis coefficient lines for each panel and their coefficients of determination are as follows: (1) $Y = -1.519X + 70.886$ ($r^2 = 0.524$), (2) $Y—1.5144X + 71.301$ ($r^2 = 0.583$), (3) $Y = -1.038X + 61.712$ ($r^2 = 0.312$), and (4) $Y = -0.403X + 16.604$ ($r^2 = 0.320$).

of seeds from plants grown on L-L soil (**Figure 12D**). A negative correlation between the subtract values and seed protein contents of L-L soil was observed. Seed protein contents in 3 low-protein CVs grown on L-L soil were higher than those of H-H soil. The reverse was true for four high-protein CVs. Differences in seed protein contents between plants producing low and high protein seeds were possibly ascribed to the amount of N that immature seeds received from nodules and soil.

Protein contents correlated with oil ones in all cases, and coefficients of determination were 0.724, 0.770, and 0.559 for seeds grown on the L-L, H-L, and H-H conditions, respectively. The coefficient between protein and oil concentrations from plants grown on H-H soil was lowest, and that of those grown on H-L soil was highest among plants grown on the three types of soils. This result suggests a higher amount of N supply from nodules or soil during seed maturation promotes protein accumulation but does not oil accumulation because plants grown on the H-L soil might have fewer nodules than those on L-L, and they absorb less soil N than those on the H-H soil during reproduction stage.

Different seed weight distributions between a low protein cultivar Tamahomare (TH) and those of a high one Fukuyutaka (FU) supported this idea. Plants of TH grown on L-L had smaller seeds than those on H-H. The reverse was true for FU (**Figure 13**). Seeds of CV FU grown on H-L had similar seed weight distribution to that on L-L. On the other hand, seeds of CV TH grown on H-L were smaller than those on L-L, judging from their seed weight distribution profiles. These results suggested that immature seeds did not receive enough amount of N from vegetative tissues including nodules for the demand of seeds in the case of CVs producing low protein content seeds grown on L-L. Such plants grown on H-H absorbed and metabolized fertilizer-derived N from soil enough for immature seeds' demand. These results suggested that plants of CVs producing high protein content seeds grown on L-L had enough amount of N in the vegetative tissues for the need of immature seeds. These results also implied that those grown on H-H where nodulation and N_2 fixation activity of plants were suppressed by soil N did not have enough amount of N in the vegetative tissues for the demand of immature seeds.

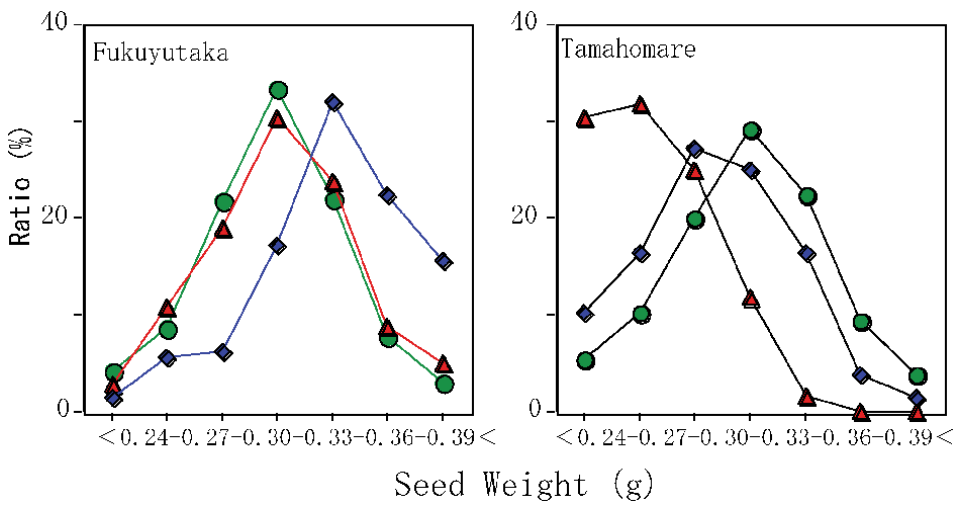

Figure 13.
Differences in the seed weight distributions from plants grown on three types of UCSLFs between two cvs producing seeds of high and low protein contents. Left and right panels show ratios of seed counts per seed weight ranges as a cv Fukuyutaka producing seeds of high protein content and a cv Tamahomare producing those of low protein content, respectively. Symbols indicate the soil types as follows; rhombus, L-L; triangle, H-L; circle, H-H.

3. Carbon metabolism in maturing seeds is affected by the supply of N from source organs-role of phospho*enol*pyruvate carboxylase (PEPC) in the accumulation of protein

3.1 Background for the research on the role of PEPC in the synthesis of storage protein in soybean seeds

Seed storage compounds in soybean, proteins and oils, are synthesized from substances transported from vegetative tissues, leaves, roots, and nodules. The primary forms of the substances are sucrose, ureides, and Asn [3]. Amino acids constituted storage protein are synthesized by introducing amino acid residues into organic acids in cotyledon during seed maturation. This fact implied two factors: amounts of organic acids and amino residues formed from the imported substances affect the protein content of soybean seeds. Since organic acids are utilized as the substrate for the synthesis of both fatty and amino acids, the inverse correlation between contents of protein and oil in soybean seeds [1] could be caused by the competition of fatty and amino acid synthesis. Non-photosynthetic type Phospho*enol*pyruvate carboxylase (EC 4.1.1.31, PEPC) was thought to play the anaplerotic role in the supply of organic acids [16]. Immature crop seeds have a high activity of the enzyme, and its role in maturing seeds was assumed to refix CO_2 formed from respiration in seeds [17].

Multiple types of PEPC isogenes were encoded in plant genomes. These enzyme genes were categorized into plant-type and bacterial-type isogenes [18]. Plant-type isogenes were further subdivided into C_4 and other (C_3) type. Subsequent sections present our research on soybean seed PEPC over time and discuss the role of PEPC isozymes in the synthesis of storage proteins.

3.2 Relationships of PEPC activity and contents of storage compounds, protein and oil, in mature soybean seeds

High CO_2 fixing activity was observed in immature cotyledons of soybean seeds under an unilluminated condition in a study [K. Tanaka, personal communication], which evaluates the photosynthetic activity of immature soybean seeds [19]. In cotyledon, ribulose 1,5-bisphosphate carboxylase (RuBPCase) gradually decreased its activity during seed maturation, whereas PEPC kept its activity high during seed maturation. PEPC enzyme rapidly decreased its activity between 3 and 9 days after germination [20]. These results implied the engagement of PEPC in the accumulation of storage compounds. As the enzyme activity was kept in matured soybean seeds, the enzyme activity and contents of storage compounds, i.e., protein and oil, were compared among seeds from plants of 13 types of seeds in 11 CVs grown in 12 prefectures of Japan (**Figure 14**) [21]. The enzyme activity was positively and negatively proportional to the protein and oil contents of seeds, respectively. This observation suggested PEPC in immature soybean seeds plays a role in the supply of carbon skeleton to synthesze of amino acids.

PEPC activity in maturing rice seeds increased its activity by the addition of N fertilizer [22]. In soybean, N fertilizer application was thought to be insufficient to the enzyme activity in seeds because soybean plants had nodules and supply N compounds in nodules to maturing seeds [3]. When N contents in leaves and PEPC activity in maturing seeds simultaneously were compared, they were proportional with each other (**Figure 15**) [10]. Leaves gradually lose their greenness by exporting amino acids to developing seeds during seed maturation, which means leaves lose photosynthetic activity. This observation suggested that PEPC in soybean seeds changed its activity responding to N supply from vegetative tissues and that PEPC plays an essential role in the amino acid synthesis for storage protein. One of PEPC

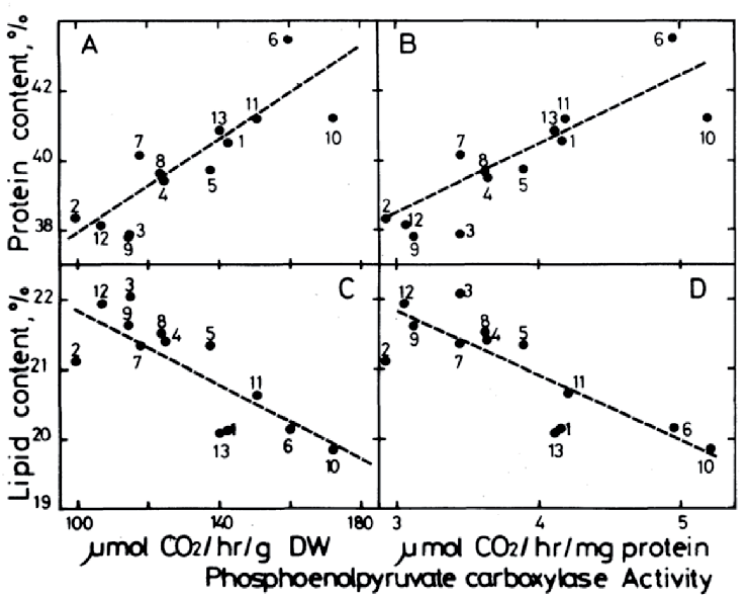

Figure 14.
Relationships between PEPC activity and contents of protein and lipid in soybean seed. Left-upper panel A: Relationship between PEPC activity per 1 g of dry seed (X) and protein content (Y) in soybean seed. Right-upper panel B: Relationship between PEPC activity per 1 mg of soluble protein (X) and protein content (Y) in soybean seed. Left-lower panel C: Relationship between PEPC activity per 1 g of dry seed (X) and lipid content (Y) in soybean seed. Right-lower panel D: Relationship between PEPC activity per 1 mg of soluble protein (X) and lipid content (Y) in soybean seed. Cultivar names and produced prefectures of soybean seeds harvested in 1986 are as follows: 1, Fukuyutaka (Fukuoka); 2, Tamahomare (Yamaguchi); 3, Akishirome (Yamaguchi); 4, Akishirome (Hiroshima); 5, Akiyoshi (Kagawa); 6, Enrei (Nagano); 7, Enrei (Niigata), 8, Tachisuzunari (Tochigi); 9, Suzuyutaka (Yamagata); 10, Miyagishirome (Miyagi); 11, Shirosennari (Akita); 12, Nanbushirome (Iwate); 13, Okushirome (Aomori). Equations and their correlation coefficients of the X-axis and Y-axis coefficient lines for A, B, C and D panels, respectively are as follows: Y = 0.062X + 31.727 (r = 0.8395); Y = 1.99X + 32.29 (r = 0.8660); Y = −0.029X + 24.884 (r = −0.8411); and Y = −0.91X + 24.35 (r = −0.8494).

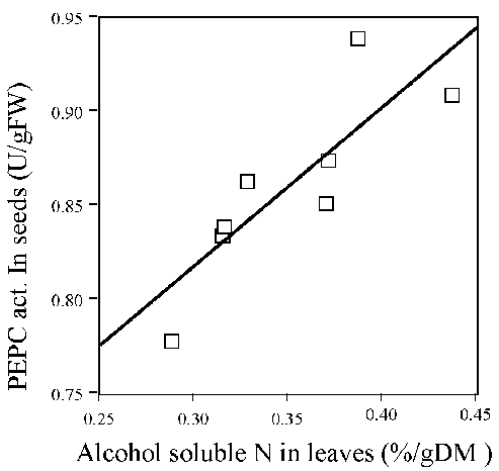

Figure 15.
Relationship between alcohol soluble nitrogen (amino acids) content in leaves and PEPC activity in immature seeds. The coefficient line and its coefficient of determination are as follows: Y = 0.846X + 0.564 (r^2 = 0.703).

isogenes was expressed in immature seeds and other vegetative tissues of soybean plants [23]. Another PEPC isogene expressed in soybean maturing seeds was identified together with the isogene mentioned above [24]. In recent, it appeared that ten

PEPC isogenes were encoded in the soybean genome [25]. It might be likely that multiple PEPC supports soybean seed metabolism during seed development.

3.3 Roles of several PEPC isoforms in maturing soybean seeds starch is a significant carbon source of carbon in protein biosynthesis during the late maturation period in soybean seeds

3.3.1 Comparison of PEPC activity, contents of protein and oil in seeds in high- and low-protein CVs using principal component analysis

We applied principal component analysis (PCA) to evaluate the interrelationships among the four factors of maturing seeds, contents of protein and oil, PEPC activity, and seed weight (**Figure 16**) [26]. The enzyme activity was significantly

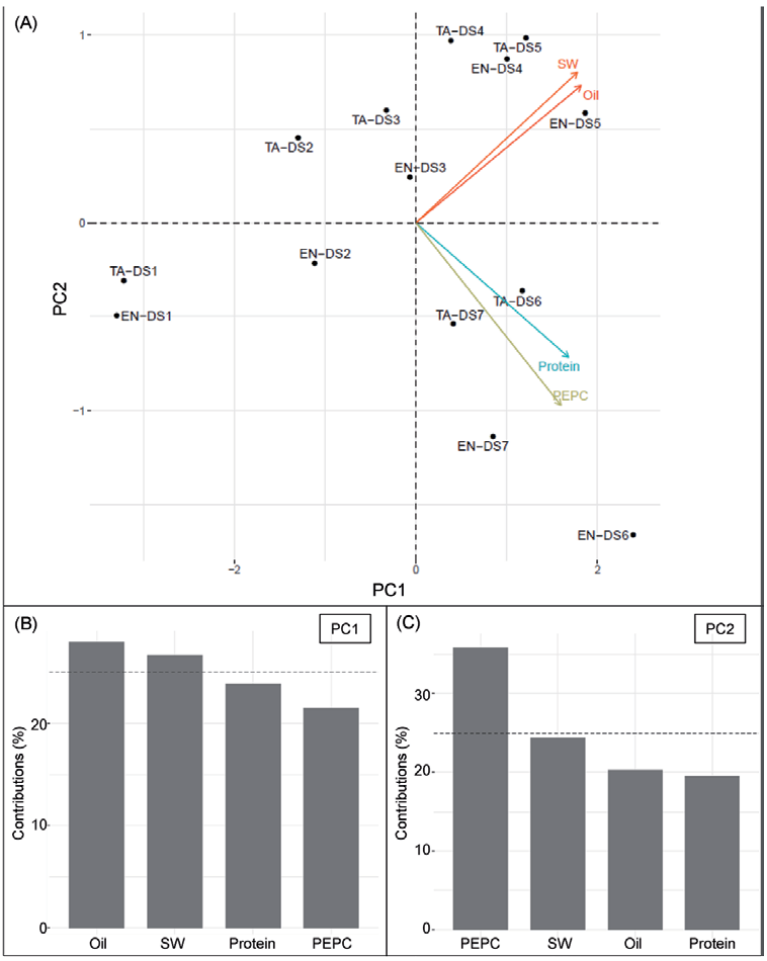

Figure 16.
The results of PCA in seed compositions, protein, oil, seed weight and PEPC activities during seed maturation. Characters and numerals show the name of cultivars and stage of samples, respectively. The characters EN and TA indicate Enrei and Tamahomare, respectively. The numerals with DS indicate the stage of samples. A: A biplot of the two major principal components, representing a distribution of the samples analyzed. Marks in black indicate samples, and arrows indicate the directions of protein content (protein), oil content (oil), seed fresh weight (SW), and PEPC activity per seed (PEPC). Horizontal- and vertical- axes represent principal component 1 (PC1) and principal component 2 (PC2), respectively. PC1 and PC2 explain 73.6 and 16.4% of the data variances, respectively. B and C: Contributions of the variables on PC1 and PC2, respectively. Dot lines indicate the averaged values.

associated with protein content but not with oil content. The oil content was associated with seed weight. Immunological assay on PEPC protein contents using antibodies for some PEPC isozymes showed plant-type ones expressed during all stages of seed maturation, and Gmppc2 expressed at the late maturation stage (**Figure 17**). The most critical period in the seed maturation period on the relation between protein content and PEPC activity was the late stage of seed maturation, DS6, in our discriminating seed growth stages.

Characteristic physiological changes of soybean plants are the loss of leaves and decrease in starch contents of seeds [27]. We observed that oil accumulation ceased at the late seed maturation period, and protein accumulation continued till seed maturation. Together, we proposed that PEPC plays a role in the supply of carbon skeleton of amino acids (organic acids) formed by the degradation of starch in seeds.

3.3.2 PEPC isogenes exhibits divergent expression patterns during seed maturation- Gmppc2 isogene is possibly a useful marker for improving seed protein content

The ten soybean PEPC isogenes showed different gene expression characteristics in developing seeds from each other [28]. Notably, one PEPC isogene *Gmppc2*

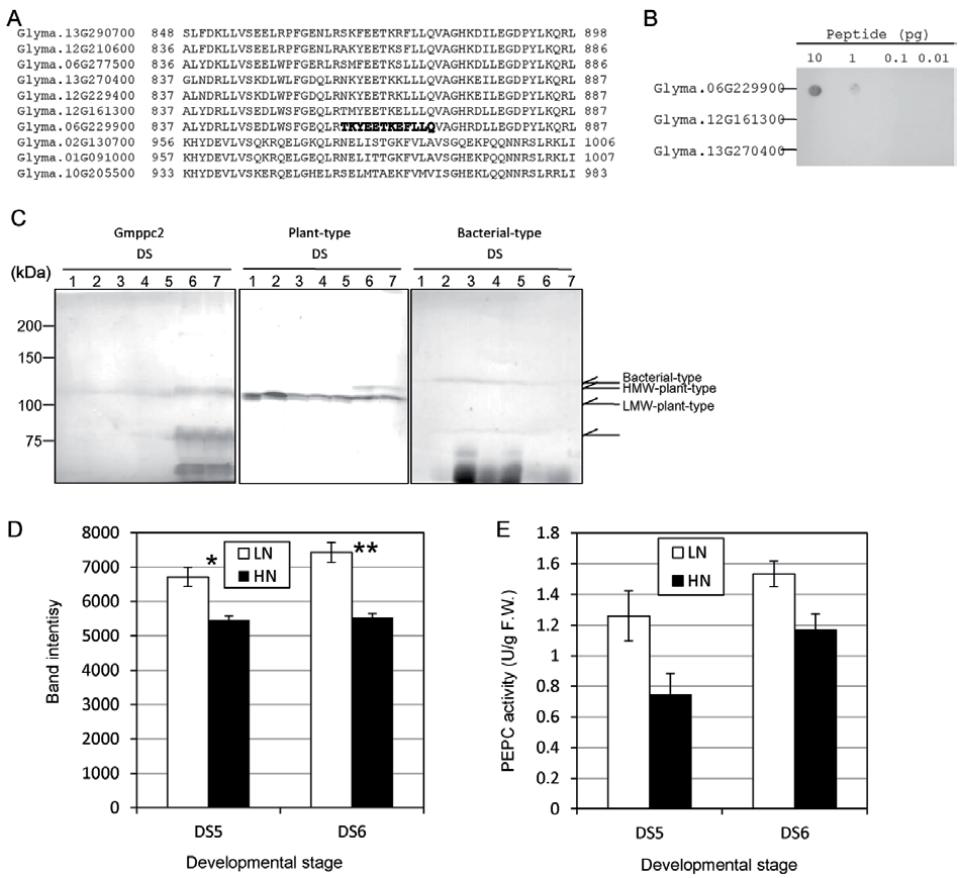

Figure 17.
*The expression of PEPC in immature soybean seeds. A: Partial sequence alignment of the ten PEPC isoforms of the C-termini. Bold letters: The region that was used to raise a Gmppc2-specific polyclonal antibody. B: Dot blot assay to determine the specificity of the Gmppc2 antibody. C: The protein expression patterns of Gmppc2, plant-type PEPC, and bacterial-type PEPC in developing whole seeds during seed maturation from DS1 to DS7. D: The effect of nitrogen application on the expression of Gmppc2 protein. The measurement was duplicated, and the average of the values is shown. Error bar: Standard error (SE). Significance at *10% and **5% by Student's t-test. E: The effect of nitrogen application on PEPC activity. Error bar: SE.*

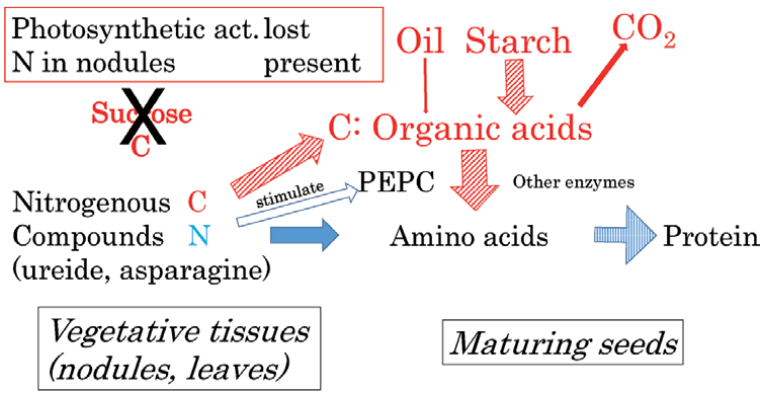

Figure 18.
Schematic presentation on the role of PEPC in the synthesis of storage protein on the late stage of seed growth maturation. Events in vegetative tissues and seeds at the late maturing stage of a CV, Enrei were drawn. Photosynthetic activity of leaves dramatically decrease by the decreases of N and chlorophyll contents, and resultantly supply of photosynthate (sucrose) to maturing seeds cease. On the other hand, nitrogenous compounds are supplied from degrading nodules to maturing seeds. Nitrogenous compounds are once separated into organic acids and amino residues in maturing seeds. Amino residues are introduced to newly synthesized organic acids of which carbon source is starch in maturing seeds to form amino acids which are substrates for the synthesis of storage protein. Carbon metabolizing enzymes including PEPC work to synthesize those organic acids. Activity of PEPC respond to the N supply to maturing seeds, thus affecting the protein content of seeds. Plant-type isozymes of PEPC work in the synthesis of amino acids through maturation period of seeds, an isozyme of Gmppc 2 was expressed at the late maturing stage of seeds.

exhibited a distinct expression pattern during soybean seed maturation. Namely, soybean seeds kept the expression of *Gmppc2* at a low level until the soybean seed is going to be matured (until DS4). In contrast, soybean seeds let the expression levels of *Gmppc2* at DS4 up-regulated drastically until seed maturation. Especially the expression level at DS6 in cotyledon was high. As mentioned in the previous section [3.3.1], DS6 is the critical stage at which seed protein accumulation varied between the two representative CVs [26]. To verify whether *Gmppc2* attributes to the different PEPC activity and protein content in the two CVs, expression of Gmppc2 protein was analyzed in soybean maturing seeds with the presence and absence of a nitrogen fertilizer, which suppressed nodulation and nitrogen fixation activity (**Figure 17**). The results indicated that soybean seeds highly expressed Gmppc2 protein at DS5 to DS7, and the expression was concordant with PEPC activity at DS5 and DS6 in response to the nitrogen fertilization. We illustrate the role of PEPC isozymes in the accumulation of protein (**Figure 18**). *Gmppc2* is the potential PEPC isogene, explaining the variation of seed protein content and PEPC activity among soybean CVs observed previously (**Figure 14**).

4. Conclusion: Our views of how nodules and their N_2 fixation activity affect protein and oil contents in soybean seeds

We carried out physiological experiments on the response of soybean plants to different types of N fertilizers. These experiments were designed from the viewpoint that there might be differences on the accumulation of storage protein and oil between soybean plants having different activities of N_2 fixation and N assimilation. We summarize our views on the characteristics of the accumulation of protein and oil in soybean seeds as follows.

1. Storage protein accumulation is independently regulated from storage oil accumulation in seeds.

We showed the accumulation profiles of protein and oil during seed maturation were quite different from each other (**Figures 9** and **10**). In immature seeds, PEPC plays a role in the protein accumulation (**Figure 16**), and its activity responds to the supply of N (**Figure 17**).

2. N_2 fixation results in decreasing the amount of oil per seed and leads to the decrease in oil content of seeds.

Seeds of nodulated CVs with high N_2 fixation activities have less oil content than those with low N_2 fixation activities (**Figure 4**; **Table 4**).

3. A high pseudo negative correlation between protein and oil was observed in seeds from plants which were grown on soil applied no N fertilizer.

High negative correlations were observed between contents of protein and oil in seeds of both nodulated and non-nodulated CVs grown under low N supply at the late maturation period of plants (**Figure 6**). Amounts of oil per seed were almost constant, and those of protein per seed were variable among plants in the cases of nodulated plants grown on L-L and non-nodulated ones grown on H-L (**Figure 7**). These results suggested that the observed high negative correlations between protein and oil contents in seeds were caused by the differences in the amount of accumulated protein in seeds among respective plants, and not related to oil accumulation in seeds.

4. Plants utilize soil N during the late growth stage as the source for seed protein, which implies the importance of N fertilization during the reproductive phase of soybean plants.

Based on the estimated amounts of fertilizer-derived N in seed protein, N excreted at the late period of plant growth was highly incorporated to seed protein in plants of both the nodulated and non-nodulated CVs (**Tables 2** and **3**). The higher seed yield of plants of regular nodulated CV grown on L-H plot than that grown on L-L and H-H plots suggested N supplied from both nodules and fertilizer at the late plant growth period increased seed yields (**Figure 2**).

5. Plant-type PEPC gene family plays a role in the synthesis of amino acids for storage protein. Gmppc2 isogene may have a crucial role in accumulating protein on the late maturation period of seeds.

Pattern analysis suggested that PEPC promotes protein accumulation but not oil accumulations (**Figure 16**). As the increase of acetyl Co-A carboxylase activity in immature rapeseed by gene engineering increased seed oil content [29], this enzyme might play a key role in oil accumulation in soybean seeds. These enzymes might work to synthesize respective storage compounds, protein and oil, independently with each other. Plant-type PEPC isogenes were expressed in immature seeds through the seed maturation period (**Figure 17C**). *Gmppc2*, an isogene of plant-type PEPC, was expressed in seeds at the late maturation period, and its PEPC protein expression level was higher in seeds of low N condition than in those grown on high N one (**Figure 17D**). These observations coincides with the observations that seeds of low N soil (L-L plot) had higher protein content than those grown on high N soil (H-H plot) (**Figures 3** and **6**).

We observed that the ratio between changing amounts of protein and oil in seeds was almost the same among plants grown on soils fertilized with different types of CUSLNFs (**Figure 7**). This observation implied that the allocation ratio of C to protein and oil was controlled by some mechanism.

Genetic studies on the storage compounds of soybean seeds have made progress. A quantitative trait locus analysis mapped regions controlling the contents

of storage compounds in soybean seeds on the contents of protein and oil on the soybean genome [30]. Concerts between the physiological and genetic approaches are useful to elucidate the mechanism of how contents of storage compounds are controlled in soybean seeds.

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Chapter 7

Modeling of Nitrogen Use Efficiency in Lettuce Culture (*Lactuca sativa*): Isotopic Nitrogen (15 N) and AquaCrop

Mawhoub Amirouche, Dalila Smadhi and Lakhdar Zella

Abstract

The present study is highlighted through an experiment carried out over two consecutive years 2014–2016, in the sub humid region of Algiers. The methodology adopted concerns the variation of optimal nitrogen doses and their effects on the evolution of lettuce (*Lactuca sativa* L.) cultivation, whose socio-economic impact is proven, using isotopic nitrogen (15 N) and the AquaCrop model. The experimental design adopted is of the complete randomized block type, with four (04) levels: 0 (control), 60, 120, and 180 kg N/ha with four (04) replicates. The results obtained showed that the 120 kg N/ha dose is the efficient dose to cover the nitrogen requirements of lettuce with an efficiency of 74.48%. The accuracy of the model in calibration was tested using the following statistical indicators: R2, nRMSE, and d, which are, respectively, $0.64 < R^2 < 0.81$; $18 < nRMSE < 46.3$ and $0.78 < d < 0.94$ for canopy coverage and $0.92 < R^2 < 0.98$; $21.6 < nRMSE < 34.5$ and $0.91 < d < 0.96$ for dry biomass. The AquaCrop model could be recommended as a practical tool to better manage agricultural practices including fertilization.

Keywords: lettuce, AquaCrop, isotopic nitrogen 15 N, nitrogen use efficiency, fertility stress

1. Introduction

In the coming years, agricultural production will have to face a double challenge, meeting the growing needs of the world's population while preserving the environment and natural resources. According to [1], the world's current population of about 6.3 billion people will reach nearly 8.6 billion in 2030. Agricultural production will then have to be significantly higher. This will be achieved by increasing yields. This has been achieved mainly through varietal improvement and associated cultivation techniques, including nitrogen fertilization.

In Algeria, 20% of the agricultural potential is located in the north of the country, which is characterized by poorly fertile soils. These soils are low in nutrients and have a very low rate of organic matter. Fertilization has remained archaic in the country. According to [2], in Algeria, the use of fertilizers in agriculture is not under control, despite the efforts made by farmers in charge of the cereal intensification program and potato farmers.

According to [3], fertilizers are applied in the absence of technical standards, neglecting the initial soil content; consequently, inputs are often poorly fractioned, leading to waste, which is a source of soil and water pollution. As several researchers have shown in their work on Algerian soils. In this context, [4, 5] conducted trials in the same semi-arid climate, respectively, on durum wheat and barley seed production, obtained maximum yields with similar rates (150 kg N/ha). These yields reached the respective values of 33.82 and 33.25 q/ha, i.e. gains of 11.52 and 9.76 q/ha. Halilat [6] showed that the interaction of potassium (P) and nitrogen (N) fertilization significantly affects wheat grain yield in the Saharan zone. The maximum yield reached 6.780 Mt./ha with the N250 P180 dose. With regard to nitrogenous fertilization, the observation highlights the need to promote adapted and balanced fertilization. Since urea is the most widely used nitrogen fertilizer in the world [7], it is crucial to assess the nitrogen use efficiency (NUE) by crops, since it is always aimed at achieving higher yields with a minimum application of fertilizer. This indicator (NUE) has been widely studied by several researchers around the world on various crops, including cereals, e.g. rice [8], maize [9], durum wheat [10, 11]; leafy vegetables, e.g. lettuce [12–14], spinach [15], cabbage [16] and vegetable crops, e.g. Potato [17, 18]; beans [19], tomato [20, 21].

In this perspective, this study uses the isotope approach 15 N to evaluate the nitrogen use efficiency. This new method, used by [22], highlights 15 N isotopic nitrogen, which is the most commonly used stable isotope in agriculture-related studies. It is the direct way to measure nitrogen uptake by applied fertilizer, and the most reliable way to monitor the flow and fate of nitrogen in the soil–plant system [23, 24]. To highlight the monitoring of this system, the chosen plant material is lettuce (*Lactuca sativa* L.), due to its short growing cycle. But also, because of the socio-economic impact that is beginning to dominate, at the national level. It is a source of wealth and income for producers. The search for decision support tools is essential in order to master agricultural practices and to plan for a sustainable agriculture that respects the environment. In this respect, the AquaCrop model, designed by the FAO, has been chosen as a decision support tool. The objective of this study is essentially oriented toward the search for optimum doses of nitrogenous fertilizers with the aim of contributing to the production of technical references for the efficient use of fertilizers.

2. Materials and methods

2.1 Study site

The study was conducted at the National Institute of Agronomic Research of Algiers (36°68′ N and 3°1′ E, at an altitude of 18 m), located south-west of Algiers in the eastern part of the Mitidja (**Figure 1**).

2.2 Climatic and soil data

Climatic conditions in the study area are characterized by pronounced seasonal variations with mild, wet winters and hot, dry summers. The meteorological data used are from the automatic weather station installed in the field. The measurements taken at daily time steps are: minimum and maximum temperatures (°C), rainfall (mm), wind speed (m/s) at 2 m above ground level, solar radiation (W/m^2) and relative humidity (%). The reference evapotranspiration (ET0) was calculated according to the FAO Penman-Monteith method [25]. A soil profile was carried out

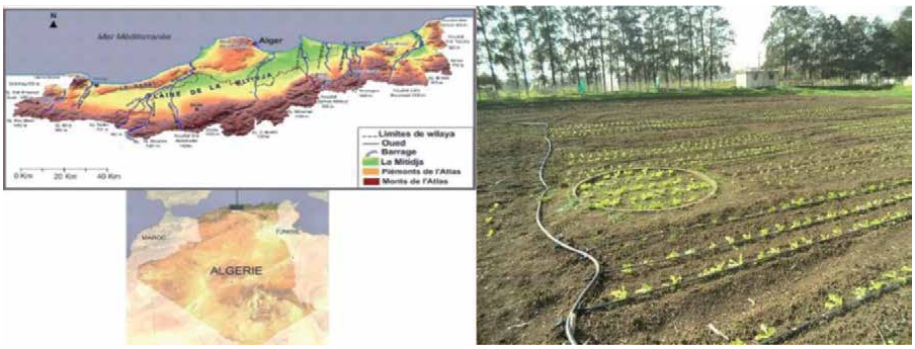

Figure 1.
Location of the study area.

over a depth of one meter, comprising three horizons. Soil samples were taken from each horizon with an auger for analysis physico-chemical.

2.3 Crop data

The crop taken into consideration is variety lettuce, stubborn from Nîmes, belonging to the lettuce to be applesauce class, which is eaten young, before it goes to seed. Lettuce seeds were sown in the honeycomb plates for 19–25 days in the nursery before being transplanted. The young lettuce plants were transplanted at the 3–4 leaf stage onto well plowed soil in the field.

2.4 Experimental protocol

The experiment was carried out in the open field using a complete randomized block experimental design with four levels of nitrogen, namely: T1 (0 N kg/ha), T2 (60 N kg/ha), T3 (120 N kg/ha) and T4 (180 N kg/ha) arranged in four blocks. Each block has four sub-plots. Each micro plot is 6 m long and 3 m wide, giving a total area of 18 m^2, of which 4.5 m^2 was used for the 15 N. The trial was repeated for two consecutive years (2014–2015) and (2015–2016). Isotopic nitrogen was used only in the first year because of its high cost. The amounts of nitrogen used were distributed along the crop development cycle, namely: 10% at 15 days after transplanting (DAT), 30% at 40 DAT, 40% at 60 DAT and 20% at 75 DAT. The growing season is from January to April for both companions, coinciding with the winter season, during which irrigation is not necessary.

2.5 Measured parameters

The parameters measured in the field are essentially the above-ground biomass (B), which represents a parameter that best allows verification of fertilizer efficiency in lettuce where the growth of the above-ground part is a determining factor in agricultural value [26]. Every 10 days, samples of 6 plants/subplot are taken and brought back to the laboratory where they are dried in the open air for 24 h and then in an oven for 48 h at 70 C°. In addition to this, the evolution of the green canopy (CC) cover is monitored by reference to photos taken vertically at a height of 1.8 m above the crop, using a photometric device. The photos were analyzed using ARCgis 10.1 software using the supervised classification by maximum likelihood method (**Figure 2**). Harvesting was done when the apples were tightly packed and full for each subplot of 1 m × 1 m.

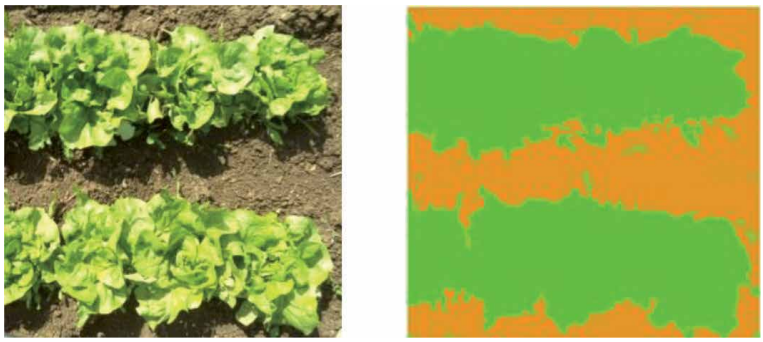

Figure 2.
Analyses of the fraction of the green canopy for the growth stage.

To determine the isotopic composition of lettuce plants, lettuce heads receivingan15N were divided into two parts (roots and leaves). Fresh weight was assessed for all parts of the crop. The samples were dried at 70°C for 24 hours, weighed for dry weight determination, ground into a fine powder using a 0.3 mm sieve and homogenized for total nitrogen and excess N15. The isotopic analysis of the lettuce culture samples was carried out at the National Centre for Energy, Science and Nuclear Techniques (CNESTEN-Morocco).

The quantification of fertilizer nitrogen was measured on the basis of the isotope dilution method from fertilizer nitrogen and the rate of nitrogen fertilizer applied, according to the following equation defined by [22]:

$$\%\ \text{Fertilizer N utilization} = \frac{Fertilizer\ N\ yield}{RateofNapplication} * 100 \quad (1)$$

2.6 Description and evaluation of the data by AquaCrop

AquaCrop requires five important components to be functional: climate, with its thermal regime, rainfall, evaporative demand (ETP) and carbon dioxide concentration; then crop characteristics, including development, growth and yield formation processes (**Table 1**); then soil, with its hydraulic characteristics (hydraulic conductivity at saturation, moisture at saturation, field capacity and permanent wilting point); and finally management practices, which are divided into two categories: plot management and irrigation practice management; and finally initial conditions.

2.7 Model calibration for soil fertility stress

Calibration of the model to fertility stress requires coverage of the green canopy (CC) and biomass production (B), recorded on the fertility stressed plot 'stressed plot' and the unstressed plot 'reference plot' (**Table 2**). The soil fertility stress in the AquaCrop model is given as follows:

$$\text{Stress} = 100\left(1 - B_{rel}\right) \quad (2)$$

Where: B_{rel} is the ratio of total dry above-ground biomass at the end of the growing season in the reference plot (B_{ref}) to that under stress (Bstress). Soil fertility

Description	Units	2015–16	Source
Conservative crop parameters			
Base temperature	C°	7	Calibrated
Upper temperature	C°	30	Calibrated
Upper threshold for canopy expansion, Pexp,upper	—	0.25	Simulated
Lower threshold for canopy expansion, Pexp,lower	—	0.55	Simulated
Shape factor for the stress coefficient for canopy expansion	—	3	Calibrated
Upper threshold for stomatal closure, Psto,upper	-	0.50	Calibrated
Shape factor for the stress coefficient for stomatal closure	—	3	Calibrated
Water productivity (WP)	g m^{-2}	19	Calibrated
Reference harvest index (HIo)	%	95	Measured
Crop coefficient when canopy is complete	—	0.85	Simulated
Non conservative parameters		2015–16	
Number of plants per m^2	Plant m^{-2}	15	Measured
CC0	%	2.25	Simulated
Maximum canopy cover CCx	%	81	Measured
Canopy size of the transplanted seedling	cm^2 $plant^{-1}$	15	Measured
Time from transplantation to emergence	Days	7	Observed
Time from transplantation to senescence	Days	80	Observed
Time from transplantation to maximum (CCx)	Days	50	Observed
Time from transplantation to maturity	Days	95	Observed
Minimum effective rooting depth	m	0.20	Measured
Maximum effective rooting depth	m	0.40	Measured
Transplantation time at maximum depth of rooting	Days	55	Observed
Date of transplantation		11/01/16	
Harvest date		14/04/2016	
Canopy growth coefficient (CGC)	% $days^{-1}$	14.30	Simulated
Canopy decline coefficient (CDC)	% $days^{-1}$	8.0	Simulated

Table 1.
Input culture parameters to calibrate the AquaCrop model.

Treatments	Brel (%)	CCx under fertility level (%)	Canopy Decline (-)
T1	51	51	Strong
T2	73	55	Medium
T3	100	61	Little
T4	100	58	Little

Table 2.
Input data to calibrate the AquaCrop model for soil fertility stress.

affects water productivity (WP), canopy growth coefficient (CGC), maximum cover (CCx) and canopy senescence.

The evolution of canopy cover, dry above-ground biomass and yield were taken into account in the evaluation of the AquaCrop model, while using the following statistical indicators: the coefficient of determination (R^2) of the linear fit, the square root of the normalized root mean square error (nRMSE) and the Willmott's agreement index (d).

3. Results and discussions

3.1 Analysis of climate data

Variations in rainfall and ETP are shown in **Figure 3**, which illustrates the rainfall distribution during the two years of experience 2014–2015 and 2015–2016. The cumulative rainfall received between September and August is, respectively, of the order of 552 and 551 mm. Those corresponding to the experimental seasons (January to April), they are close to the averages of 211.4 and 303.4 mm. The corresponding potential annual evapotranspiration is of the order of 744.3 and 782.6 mm. Those corresponding to the growing seasons are, respectively, 195.4 and 196.5 mm.

3.2 Physical and chemical characteristics of the soil

The study site is characterized by deep and heavy soils with high clay content. Soil analysis revealed the existence of 3 horizons with a silty-clay texture with high clay rates increasing with soil depth. At profiles of 0–25 cm, 25–55 cm and beyond 55 cm depth, these rates are 43, 49, and 52%, respectively. The pH of the station soils is generally slightly basic at 7.8, CEC varies between 17.9 and 15 meq/100 g and total limestone has a rate between 7.9 and 7.8%. The organic matter rate is 1.57% on the surface and 0.49% at depth.

3.3 Effect of fertilization on dry above-ground biomass

Figure 4 shows the evolution of the nitrogen doses applied at different phenological stages of the plant. This evolution is supported by the analysis of variance, which showed a very highly significant effect ($p < 0.001$), of the dry biomass, in relation to the increase in the doses of nitrogen supplied. A maximum of dry biomass is reached at the dose of 120 kg N/ha. Above this level, the increase in nitrogen rate is not significant. This result is consistent with that of [27], which showed that fertilization at high doses leads to a decrease in above-ground biomass. This is the case in the first year (2014–2015).

3.4 Effect of fertilization on yields

Figure 5 shows lettuce yields as a function of applied nitrogen rates. In fact, the graph shows that, during the two experimental campaigns, the highest lettuce yields (55.24 and 57.96 t/ha) were obtained by applying the 120 and 180 kg/ha rates. These doses are very highly significant ($p < 0.001$) compared to those obtained (30.19 and 45.49 t/ha) by applying the minimum doses of less than 60 kg/ha. This result is consistent with those of [28–30], who reported that increasing the N level from 0 to 120 kg N/ha had a positive effect on lettuce production. Nevertheless, in detail, the T4 treatment from the 2014–2015 trial shows a relatively lower yield of 50.25 t/ha

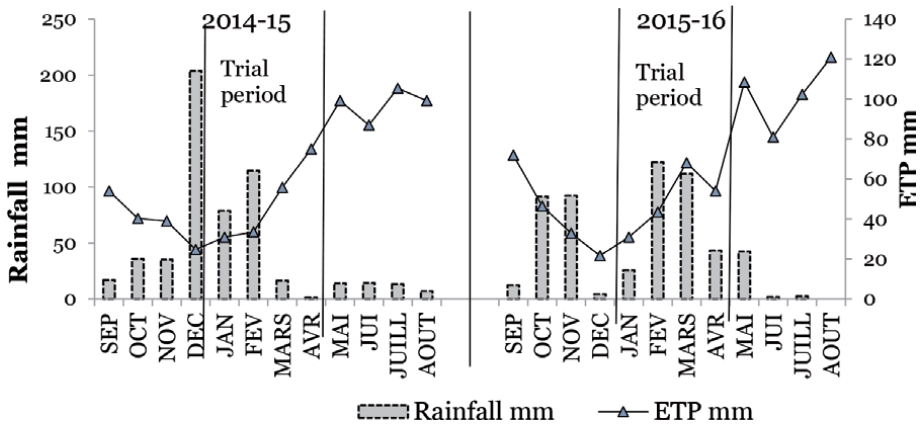

Figure 3.
Precipitation, potential evapotranspiration (ETP) on a monthly scale for test years 2014–2015 and 2015–2016.

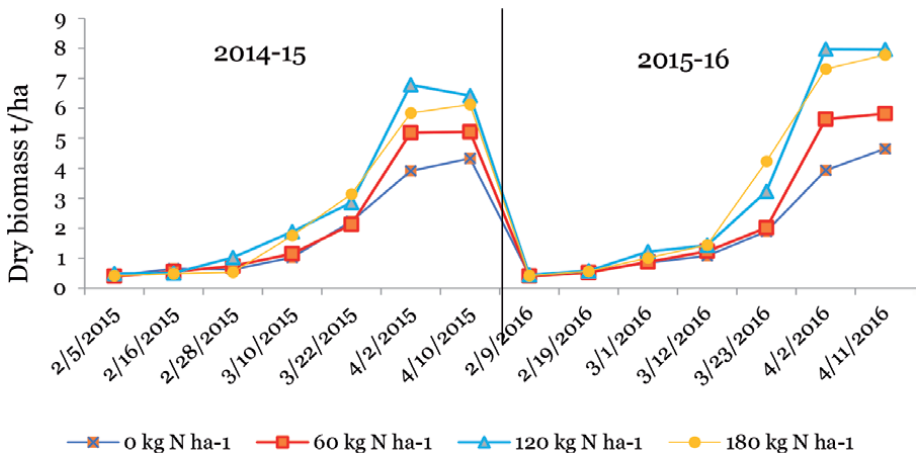

Figure 4.
Effect of different levels of fertilization on the evolution of dry above-ground biomass for the two growing seasons.

compared to the T3 treatment (54.25 t/ha) from the same year. The difference, evaluated at 3.08 t/ha, can be explained by the toxicity of the plants or by the nonattraction of nitrogen by the plants resulting from the consumption of excess nitrogen fertilizer, as pointed out by [31]. The response of lettuce for yields is considerably higher in 2016 than in 2015. This result is related to the higher rainfall amounts.

3.5 Nitrogen use efficiency

Nitrogen Use Efficiency (NUE) is an important indicator in the application of nitrogen fertilizers. Achieving a higher NUE always becomes a priority in agriculture [8]. In this context, **Figure 6** illustrates the variation in the percentage of NUE as a function of defined thresholds. For rates ranging from 60, 120 to 180 kg N/ha, the NUE varies from 65.42, 74.49 to 68.38%, respectively. The NUE decreased from 74.49% to 68.38% by increasing the rate from 120 to 180 kg N/ha. These results are similar to those reported by [32, 33]. The 120 kg N/ha rate provides the best efficiencies. This means that 74.48% of the fertilizer applied is consumed by the lettuce crop. The remaining 25.52% of N is either in the soil or lost through leaching. Lettuce is a short-cycle crop, making the best use of available nitrogen, as reported by [34].

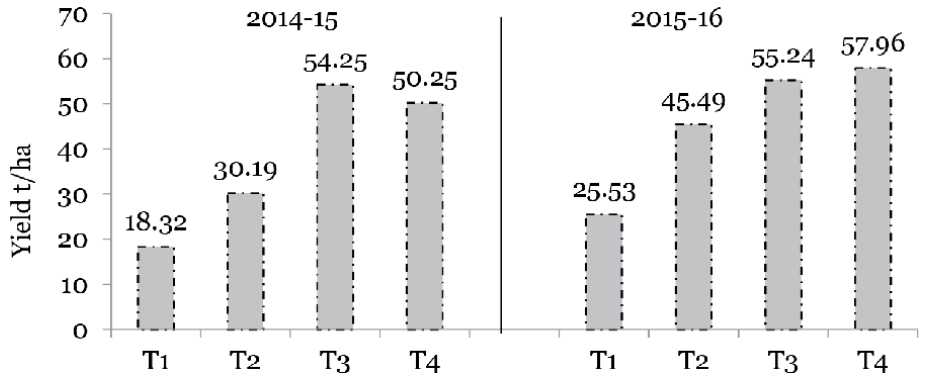

Figure 5.
Effect of different levels of fertilization on yield for the two growing seasons.

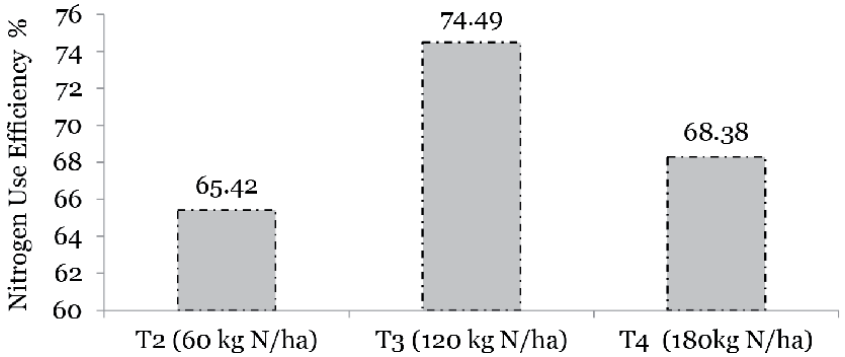

Figure 6.
Variation in nitrogen use efficiency by lettuce, expressed as a percentage (%).

3.6 Effect of fertilization on water productivity

Figure 7 shows the variation in water productivity (WP), soil evaporation (Es) and transpiration (Tr) of the lettuce crop under different levels of fertilization. This variation is supported by the analysis of variance, which showed a very highly significant effect ($p < 0.001$) of these parameters (WP, Es and Tr), in relation to the increase in the doses of nitrogen applied. The maximum values of WP and Tr are reached at the dose of 120 kg N/ha, for the two companions 2014–2015 (WP = 8.95 kg/m3; Tr =51.4 mm) and 2015–2016 (9.57 kg/m3; Tr = 55.80 mm). Above this level, the increase in the nitrogen rate is not significant.

3.7 Calibration of the AquaCrop model

3.7.1 Canopy cover and dry biomass

Experimental results of yield, canopy cover and dry above-ground biomass under different levels of fertilization are presented in **Table 3**. The AquaCrop model (V. 6.1) was calibrated using the crop data set obtained from the T3 treatment (120 kg N/ha). The lowest dry yield and dry aboveground biomass observed were 4.021 t/ha and 4.125 t/ha under the T1 treatment (0 kg N/ha), and the highest were 8.854 t/ha and 9.320 t/ha under the T3 treatment (120 kg N/ha), respectively.

The AquaCrop model is capable of simulating these parameters. Overall, the agreement between simulated and observed vegetation cover and biomass

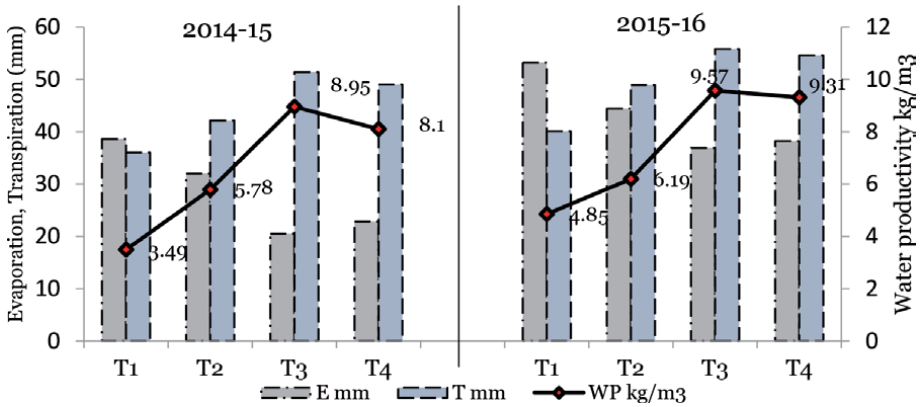

Figure 7.
Variation in water productivity of lettuce under different levels of fertilization.

Treatments	Biomass (t/ha)		Dry yield (t/ha)			CCx (%)		
	Obs	Sim	Obs	Sim	SD (±%)	Obs	Sim	SD (±%)
T1	4.125	4.785	4.021	4.546	(6.70)	51	44.80	(1.68)
T2	5.872	6.806	5.234	5.785	(11.68)	55	54.10	(5.76
T3	7.969	9.320	7.834	8.854	(8.27)	61	63.90	(2.67)
T4	7.788	9.100	7.626	8.645	(8.40)	58	63.70	(3.55)

Table 3.
Biomass calibration results, yield, and maximum canopy cover under different levels of fertilization in 2015–2016.

is satisfactory with 0.64 < R2 < 0.81, 18 < nRMSE > 46.3 and 0.78 < d < 0.94; 0.92 < R2 < 0.94, 21.6 < nRMSE <34.5, 0.91 < d < 0.96 (**Table 4**).

Figure 8 shows the comparison between simulated and observed canopy cover (CC) and dry above-ground biomass (B) for the calibration period (2015–2016). This figure shows that there is a close correspondence between observed and simulated CC and B. It is also important to note that the AquaCrop model correctly simulates CC from seeding to the maximum growth phase at which CCx is reached. This observation has been reported in several studies [35–37]. From **Figure 8**, it is clear that both parameters (CC) and B were overestimated by the AquaCrop model. In a recent study [38], it was shown that the AquaCrop model overestimated the cabbage canopy under different irrigation regimes. Nikolaus [39] also noted a slight (10%) but systematic overestimation of the amount of rice biomass conducted under different levels of irrigation and fertilization.

3.7.2 Yield

Observed and simulated lettuce yields are shown in **Figure 9**. The observed yields for treatments T1, T2, T3 and T4 are, respectively, 4.214; 5.187; 6.942 and 6.214 t/ha, while the simulated yields are 4.897; 5.981; 7.414 and 6.987 for the trial period (2014–2015), with a correlation coefficient R^2 = 0.92. On the other hand, the yields observed and simulated under the four treatments for the trial period (2015–2016) are of the order of 4.021; 5.234; 7834 and 7.626 t/ha, while those simulated are of the order of 4.546; 5.785; 8.854 and 8.645, with a correlation coefficient R^2 = 0.99. Analysis of statistical tests and linear regression indicated that the values

Indicators	CC (%)				Dry biomass (t/ha)			
	T1	T2	T3	T4	T1	T2	T3	T4
R^2	0.81	0.71	0.66	0.64	0.92	0.98	0.94	0.94
NRMSE	18	35.5	41.4	46.3	34.5	21.6	25.6	25
EF	0.79	0.03	−0.13	−0.06	0.55	0.85	0.82	0.82
d	0.94	0.81	0.78	0.80	0.91	0.96	0.96	0.96

Table 4.
Indicators of goodness of fit in estimating canopy cover and dry biomass.

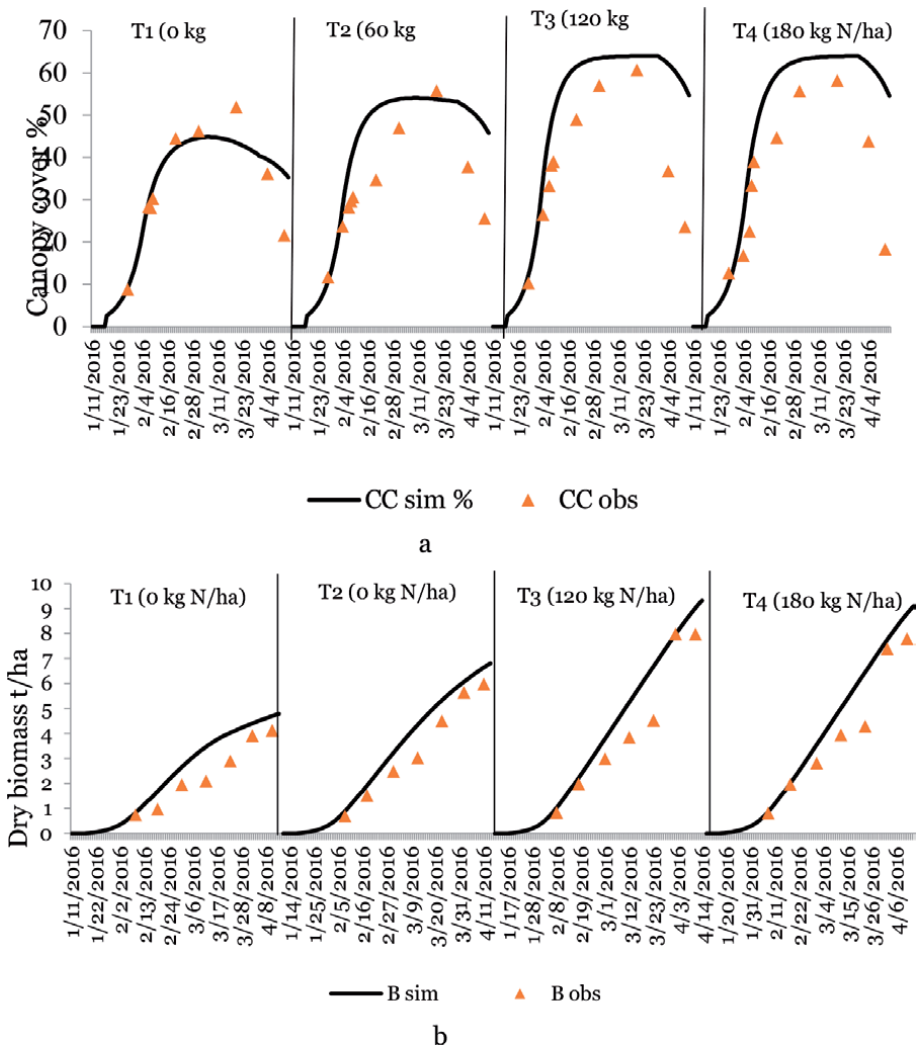

Figure 8.
Canopy coverage (a) and dry biomass (b) simulated and measured for the calibration period (2015–2016) under different fertilization levels (T1, T2, T3, and T4).

simulated by the AquaCrop model are in good agreement with those observed. Araya et al. [40] reported R^2 values >0.80 when simulating above-ground biomass and barley grain yield using AquaCrop.

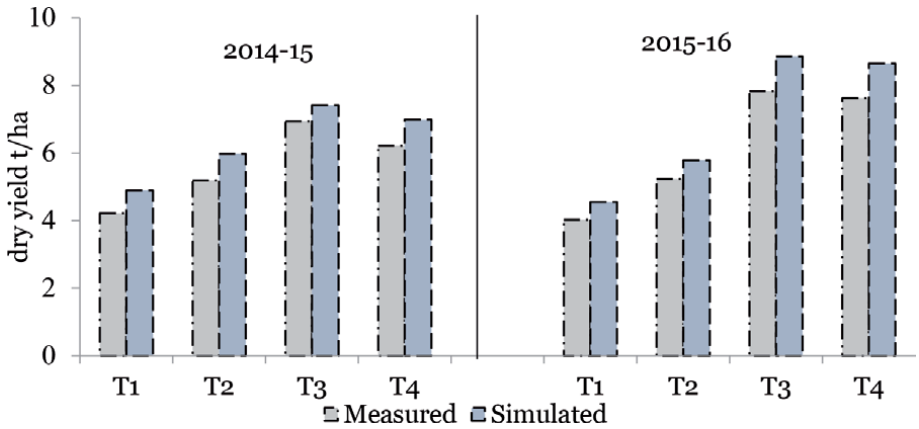

Figure 9.
Simulated and observed lettuce yields under different levels of fertilization.

4. Conclusion

The management of nitrogen fertilization is a major issue for agricultural production while contributing to water and soil pollution. In this situation, the adoption of fertilization management strategies aimed at using efficient doses and increasing the effectiveness of their use becomes necessary. Crop models simulating yield under such conditions could be important tools for fertilizer management planning. To this end, the parameterization of the AquaCrop model to estimate the effect of fertility constraints on lettuce yield under different levels of fertilization was investigated. The model tended to overestimate canopy coverage for T3 (120 kg N/ha) and T4 (180 kg N/ha) treatments, but with reasonable statistical indices (nRMSE: 14.80 for T3 and 12.50 for T4). AquaCrop has confirmed that it is a very useful tool that can be used to optimize the N rates applied to the crops, to play on the management of the plot in order to maximize yields.

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Chapter 8

Nitrogen in Flowers

Soraya Ruamrungsri, Kanokwan Panjama, Takuji Ohyama and Chaiartid Inkham

Abstract

This chapter explores the literature and research on nitrogen in flowers. An overview of nitrogen deficiency symptoms in some flowers, i.e., *Curcuma alismatifolia* (ornamental curcuma), *Tagetes erecta* (marigold), *Zinnia violacea* (zinnia), and *Gomphrena globose* (gomphrena) were presented. Additionally, nitrogen uptake, translocation, and application in some flowers, i.e., ornamental curcuma, narcissus, orchids, and rose, were discussed in this chapter. Nitrogen affects the life cycle of flower, including vegetative and reproductive phases. Flower size, stem length, number of flowers per plant, and color were reduced by nitrogen deficiency. Therefore, the optimum level of nitrogen supply in each growth stage is important for flower crop production.

Keywords: nitrogen deficiency, nitrogen uptake, nitrogen application, nitrogen translocation, flowers

1. Introduction

Flower crops, similar to other horticultural crops, require optimum fertilizer for a good quality flower size, stem length, number of florets, stem, and petal color. Essential elements, especially nitrogen, play an important role in growth and development in each stage of the life cycle. Different genera have different nitrogen requirements. Generally, they have a nitrogen content that is enough for root emergence and shoot sprouting. However, flowers grown from seeds may require fertilizer as soon as root emergence. A lack of fertilizer supply will lead to severe nutrient deficiency. Seed germination starts with water uptake, and then food reserves, i.e., carbohydrates and storage proteins, are oxidized for the growth process [1]. Flower seedlings show deficiency symptoms when the fertilizer supply is not enough. Nitrogen is an especially important element in the life cycle of plants from seedlings to the vegetative stage and flowering until senescence. It affects flower qualities, such as size, stem length, and color. This chapter focuses on the role of nitrogen in some economic flower crops. Most information was derived from our research experiments and some are unpublished data.

2. Nitrogen deficiency symptoms in different flower species

2.1 *Curcuma alismatifolia* (ornamental curcuma)

Nitrogen deficiency (-N) affected the growth and characteristics of *C. alismatifolia* (**Table 1** and **Figure 1**). Most growth parameters, such as plant

Nutrient solution	Plant growth at flowering stage (12 weeks after planting)							
	Plant Height (cm)	No. leaves per plant	Root length (cm)	Leaf green color intensity (SPAD unit)		Leaf area (cm^2)	Total fresh weight (g)	Total dry weight (g)
				Old leaf	Young Leaf			
Complete	49.1 a	5.0 a	42.8 a	57.1 a	52.5 a	253.8 a	285.0 a	31.3 a
-N	36.0 b	4.3 a	40.6 b	34.2 b	52.8 a	191.2 b	111.4 b	14.7 b
%CV	8.7	8.8	0.9	10.8	4.9	10.6	11.0	12.4
$LSD_{0.05}$	*	NS	*	*	NS	*	*	*

**Means within the same column followed by different letters were significantly different in an LSD test; ($p \leq 0.05$). NS = not significant.*

Table 1.
Plant growth of Curcuma alismatifolia *treated with complete nutrient solution or nitrogen deficiency (-N) at the flowering stage (12 weeks after planting).*

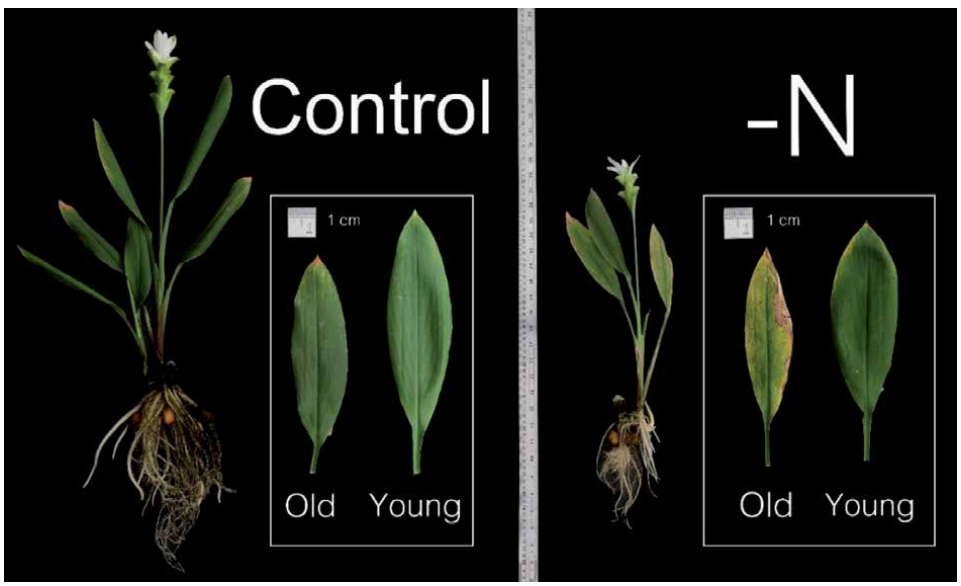

Figure 1.
Growth and flower quality of Curcuma alismatifolia *was affected by complete nutrient solution (control) and nitrogen deficiency (-N) treatment at the flowering stage (12 weeks after planting). (photo by Chaiartid Inkham).*

height, root length, leaf area, and total fresh and dry weight, were higher when plants were supplied with a complete nutrient solution, rather than -N treatment (**Table 1**). However, there was no significant difference in the number of leaves per plant between plants supplied with complete nutrient solution and -N treatment (**Table 1**). The characterization of nitrogen deficiency symptoms in *C. alismatiflolia* were evaluated at the flowering stage (12 weeks after planting). Leaves are the main plant part in which visual symptoms of the plant's response to nitrogen deficiency are usually observed. When there is a nitrogen deficit, older leaves of *C. alismatifolia* turn yellow and brown, while young leaves still appear green (**Figure 1**). The old leaves' green color intensity in -N treatment was lower than those treated with the complete nutrient solution (34.2 and 57.1 SPAD unit,

respectively). There was no significant difference among treatments in young leaves (**Table 1**). This result could explain nutrient remobilization processes in plants. Nitrogen is a macronutrient that is highly mobile in the phloem [2]; therefore, in N deficit conditions, nitrogen in old leaves of *C. alismatifolia* may be remobilized and translocate to young leaves. The remobilization of nutrients is frequently associated with foliar senescence, which makes nutrients available for younger plant organs and contributes to nutrient use efficiency [3].

Nitrogen deficiency delays flowering in *C. alismatifolia* and decreased flower quality in term of inflorescent length. However, there were no significant differences in inflorescence width, stalk length, and number of inflorescences per plant (**Table 2** and **Figure 1**). Nitrogen deficiency delayed flowering in narrow-leafed lupin [4]. The production of *C. alismatifolia,* in terms of flower quality and rhizome yield, depends on the response to N fertilizers [5]. Nitrogen-deficient plants are stunted and the quality of their flowers and rhizomes is significantly decreased. The increase of nitrogen from 0 to 50 mg L^{-1} increased the number of flowering shoots and, consequently, the number of rhizomes [6].

2.2 *Tagetes erecta* L. (Marigold)

The overall growth parameters of marigold were decreased under nitrogen deficit conditions (**Table 1** and **Figure 2**). At 8 weeks after planting, plants in the -N treatment were stunted with a plant height of only 47.5 cm, which was 42.2 cm shorter than plants in the complete nutrient solution treatment. Moreover, there was a dramatic decrease in leaf area and the total fresh weight of marigolds grown under -N treatment when compared with complete nutrient solution treatment (decreasing 82 and 90%, respectively) (**Table 3**). Leaf green color intensity of marigold was detected both in young leaves and old leaves to evaluate visual symptoms of plants grown under -N conditions. The results showed that leaf green color intensity of marigold in both young and older leaves was lower when grown under -N treatment than grown under complete nutrient solution treatment (**Table 3**). In addition, the leaves of plants under -N treatment were smaller than those under complete nutrient solution treatment. Older leaves turned yellow, red and brown, while young leaves had symptoms of chlorosis and turned light yellow (**Figure 2**). Plant height, plant spread, and the number of primary branches per plant of African marigold increased significantly with the increase in nitrogen level from 0 to 30 g m^{-2} [7]. A suitable supply of N enhanced plant growth efficiency, thus increasing plant yield and flower quality [8].

Nutrient solution	Flower quality				
	Days to flowering (day)	Inflorescence width (cm)	Inflorescence length (cm)	Stalk length (cm)	No. inflorescence per plant
Complete	70.7 b	6.9 a	11.8 a	34.3 a	1.0 a
-N	78.3 a	5.8 a	9.9 b	29.4 a	1.0 a
%CV	0.8	9.7	4.7	8.1	0
$LSD_{0.05}$	*	NS	*	NS	NS

**Means within the same column followed by different letters were significantly different in an LSD test; ($p \leq 0.05$). NS = not significant.*

Table 2.
Flower quality of Curcuma alismatifolia *treated with complete nutrient solution or nitrogen deficiency (-N) at the flowering stage (12 weeks after planting).*

Figure 2.
Growth and flower quality of marigold was affected by complete nutrient solution (control) and nitrogen deficiency (-N) treatments at the flowering stage (8 weeks after planting). (photo by Chaiartid Inkham).

Nutrient solution	Plant growth at flowering stage (8 weeks after planting)					
	Plant Height (cm)	Root length (cm)	Leaf green color intensity (SPAD unit)		Leaf area (cm^2)	Total fresh weight (g)
			Old leaf	Young Leaf		
Complete	89.7 a	28.3 b	43.9 a	45.5 a	3,990.3 a	889.8 a
-N	47.5 b	36.5 a	23.2 b	22.2 b	706.3 b	88.7 b
%CV	8.9	5.9	14.1	19.0	40.3	2.7
$LSD_{0.05}$	*	*	*	*	*	*

**Means within the same column followed by different letters were significantly different in an LSD test; ($p \leq 0.05$).*

Table 3.
Plant growth of marigold treated with complete nutrient solution or nitrogen deficiency (-N) at the flowering stage (8 weeks after planting).

Flowering of marigold was delayed under nitrogen deficiency condition (about 12 days delay when compared with complete nutrient treatment) (**Table 4**). Furthermore, the flower quality in terms of flower width, flower length, and stalk length were also reduced in plants in the -N treatment compared to those treated with the complete nutrient solution (**Table 4**, **Figure 2**). The flower yield of marigold was highly sensitive to nitrogen deficiency, since there was a 90% decrease in the number of flowers per plant when the plants were grown under -N treatment compared with the complete nutrient solution treatment (**Table 4**). In marigold (*Calendula officinalis* L. 'TOKAJ'), nitrogen fertilization had a significant impact on the number of flower heads per plant (especially on the second-rank branches) [9].

2.3 *Zinnia violacea Cav.* (zinnia)

Nitrogen deficiency caused a decrease in plant height, number of leaves per plant, and root length of zinnia at 9 weeks after planting (**Table 5**). Additionally, yields of zinnia in terms of leaves area, total fresh weight, and total dry weight

Nutrient solution	Flower quality				
	Days to flowering (day)	Flower width (cm)	Flower length (cm)	Stalk length (cm)	No. flowers per plant
Complete	55.7 b	8.5 a	4.8 a	12.7 a	10.0 a
-N	67.7 a	4.8 b	2.0 b	4.5 b	1.0 b
%CV	0.9	18.7	15.8	27.3	25.7
$LSD_{0.05}$	*	*	*	*	*

**Means within the same column followed by different letters were significantly different in an LSD test; ($p \leq 0.05$).*

Table 4.
Flower quality of marigold treated with complete nutrient solution or nitrogen deficiency (-N) treatment at the flowering stage (8 weeks after planting).

Nutrient solution	Plant growth at flowering stage (9 weeks after planting)							
	Plant Height (cm)	No. leaves per plant	Roots length (cm)	Leaf green color intensity (SPAD unit)		Leaf area (cm^2)	Total fresh weight (g)	Total dry weight (g)
				Old leaf	Young Leaf			
Complete	25.0 a	284.7 a	50.3 a	32.8 a	30.5 a	7,950.0 a	84.4 a	11.7 a
-N	17.3 b	44.0 b	24.0 b	16.7 b	27.2 a	1,155.8 b	15.7 b	1.9 b
%CV	10.7	17.4	15.7	14.3	9.4	9.6	9.9	19.4
$LSD_{0.05}$	*	*	*	*	NS	*	*	*

**Means within the same column followed by different letters were significantly different in an LSD test; ($p \leq 0.05$). NS = not significant.*

Table 5.
Plant growth of zinnia treated with complete nutrient solution or nitrogen deficiency (-N) at the flowering stage (9 weeks after planting).

also decreased when grown plant under -N condition compared with those treated with complete nutrient solution; the percentage of reduction was 85, 81, and 84%, respectively (**Table 5**). Visual symptoms of nitrogen deficiency in zinnia were observed at the flowering stage. In -N treatment, plants were stunted with less branches (**Figure 3**). Old leaves in the -N treatment turned yellow, while young leaves remained green in both treatments (**Figure 3**). Visual symptoms observed on leaves had a similar trend with leaf green color intensity detected by a chlorophyll meter (SPAD) (**Table 5**). The results indicated that there was a significantly different leaf green color intensity in old leaves between plants treated with -N and complete nutrient solution. There was not a significant difference in young leaves (**Table 5**). Growth of *Zinnia elegans* Cv. Meteor increased with increasing nitrogen concentration from 0 to 20 g N/pot, i.e., plant growth rate (42% increase), plant height (28% increase), number of lateral shoots (56% increase), length of lateral shoots (17% increase), number of leaves (59% increase), and leaf area (40% increase) [10].

The flowering of zinnia was delayed for 15 days when plants were grown under -N treatment (**Table 6**). Nitrogen deficiency decreased flower quality in term of flower width (50% decrease), number of flower buds per plant (80% decrease),

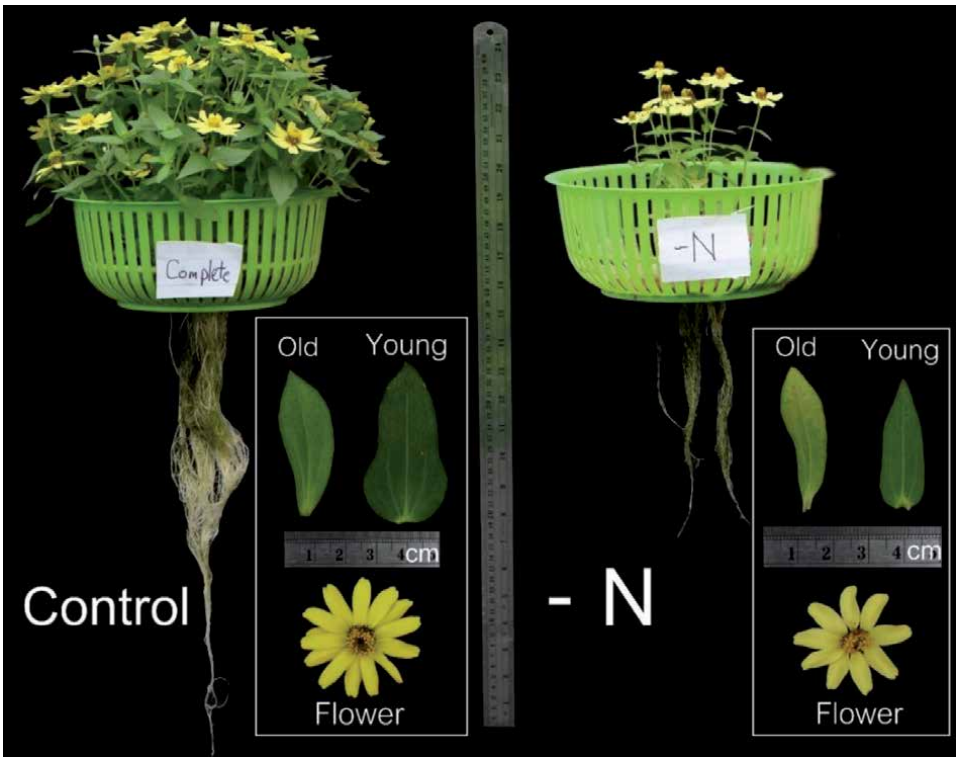

Figure 3.
Growth and flower quality of zinnia was affected by complete nutrient solution (control) and nitrogen deficiency (-N) treatment at the flowering stage (9 weeks after planting). (photo by Chaiartid Inkham).

Nutrient solution	Flower quality			
	Days to flowering (day)	Flowers width (cm)	No. flower buds per plant	No. flowers per plant
Complete	54.3 b	6.2 a	54.0 a	26.3 a
-N	69.3 a	3.1 b	10.7 b	6.7 b
%CV	3.4	9.8	11.6	18.5
$LSD_{0.05}$	*	*	*	*

**Means within the same column followed by different letters were significantly different in an LSD test; ($p \leq 0.05$).*

Table 6.
Flower quality of zinnia treated with complete nutrient solution or nitrogen deficiency (-N) at the flowering stage (9 weeks after planting).

and number of flowers per plant (74% decrease) in zinnia (**Table 6**). Visual symptoms of zinnia flowers response to -N treatment were smaller flowers and a reduced number of petals compared to those treated with complete nutrient solution (**Table 6** and **Figure 3**). In *Zinnia elegans* cv. Meteor, flower quality increased when supplied with 10 g N/pot compared with 0 g N/pot (18% increase in number of flowers per plant, 52.5% increase in flower size, and blooming stage was prolonged for 11 days) [10]. A high dose of nitrogen (20 g N/pot) negatively impacted flowering when compared with those supplied 10 g N/pot, i.e., delay emergence of first flower for 8 days, 20% decrease in the number of flowers per plant, and 17.5% decrease in flower size [10].

2.4 *Gomphrena globose* (gomphrena)

The growth of gomphrena was affected by nitrogen deficiency (**Table 7**). In the -N treatment, plants were stunted, with plant height 34.4 cm lower than those in the complete nutrient treatment (49.3 cm). Shorter root length was also observed in plants under -N treatment. Leaf area, total fresh weight, and total dry weight were dramatically decreased when plants were grown under nitrogen deficiency conditions with 80, 85, and 85% reductions, respectively (**Table 7**). Visual symptoms of nitrogen deficiency were a changed leaf color. Both old and young leaves in the -N treatment turned yellow with SPAD values lower than those under complete nutrient treatment (**Table 7** and **Figure 4**). Moreover, a decrease in the number of new branches was observed when the plant was grown under -N treatment (**Figure 4**).

Nutrient solution	Plant growth at flowering stage (13 weeks after planting)							
	Plant Height (cm)	No. leaves per plant	Root length (cm)	Leaf green color intensity (SPAD unit)		Leaf area (cm^2)	Total fresh weight (g)	Total dry weight (g)
				Old leaf	Young Leaf			
Complete	49.3 a	49.3 a	59.7 a	30.6 a	37.7 a	1,251.8 a	250.6 a	39.2 a
-N	34.4 b	21.7 b	32.6 b	22.1 b	26.6 b	254.2 b	38.0 b	6.0 b
%CV	13.2	18.0	10.0	9.67	17.8	17.9	18.0	18.0
$LSD_{0.05}$	*	*	*	*	*	*	*	*

**Means within the same column followed by different letters were significantly different in an LSD test; ($p \leq 0.05$).*

Table 7.
Plant growth of gomphrena treated with complete nutrient solution or nitrogen deficiency (-N) at the flowering stage (13 weeks after planting).

Figure 4.
Growth and flower quality of Gomphrena was affected by complete nutrient solution (control) and nitrogen deficiency (-N) treatment at the flowering stage (13 weeks after planting). (photo by Chaiartid Inkham).

Nutrient solution	Flower quality				
	Days to flowering (day)	Flower width (cm)	Flower length (cm)	Stalk length (cm)	No. flowers per plant
complete	86.7 a	2.1 a	2.6 a	14.1 a	8.7 a
-N	94.3 b	1.6 b	1.5 b	9.6 b	3.7 b
%CV	0.6	6.9	16.1	8.1	29.6
$LSD_{0.05}$	*	*	*	*	*

**Means within the same column followed by different letters were significantly different in an LSD test; ($p \leq 0.05$).*

Table 8.
Flower quality of gomphrena treated with complete nutrient solution or nitrogen deficiency (-N) treatment at the flowering stage (13 weeks after planting).

The flower quality of gomphrena was low under nitrogen deficiency conditions (**Table 8**). The flowering of gomphrena was delayed for 8 days when the plant lacked nitrogen. Additionally, flower size in terms of flower width, flower length, and stalk length were reduced under nitrogen deficit treatment. The number of flowers per plant also decreased by about 57% in the -N treatment compared with the complete nutrient treatment (**Table 8** and **Figure 4**).

3. Uptake, translocation, and nitrogen application in different flower species

Plants take up inorganic nitrogen, mostly in the form of ammonium (NH_4^+) and/or nitrate (NO_3^-). Uptake depends on the plant species and growth stage [11]. The translocation of N, including the free amino acid form, from roots to leaves could be done via the xylem. Some flowers can also utilize N via N_2-fixation by endophytic bacteria, such as *Curcuma alismatifolia* and *Vanda*. Some studies have shown the uptake, translocation, and assimilation of N-forms in flowers at different growth stages using ^{15}N-tracer feeding.

3.1 *Curcuma alismatifolia*

Curcuma alismatifolia, commonly known as the Siam tulip, is a flower bulb in the family Zingiberaceae. It is an economical flower crop in Thailand. Growers export rhizomes and cut flowers to other countries, including Japan, the Netherlands, the USA. The inflorescence of this plant is showy with pink and greenish bracts on a long peduncle (**Figure 5A**). The storage organ is the underground part of the plant and is so-called rhizome modified from the stem and attached to some storage roots (**Figure 5B**).

The rhizome is a major organ to store N, while carbohydrates are mostly stored in storage roots. The N concentration in the stubbed rhizome is 41–45 mgN gDW^{-1}, on average, while it was about 9–14 mgN gDW^{-1} in storage roots. Most of N in dormant rhizome was in PBS-insoluble form (79%), as a storage protein localized in the cytosol and cell wall, which presented as 10.6 and 12.0 kDa bands by SDS-PAGE staining. They contained five peptides and one peptide, respectively, when separated by 2D-PAGE. N in the storage roots was assimilated into different forms of free amino acids and protein compounds. The total free amino acid concentration in storage roots was higher than in the rhizome (343.8 and 109.0 μmol gDW^{-1}, respectively)

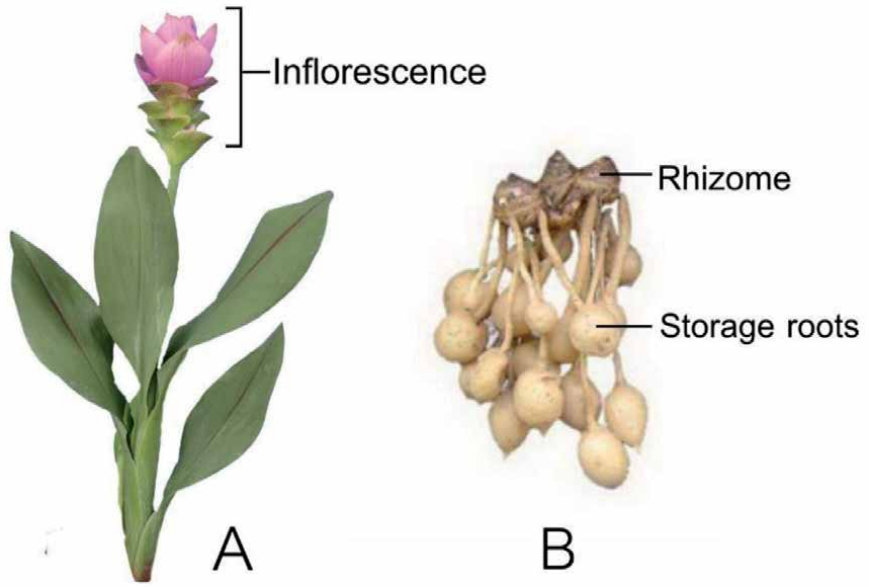

Figure 5.
Inflorescence (A) and rhizome (B) of Curcuma alismatifolia *Gagnep. (photo by Chaiartid Inkham).*

when the plants were supplied with 50 mgN L^{-1}. Most of the free amino acids in the rhizome and storage roots were arginine and glutamic acid, respectively. Proteins in the rhizome were at a higher concentration than those in storage roots with 197.4 and 46.7 mgN gDW^{-1}, respectively. A lack of N reduced the protein and free amino acid concentration in both organs. The carbon content in *Curcuma* rhizomes and storage roots was about 0.9 and 2.48 g C $plant^{-1}$, respectively, at the planting period, and it continuously decreased after planting. N and C stored in the rhizome was assimilated and utilized for root emergence and shoot sprouting [12–15].

At *Curcuma* shoot sprouting, fertilizer was generally supplied, and N was translocated via roots and leaves. Anatomical study of *Curcuma* roots and leaves showed the different sizes of cortical cells and the vascular bundle, which were larger in roots than leaves. However, the number of vascular bundles in the roots were lower than in the leaves. The abaxial leaf surface presented less barriers than the adaxial surface [16]. The N-use efficiency of fertilizer (%) via roots was 51–57%, which was higher than that via leaves (7–10%). The research by ^{15}N tracer revealed that N supply during the 1st and 2nd fully expanded leaf stage stimulated leaf growth, and N supply during the 3rd and 4th fully expanded leaf stage was translocate to utilize for flower blooming. N supply with 50 mg N L^{-1} increased the number of flowers and rhizomes compared with those at 25 mg N L^{-1} and lack of N supply. After translocation into plant organs, 81–97% of N was assimilated in an 80% ethanol insoluble fraction, mostly by proteins. N supply affected carbohydrate concentration in this plant, since nitrate reduction in roots requires carbohydrates for photosynthesis. The starch and sugar concentration in the rhizome and storage roots was high when *Curcuma* was grown under N deficiency. The translocation of carbohydrates to both storage organs was related to N deficiency conditions. From the vegetative stage until flowering, C content in leaves increased from 1.37 to 5.31 g C $plant^{-1}$. The ^{13}C exposure experiment revealed that C was accumulated in leaves during the vegetative stage and flowering, then it translocated to new storage organs (new rhizomes and storage roots) before plant dormancy [13, 15, 17, 18].

3.1.1 Effect of temperature on N uptake

Usually, N uptake and assimilation occur under normal temperature conditions. The optimum temperature for ^{15}N uptake in *Curcuma alismatifolia* was 25–35°C [19]. Day and night temperature also affected N uptake and assimilation. The nitrate concentration in leaves, nitrate reductase activity in roots, and total free amino acid content was higher in a plants grown at 30/18°C (day/night temperature) than at 30/25°C. However, a low night temperature reduced the number of shoots per plant and inflorescence quality (spike length and stalk length) in this plant [20, 21].

3.1.2 Response of curcuma to N application

Plant dry matter contains 2–4% N. The most important inorganic forms of N are ammonium (NH_4^+) and nitrate (NO_3^-), which are converted to an organic form, such as proteins, amino acid, and nucleic acids. There are three steps of N turnover in plants: 1) the conversion of inorganic N to organic N; 2) synthesis of high molecular weight N, such as protein and nucleic acids; and 3) breakdown of nitrogenous macromolecules by hydrolyzing enzymes [1]. Therefore, N supply is essential for growth, flower quality, and yield. However, the response to N was dependent on plant species, soil condition, temperature, and nutrition level. A lack of N reduced growth and development of *Curcuma*. The number of flowers and rhizome yield also decreased under 0 mg N compared with 25 and 50 mg N [13]. A field experiment was carried out with different N application rates at 3.75, 7.5, 15, 30, and 60 g N/plant, and the results demonstrated that supra-optimal N application at 60 g N/plant reduced plant height, number of shoots/plant, leaf area, and plant dry weight, but leaf N and leaf chlorophyll content increased. The research revealed that the leaf critical N for *C. alismatifolia*, calculated by the Mitscherlichs model, was 1.51% [21] The optimum fertilizer rate was different depending on the growth stage. The optimum N rates were 234, 937, and 468 kg N/hectares at the vegetative stage (45–75 days after planting), flowering stage (105 days after planting), and before rhizome harvest (135–165 days after planting), respectively (**Figure 6**) [22].

3.1.3 N_2 fixation and IAA synthesis in curcuma by endophytic bacteria

Nitrogen in the atmosphere that is fixed and converted to the organic form by microorganisms is termed N_2 fixation. In *Curcuma*, N_2 fixation by endophytic bacteria was first reported by Ruamrungsri et al. in 2009 [23]. The N-fixing rate varied depending on plant species. Eleven isolates were selected from *Curcuma alismatifolia* organs, such as the leaf, leaf base, and rhizome, and the N-fixing rate was 0.02–4.20 nmole $C_2H_4/10^6$ cells/hr. Seven isolates were derived from leaves, four isolates from the leaf base [23]. Isolates from the leaf base, i.e., ECS 202, identified using 16SrDNA, were *Sphingomonas pseudosanguinis* (99.2% similarity), ECS 203 was *Bacillus drentensis* and ECS 204 was *Bacillus methylotrophicus*. The colonization of these isolates was found in the intercellular spaces of different organs, i.e., roots, leaf base, and rhizome (**Figure 7**). Re-inoculation with these isolates into *Curcuma* plantlets derived from in vitro propagation was done by soaking roots in 10^6 cells/ml of these bacteria. Results showed that plant height, total leaf area (cm^2), and N content in roots and leaves of plants inoculated with ECS 203 was higher than the control [24].

3.2 *Narcissus*

Narcissus is a bulbs that has a storage organ that is modified from the leaf base in scales (**Figure 8**). Generally, the grower grows bulbs in autumn, and flowering

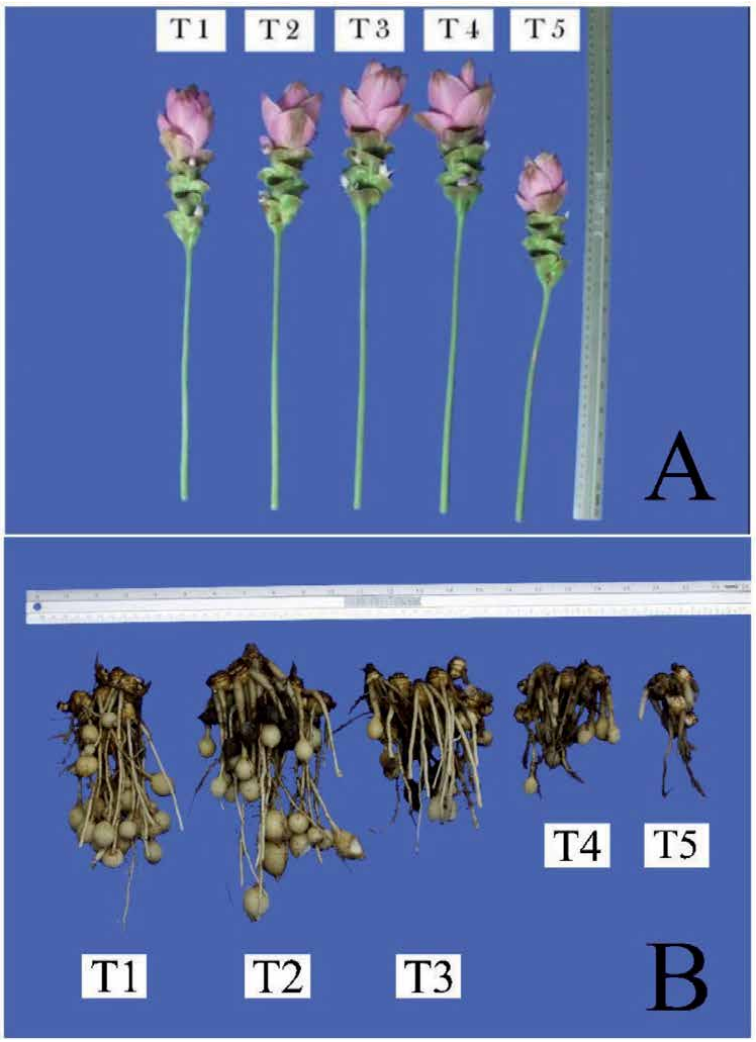

Figure 6.
Nitrogen application affected flower quality (A) and rhizome yield (B) of Curcuma alismatifolia *(T1–T5 were applied N at 234, 468, 937, 1,875, and 3,750 kg N/hectare). (Ruantip et al. [22]).*

occurs in spring. Its roots are an unbranched system that emerges under low temperatures of 9°C. A lack of nitrogen decreased shoot height, root length, chlorophyll intensity, and dry weight of the plant. N-deficient leaves were yellow and small. N concentration was about 12.47 mg gDW^{-1}, which was lower than the control plant (84.09 mgN gDW^{-1}). Sugar content in N-deficient roots was also higher than the control (with N supply), indicating that N metabolism required carbohydrates as an energy source for nitrate reduction and assimilation. Nitrogen was absorbed from fertilizer application in the winter. The N absorbed by roots was translocated to other organs after shoot emergence to promote growth and development. Therefore, N supply was required after root emergence, although the N in the mother bulb was utilized for root growth and shoot sprouting. After shoot sprouting, leaves were the sink organ to derive N from the mother bulb and fertilizer until flower senescence. The uptake of ammonium and nitrate was studied in *Narcissus* using a ^{15}N tracer. The results showed that at 2 days after fertilization with 1.0 mM of $^{15}NH_4^+$-N compared with 1.0 mM of $^{15}NO_3$-N and 0.5 mM of $^{15}NH_4^+$-N plus 0.5 mM of NO_3^--N, *Narcissus* 'Garden Giant' roots could more rapidly uptake NH_4^+-N than NO_3^--N at 2 days after

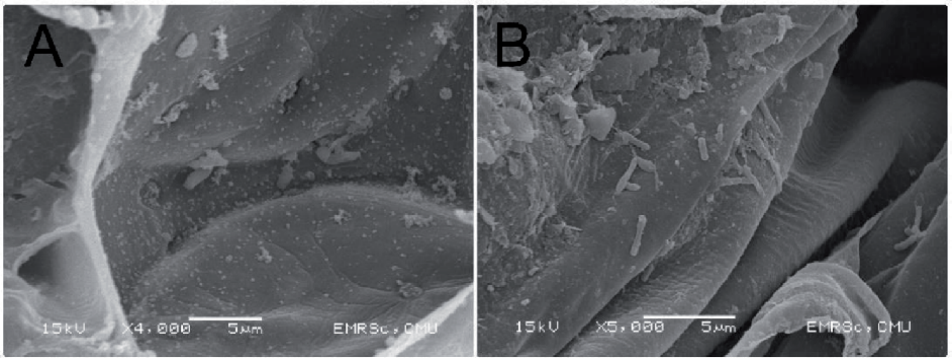

Figure 7.
Endophytic bacteria (A) isolate ECS 202 in the rhizome and (B) ECS 203 in the roots of Curcuma alismatifolia*. (photo by Soraya Ruamrungsri).*

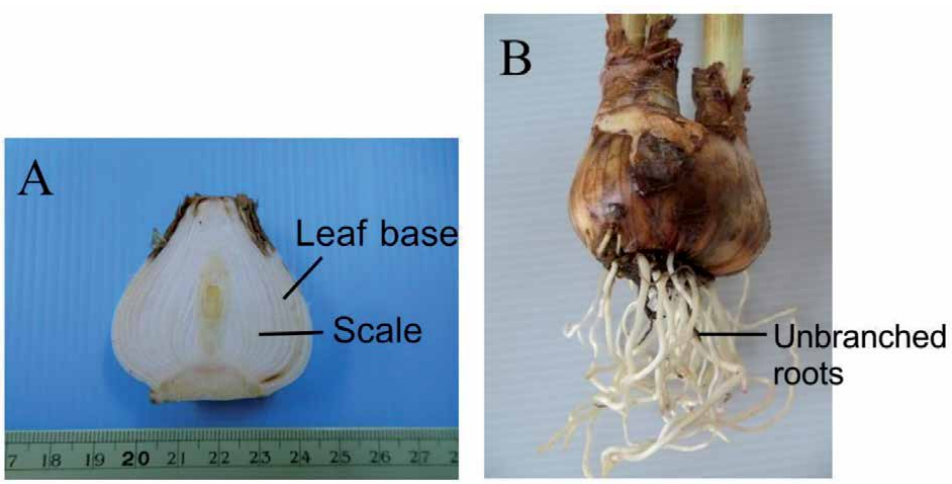

Figure 8.
Tunicated bulb of narcissus with young flower inside (A) and unbranched roots (B). (photo by Soraya Ruamrungsri).

fertilization, and the rate was equal between NH_4^+-N and NO_3^--N for 4–7 days after fertilization. Moreover, the assimilation of N into free amino acids was also different based on the N-form. At 2 days after $^{15}NH_4^+$-N fertilization, N in the roots was incorporated into free amino acids, mostly as glutamine, while asparagine and glutamine were the major assimilation forms of $^{15}NO_3^-$-N supply. [25–28].

3.3 Orchids

The growth of orchids can be presented in two ways, sympodial and monopodial habits, depending on the genus. Some orchids have enlarged bulbous organs at the base of their leaves, called a pseudobulb, with a different shape. These organs store food and mineral nutrition for plant growth and development [29].

3.3.1 Vanda

Vanda is a tropical orchid comprised of 40 species. It is an economical orchid with a high export value. The roots of *Vanda* are aerial roots, freely hanging in the

air. Growers use fertilizer applications to control flowering; however, research on plant nutrition in this plant is still rare.

The experiment on N uptake and assimilation in *Vanda* was carried out within 7 and 30 days after fertilization. The results revealed that plant uptake of $^{15}NO_3$-N occurred more rapidly than $^{15}NH_4^+$-N or their combination. However, ^{15}N use efficiency was higher in the mixed N source than the sole $^{15}NO_3$ or $^{15}NH_4^+$ form. N source affected the uptake and translocation of N to various organs, and *Vanda* prefers NO_3^--N to NH_4^+-N. N assimilation in *Vanda* was different among organs. Alanine distribution was high in leaves and roots at 7 days after feeding. Tyrosine distribution was predominant in leaves, while glutamine distribution was high in stems and roots at 30 days after fertilization [30].

3.3.2 Dendrobium

In Thailand, *Dendrobium* is an important orchid genus for export. The main area for *Dendrobium* production is in the central region of Thailand, such as Ratchaburi, Nakhon, and Pathom provinces. Fertilizer application affected the growth and development of this orchid. A combination of N sources (NO_3^- and NH_4^+) promoted the height of psuedobulbs, number of leaves and canes, spike length, and flowering percentage. The N concentration in leaves, roots, and psuedobulbs was 1.24, 0.97, and 0.61%, respectively. *Dendrobium* prefers a mixed N-source (ammonium and nitrate) to a sole N source [31].

3.3.3 Phalaenopsis

N level affected the growth of *Phalaenopsis* orchids. A concentration of 150–200 mg N L^{-1} increased the inflorescence length, stalk length, and number of flowers per stem. Supplying fertilizer with 21 N-21P_2O_5-21K_2O at 150 mg L^{-1} once a week to young plants increased the leaf area, leaf dry weight, and N concentration [32]. However, when a high N concentration (200 mg N L^{-1}) was supplied, the K concentration should also be supplied at 200 mg K L^{-1} to obtain healthy leaves and good quality flowers [33].

3.4 Rose

Rose is the highest potential cut flower in the world market. Rose quality is graded by stem length and flower size. N levels affected shoot height, shoot and flower diameter, flower fresh and dry weight, number of petals per flower, and flower yield. The N concentration in aboveground organs and roots was 19.4 and 20.9 mg N g DW^{-1} higher, respectively, when the plant was supplied with 200 mg N L^{-1}. The lack of N decreased all quality parameters and showed deficiency symptoms. The optimum N concentration for roses was 200 mg N L^{-1} $plant^{-1}$ once a week [34].

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 9

Nitrogen Storage in Crops: Case Study of Zeins in Maize

Marija Duvnjak, Kristina Kljak and Darko Grbeša

Abstract

Crop grains accumulate significant amounts of nitrogen in the form of storage proteins. Grain storage proteins are not only important in the aspects of germination but also, storage proteins are a valuable food source in human and animal nutrition. This chapter will give insight into genotype and growing conditions influencing the quantity and quality of storage proteins, primarily maize storage proteins the leading cereal by world production. Main storage proteins in cereals are prolamins, and in maize prolamins are called zeins located within the endosperm in protein agglomerations called protein bodies. Four main classes of zein proteins are: alpha, beta, gamma and delta zein. Each of four zein classes has a distinctive position and role within protein bodies. Prolamin proteins define nutritional value of maize grain not only via amino acid quality but also via starch availability. Starch, the most important energy component of maize grain, is located within starch-protein matrix. Within this matrix, starch granules are surrounded by protein bodies that limit starch availability. In this chapter, we will describe how zein proteins influence characteristics of maize grain and nutritional value of maize.

Keywords: maize grain, zein proteins, starch, amino acid quality, starch digestibility, maize nutritional value, animal nutrition

1. Introduction

The protein content in cereal grain can vary greatly, from less than 6% to more than 20% in dry matter (DM), and this content depends on several factors such as the type of cereal, variety, agrotechnical conditions and others. These factors can be divided into two major groups, genotype and environment. Today, producers manipulate these factors to obtain grain of good quality and high protein content. For example, it has been found that the rate of maize yield gain is significantly higher after application of 220 kg N ha^{-1} (0.12 Mg ha^{-1} $year^{-1}$) than without fertilization (0.05 Mg ha^{-1} $year^{-1}$) [1].

In terms of their functions, there are three types of cereal grain proteins: structural proteins (as membrane proteins), metabolic proteins (as enzymes and enzyme inhibitors) and storage proteins, the largest fraction occurring primarily in starchy endosperm. Storage proteins account for 70–80% of the total protein content in grain and have a unique structure. The primary function of storage proteins is to supply grain embryo with nitrogen and amino acids during germination. However, these proteins are also a valuable food and feed source in human and animal nutrition.

The major storage proteins in cereal grains are called prolamins; the name is derived from their high content of the amino acids proline (Pro) and glutamine (Gln). However, the exact name of prolamins differs in different cereals – in maize prolamins are called zeins, in wheat gliadins, in oat avenins, in barley hordeins, in rye secalins, and in sorghum kafirins.

In human and animal nutrition, cereal crops are an important diet ingredient, and maize is the most commonly used cereal crop. Maize grain is palatable, highly digestible both by humans and animals, and it is an excellent source of metabolizable energy (ME). Although maize grain is low in protein content, the amount used in livestock production makes it an important source of protein in animal diets [2].

2. Maize storage proteins - zeins

Several types of storage proteins are classified in maize depending on their extraction in different solvents. About 70% of maize grain proteins are storage proteins, of which more than 60% are zein (prolamin) proteins. Except zeins, other types of storage proteins include albumins, globulins, and glutelins [3].

First reports describing zeins date back to 1821 when J. Gorham named proteins isolated from maize 'zeine'. T.B. Osborne, the founder of seed storage protein research, classified zeins as prolamins and developed first extraction methods based on their hydrophobic nature at the beginning of 20 century [4].

Maize zein proteins are located in endosperm within protein aggregates called protein bodies (PBs) consisting of four distinctive zein proteins: alpha (α), beta (β), delta (δ) and gamma (γ) zein (**Figure 1**). The PBs are part of starch-protein matrix, where starch granules are surrounded with abundant zein proteins in PBs embedded in a matrix of glutelin proteins [7].

As shown in **Figure 1**, the location of each distinctive zein differs within PB. Alpha zeins are the most abundant group of zein proteins located in the central part of PB together with delta zeins. They account for up to 70% of total zeins. Based on apparent migration rates on SDS-page, two distinctive alpha zeins are defined, namely relative molecular mass (Mr) 19-kDa and Mr 22-kDa alpha zeins [8].

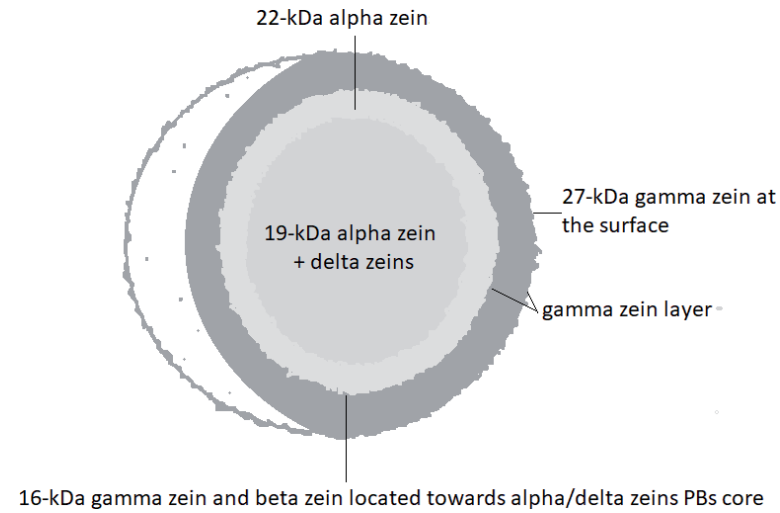

Figure 1.
Shematic presentation of zein proteins location within protein bodies. Adapted data from the [5, 6].

Gamma zeins, the second most abundant zeins (up to 20% in the total zein fraction), are considered to be the most important zeins. This zein class is located on the surface of PB and in small spots within where they stabilize alpha zein core together with beta zein. Gamma zeins are responsible for number and features of PBs and consist of three distinctive proteins: 16-kDa gamma zein, 27-kDa gamma zein and 50-kDa gamma zein [8, 9]. The 27-kDa gamma zein is the most abundant gamma zein, followed by 16-kDa gamma zein and 50-kDa gamma zein, with the latter being in low abundance and for long misidentified as a dimer of the 27-kDa [10]. Based on the apparent migration rates on SDS-page, two distinctive delta zeins (10-kDa and 18-kDa) and one beta zein (15-kDa) are defined [8, 9].

Beta zein and delta zeins are expressed in much lower amounts in maize endosperm. Transcripts encoding alpha zeins account for about 30%, for gamma zeins about 10% whereas for beta and delta zeins 5% and 1.5% of total transcripts, respectively. In the same study, zein specific sequences accounted for almost 50% of the total cDNAs obtained from development of the maize endosperm [10] demonstrating the importance of zein proteins during nitrogen accumulation in maize grain.

2.1 Zein genes

Zein proteins are encoded by a large superfamily of genes. Gamma, beta and delta zeins are encoded by single-copy genes (3 for gamma, 1 for beta, 2 for delta). With some exceptions, two members of delta zeins originate from diploidization, and in quality protein maize (QPM) the genes encoding for 27-kDa gamma zein are duplicated [5]. Alpha zeins are different from the other zein proteins; this group of zein proteins are encoded by four multimember gene families, three for 19-kDa alpha zeins and one for 22-kDa alpha zeins [5]. Studies show large gene clusters that are sometimes disrupted by transposons or other genes [11, 12]. Both 19-kDa and 22-kDa alpha zeins share a common ancestor [12] which underwent amplification and chromosome translocation during evolution [13]. Alpha zeins gene families are located at seven chromosomal sites, but the exact location, and number and organization of genes varies greatly between different inbred lines. Furthermore, not all alpha zein genes are expressed, but only selected members of each alpha zein family [5]. For example, a detailed expression analysis of inbred line of B73 maize showed that only 18 of the 41 alpha zein genes were expressed [14].

2.2 Zein amino acid structure

Zein proteins, and prolamin proteins in general, are rich in amino acids Pro and Gln. However, zeins are devoided of essential amino acids lysine (Lys) and tryptophane (Trp). Thus, a great deal of research has been done to increase the amount of essential amino acids in zein proteins. Besides, the nutritional properties, solubility and chemical structure of zeins are influenced by amino acid characteristics. Zein proteins contain only a few charged amino acids and as a consequence have a hydrophobic nature and thus are insoluble in water. Solubility and chemical structure are important because they define and influence processing and manufacturing of food and feed [15].

Alpha zeins are encoded by large multigene families, and besides Pro and Gln, contain a high proportion of hydrophobic amino acids alanine (Ala) and leucine (Leu). Their structure is largely defined by a series of tandemly repeated peptides of 20 amino acids with nine repeats in the 19-kDa and ten in the 22-kDa alpha zeins. Each repeat is flanked by clusters of Gln residues [6]. Due to their structure, alpha zeins can be extracted with aqueous alcohol [4].

In addition to Pro and Gln, gamma zeins are rich in the cysteine (Cys) which has strong disulfide bonds and thus influencing stability and extractability of zeins [16]. Six highly conserved Cys-rich domains are found in gamma zein proteins [6]. All zein proteins, with the exception of alpha zein proteins, have a high content of sulfur-rich amino acids, which can vary in expression levels among maize cultivars. As mentioned above, gamma zeins are rich in Cys, delta zeins in methionine (Met), and beta zein in both Cys and Met [3].

The amino acid compositions in the most abundant zein proteins are shown in **Figure 2**.

2.3 The formation of protein bodies

Zein proteins form PBs, insoluble protein aggregates located in the starch-protein matrix. The expression of distinctive zein proteins controls the initiation and development of PBs. Immunogold staining showed that PBs start to aggregate approximately 9 days after pollination as small accretions mainly of gamma and beta zeins. During the PBs growth, alpha zeins and delta zeins enter the PB core and are responsible for the growth and the expansion of the PBs [6]. Each zein protein has a proposed distinctive role in initiation, formation and growth of PBs. The RNA interference (RNAi) technique was used to reduce the expression of a specific zein gene, and it showed that reduction of 22-kDa alpha zein led to PBs with an unusual budding structure [16]. RNAi suppression of both 19-kDa and 22-kDa alpha zein resulted in smaller PBs and with their typical number [17], indicating that 22-kDa can function in PBs morphology and 19-kDa alpha zein can function in PBs growth [5]. RNAi suppression of 27-kDa gamma zein resulted in fewer PBs,

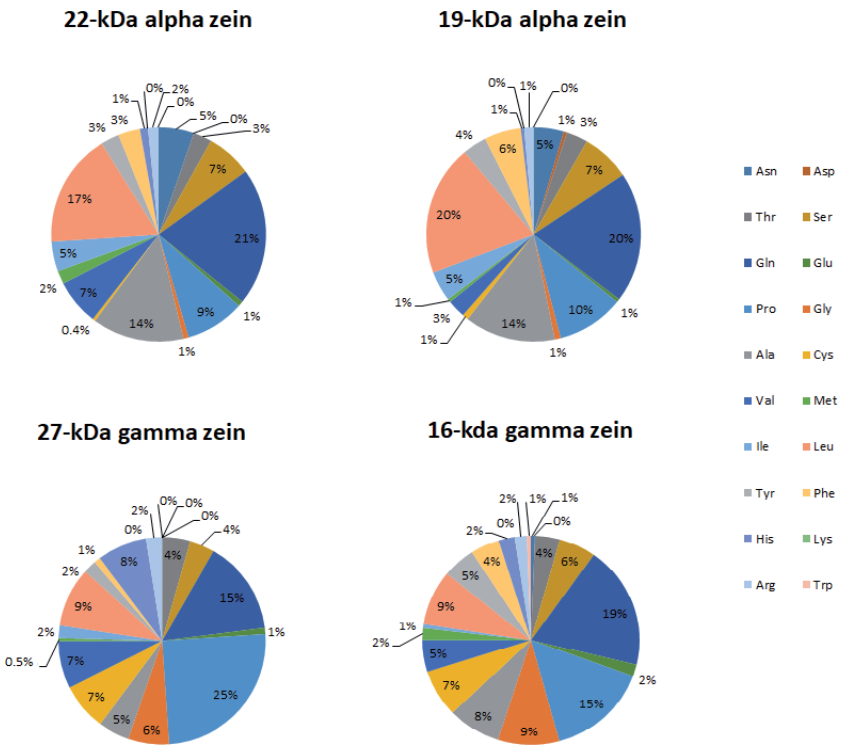

Figure 2.
Amino acid composition in most abundant zein proteins. Adapted data from the [6].

while suppression of 16-kDa and 50-kDa gamma zein resulted in smaller PBs but with their normal numbers. Results indicate a significant role of 27-kDa gamma zein in PBs initiation whereas 16-kDa and 50-kDa gamma zeins have a function in PBs expansion [17]. As the inbred line A654, deprived of both delta zeins, has PBs similar to other inbred lines with normal delta zein quantities [16], Li and Song assumed that delta zein has no essential role in the formation of PBs [5]. A study using a yeast two-hybrid system showed strong protein–protein interactions between all gamma zeins and beta zein, weak within alpha zeins, although they both interact strongly with the 10-kDa delta zein, 16-kDa gamma zein and beta zein whereas interacting poorly 27-kDa and 50-kDa gamma zeins [18]. The affinities shown here are consistent with the proposed location and role of each zein protein: 27-kDa gamma zein is responsible for the initiation and is located at the surface of PBs and 16-kDa gamma and beta zein are located towards the inner parts and stabilize alpha/delta zeins of PBs core (**Figure 1**).

2.4 Analysis of zein proteins

Biologically, zein proteins make a mixture of proteins varying in molecular size, solubility and charge [4]. Solubility of most zeins is good in aqueous-alcohol solutions, 60% isopropanol, 70% ethanol, 95% methanol. However, due to the high disulphide bonds in gamma zeins, this type of zein protein is only extractable when a strong reducing agent, such as 2-mercaptoethanol, is added [19].

When analyzing zein proteins, different extraction procedures are applied: wide range of aqueous ethanol solutions [20–22] or aqueous isopropanol solutions [23, 24], highly concentrated alkali solutions (pH 11 or above) [25], highly concentrated aqueous urea solutions (8 M) [26], or anionic detergent-containing solutions [20, 26, 27]. The extractions are performed on wide range of extraction temperatures, ranging from 25 to 130°C [20, 21, 26], with or without addition of reducing agents as 2% 2-mercaptoethanol [27] or 10 mM DTT in 25 mM ammonium hydroxide [22]. Solubility-enhancing ingredients, such as 0.0125 M sodium borate [27], 0.5% sodium hydroxide [28], 0.5% sodium bisulfite [24] are also often added.

As zein proteins are a divergent group of proteins, extraction procedures vary significantly. Hence, analysis of zein extracts is complex and include the application of various techniques often used in protein separations. Combination of electrophoresis methods, such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which only provides the molecular weights (MW) [22, 27, 29, 30], or the two-dimensional gel electrophoresis (2DE), which provides insight into MW and charge [22], with or without the mass spectrometry (MS) for protein identification [22, 31], are used in zein analysis. Capillary electrophoresis (CE), the electrophoretic method also used for zein separation, has enabled the identification of more fractions by MS [32]. Other methods of zein separation include chromatographic methods such as reversed-phase HPLC method on C18 column with a gradient of 45 to 75% acetonitrile-ultrapure water, both containing 0.01% trifluoroacetic acid [33] or with acetonitrile-trifuoroacetic acid gradient [34]. Liquid chromatography-mass spectrometry (LC–MS), a method widely used in proteome analysis due to its high sensitivity, was for the first time adopted for zein evaluation in 2020; the authors coupled multi-enzyme digestion with nano-LC–MS/MS [35]. Separation techniques mentioned here are used for the analysis of protein compositions in biological samples of high complexity [22]. Regardless of numerous approaches in zein analysis, the most suitable method will be selected based on desired trait of maize zein proteins.

3. Conditions influencing maize zein content

Factors affecting zein proteins in maize grain can be divided into two main groups – genotype and environment. The genotype is regarded as a primary factor influencing zein properties, while environment (nitrogen fertilization, irrigation, high temperatures, etc.) affect to a lesser extent causing small variability within the same variety.

3.1 Genotype

Maize is usually subdefined according to the kernel characteristic determined by grain vitreousness. Grain vitreousness is an important agronomic trait that influences hardness and post-harvest resistance to insects and fungi, rate of starch digestibility, and semolina yield for food production [36]. It is defined as a ratio of vitreous to floury endosperm and is strongly affected by the type and quantity of zeins within the starch-protein matrix in maize endosperm. The endosperm of flint maize consists mainly of vitreous while floury maize contains almost exclusively floury endosperm [37]. Dent maize hybrids, which are derivatives of flint-floury classes, differ in their ratio of vitreous to floury endosperm.

Zein proteins surround starch granules in the starch-protein matrix in both types of endosperm but with different interactions. In vitreous endosperm, smaller starch granules are tightly packed in multiple and well developed PBs. In contrast, in the floury endosperm, starch granules are larger, and the protein layer is thinner with lesser PBs and with numerous air-filled spaces (**Figure 3**). The texture differences between vitreous and floury endosperm lead to differences in physical properties of maize samples varying in vitreousness [38]. Kljak and coauthors showed, in a study of 22 maize samples varying in kernel vitreousness (50.23% – 76.41%) and zein content (53.86–86.37 g/kg endosperm DM), that zein proteins, acting through starch-protein interactions, have the most important influence on the maize virtuousness in comparison to the other characteristics of endosperm as amylose content and starch granule size and shape [39].

Zein proteins, as the largest protein fraction in grain, define the quality of maize protein. As mentioned above, zein proteins are devoided of essential amino acids

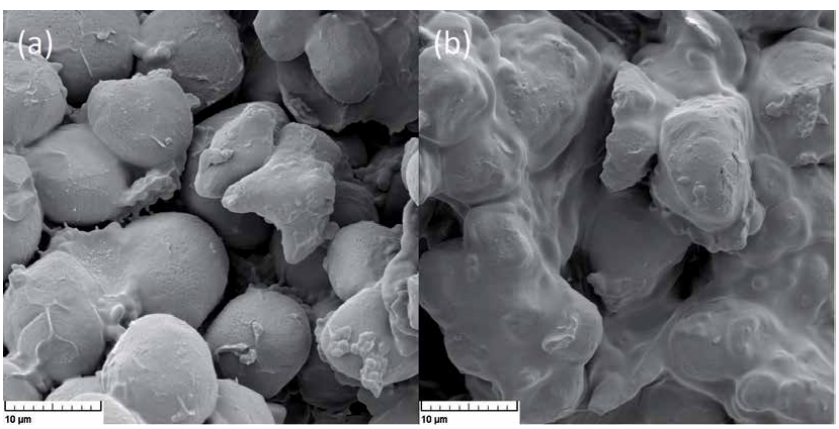

Figure 3.
Scanning electron micrographs of ground samples of maize hybrids varying in endosperm texture. Larger starch granules with thinner protein layer in the floury endosperm (a) opposite to smaller starch granules with well-developed protein layer in the vitreous endosperm (b). The maize grain endosperm morphological features (starch-protein interactions) were examined visually on 1 mm ground samples using a scanning electron microscope (SEM) (FE-SEM/Mira, Tescan, Brno, Czech Republic) with magnification 5000x.

Lys and Trp, and to some extent Met due to the low Met content in alpha zeins. Therefore, despite high yields and sufficient production quantities, amino acid composition limits the usage of maize in nutrition of both humans and monogastric animals. Maize alone cannot provide a balanced meal, and it must be supplemented with sources of essential amino acids, as environmentally questionable soya or other amino acid supplements in the diet of monogastric animals. As a result, maize deficiency in amino acids is increasing costs of food and feed supply worldwide. In order to counteract this, researchers in the last century have focused on regulating the expression and accumulation of zeins in maize.

High Lys and Trp maize mutants have been developed. In the *opaque2* (*o2*) mutant regulating zein O2 transcription factor (TF), alpha zeins have been reduced with compensatory higher amount of non-zein maize proteins. However, those mutants have undesirably soft, opaque and brittle endosperm and their use is limited due to the poor agronomic performance [40]. On the other hand, later developed quality protein maize (QPM) with higher Lys and Trp regained vitreous endosperm and agronomic performances of normal maize [41]. The QPM has zein content different from wild-type or other maize mutants. This maize has a low content of alpha zeins and accumulates a high amount of 27-kDa gamma zeins, which confirms that gamma zeins are essential in PB functionality and preserved hardness in QPM [29]. Liu and coauthors, in their study using genome-wild association study analysis, linkage mapping analysis, and map-based cloning, showed that duplication of gene encoding 27-kDa gamma zein resulted in overexpression of this class of gamma zein protein [30].

The expression of most zein proteins is regulated by one or more TFs, however, maize mutations involving zein proteins may include mutations that can alter the accumulation of zeins in PBs, resulting in abnormal PBs and opaque endosperms or mutations in genes involved in amino acid biosynthesis [5]. For example, affecting myosin XI proteins in *opaque1* (*O1*) leads to an increased number of misshapen PBs [42]. The *floury1* (*Fl1*) mutation leads to a disrupted accumulation of 22-kDa alpha zein in the outer gamma zein region and 19-kDa alpha zein core rather than in the proposed discrete ring at the outer edge of PBs core. However, without changing the PBs size, shape or abundance [43]. Mutant *O10* with mutation in *opaque10* leads to misshapen PBs, probably caused by disruption of the discrete ring-shaped outer core of PBs containing 22-kDa alpha and 16-kDa gamma zeins [44]. Mutations that lead to altered retention of zeins in PBs include *fl2, DeB30, fl4, mucronate1 (mc1)* mutation affecting 22-kDa alpha zein, 19-kDa alpha zein, 19-kDa alpha zein and 16-kDa gamma zein, respectively [5].

Mutations affecting amino acid biosynthesis include the *Pro1* mutant with inhibited Pro biosynthesis resulting in a lower amount of Pro while the *mto140* mutant inhibits the Tyr and Phe biosynthesis. However, both mutations lead to a general reduction of the accumulation of zein proteins and not to a specific zein reduction. The characterization of this type of maize opaque endosperm mutants suggests that amino acid limitations repress zein protein biosynthesis [5]. Other mutations affecting the expression and accumulation of zein proteins on the translation level are *opaque 7 (o7)* mutant with defective Acyl-CoA synthetase [45] and *zmocd1* mutant affecting oxalyl-CoA decarboxylase [46]. Both *o7* and *zmocd1* have a major impact on amino acid biosynthesis by affecting α-ketoglutaric acid and oxaloacetic acid leading to altered endosperm metabolome and opaque endosperm with reduced zein content [5].

3.2 Environment

Duvick compared the yield of American hybrids grown from 1934 to 2004 and showed that the average annual increase of maize grain yield is 115 kg/ha. The

comparison also showed that genotype contributes 50–60% to variability of hybrids (hybrids more resistant to abiotic and biotic stresses) while remaining 40–50% is affected by agricultural technology (fertilizers application, control of diseases and pests, etc.), which demonstrates the importance of not only genetic maize characteristics but also agronomic technology improvements [47].

Agronomic improvements in maize yield have been extensively evaluated; major factors are water management (irrigation) and the application of nitrogen fertilizer depending on environmental conditions. In the United States, for example, the importance of irrigation practices and plant population in the low rainfall Western region of the U.S. corn-belt has been emphasized. In contrast, nitrogen fertilizer use and plant population have been emphasized in the high rainfall Central and Eastern regions [48]. Nitrogen fertilizers are important in modern maize production; the rate of maize yield gain is much higher when 220 kgN ha^{-1} is applied compared to when no fertilizer is applied (0.12 vs. 0.05 Mg ha^{-1} $year^{-1}$, respectively) [1].

Nitrogen fertilizers are required to maintain maize production and soil fertility, however, negative effects of their use on the environment are a global concern, and thus, nitrogen use efficiency (NUE) is an extremely important issue. Nitrogen use efficiency is defined as the amount of grain produced per unit N accumulated above what is provided by soil N mineralization [1]. In modern maize hybrids, the increase in grain yield is often accompanied by a reduction in grain protein concentration [49]. However, Mueller and coauthors in their analyses of NUE in maize hybrids showed that grain yields increased faster than grain N concentration decreased. The same authors concluded that although previous research indicated that maize grain yields and NUE gains over time were primarily due to greater total N accumulation and dilution of grain N accumulation, changes in N accumulation within the plant itself are important to achieve efficient N conversion into grain yield. Key plant factors are increased stem N remobilization and retention of leaf N during reproductive growth [1].

Precipitations and temperature are two key environmental factors affecting maize grain yields and N accumulation. The warm climate will accelerate the phenological development (e.g. leaf appearance), however, drought and high temperatures at pollination as well as during the grain-filling period will reduce yields [50]. In controlled environmental studies when temperature exceeds normal temperatures by only 3°C, maize grain yields were reduced by half [51]. In field studies, rise in 6°C during grain filling period resulted in 13-88% reduction of maize grain yields and yield loss was much larger under fertilization (authors compared application of 200, 100 and 0 kg N ha^{-1}) [52]. Some studies show that protein concentration is positively correlated with high temperature during cereal grain growth [53]. However, Monjardino and coauthors concluded that heat stress during early stages of endosperm development reduces zein accumulation at synthesis level while later in development had no significant effect on zein quantity. Later during kernel development, the reduction in zeins was mainly result of protein degradation, which appears to be a part of the natural progression of kernel development [54].

4. Zein proteins influence on maize nutritional value

In maize, starch and proteins account for approximately 70% and 10% of grain DM, respectively. Depending the cultivar, starch has the potential for complete digestion in livestock digestive tract [2], and thus, it is the most important energy component in animal diets. Opposite to high energy potential of starch, zein proteins are devoided of essential amino acids Lys and Trp, with the exception for

50-kDa gamma zein which contains 2.7% of Lys. The lack of essential amino acids, Lys, Trp, and to some extent of Met is important characteristic of zein proteins which limits the use of maize protein in food and feed industries. As mentioned earlier, new maize mutants, with different amino acids ratios and protein quality have been developed by maize breeders' worldwide. The change in amino acid ratios is mostly due to the change of zein proteins compared to other grain proteins [5]. Of maize mutants, QPM, shows excellent characteristics due to the higher Lys and Trp contents and agronomic performances of normal corn. With the use of QPM or high-lysine maize, the need for Lys supplements (soybean meal, synthetic amino acid supplements) is reduced. Although QPM was primarily intended for human nutrition in developing countries, it is nowadays implemented in swine and poultry nutrition as well [2, 41].

In standard diet of monogastric animal, maize grain and soybean meal complement each other. Zein proteins have adequate quantities of sulfur amino acids, Cys and Met (depending on the quantities of alpha zeins) and low Lys and Trp whereas soya has adequate quantities of Lys and Trp and is a relatively poor source of sulfur amino acids [2]. However, it should be noted that higher protein content in maize is often connected with higher zein content, resulting in even lower quantities of Lys and Trp compared to normal maize. Thus, when using maize in monogastric animal nutrition, the amount of essential amino acids relative to energy and total feed intake is more important than the quantity of total protein [55].

The second important aspect of zein influence on maize nutritional value is related to starch rather than to the proteins. As starch granules are embedded in protein layer with PBs, zein poses a physical barrier that can limit the starch availability to digestive enzymes and rumen microorganisms [56]. As a result, starch digestibility in maize varieties with higher zein content will be slower. Maize grain with higher vitreousness has lower digestibility than grains of lower vitreousness containing less zein [7, 56–58]. Kljak and co-authors in their study on eight yellow high-yield maize hybrids varying in zein content (from 70.3 to 88.7 g kg^{-1} of total starch) showed that fractional starch digestion rate (kd) in *in vitro* poultry digestibility experiment correlated negatively to zein content (-0.36, $P < 0.05$). The authors concluded that when starch granules are embedded in a complex protein matrix, zein limits their accessibility to enzymes and affects the starch digestibility rate to a greater extent than starch properties [59].

Furthermore, when maize grain is subjected to different processing methods as silage production [33, 60] or steam flanking [61], reduction in zein content and destruction of the starch-protein matrix will occur [33]. As a result, starch granules will become easily accessible to digestive enzymes and amylolytic bacteria [61], starch digestibility increases, and starch will be digested in a higher rate [62–65].

The influence of zein proteins on starch digestibility is becoming a key factor when determining starch efficiency in poultry and swine. Since starch is almost completely digested in their digestive tract, the rate of starch digestibility is the key determinant of animal performance. Weurding showed the importance of evaluation of starch digestion rate for poultry [66]. Higher content of slowly digestible starch appears to support faster, more efficient poultry growth, and the feeds with starch digestibility rates closer to 1.26 h^{-1} seem to be the most efficient [59]. The results of studies showed that lower digestibility rates are related to a higher amount of zein proteins, and thus, maize hybrids with a higher content of slowly digestible starch are desired. Consequently, when selecting the appropriate maize hybrid for production, in particular for use in animal nutrition, it would not be efficient to know only the quantity of starch but also the quantity of the zein proteins surrounding the starch, since the zein proteins determine the availability of starch and the efficiency of starch digestion.

5. Concluding remarks

Zein proteins, maize storage proteins located in starch-protein matrix of endosperm are paramount for maize nutritional value. They not only define amino acid characteristics of maize grain but are a primary factor affecting starch availability and digestibility. Therefore, genotype and environment effects on zein protein composition and content in grain present a basis to regulate maize nutritional value.

Conflict of interest

The authors declare that there is no conflict of interest.

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Section 4

Plant-Microbe Interactions

Chapter 10

Conservation of Edible Ectomycorrhizal Mushrooms: Understanding of the ECM Fungi Mediated Carbon and Nitrogen Movement within Forest Ecosystems

Lu-Min Vaario and Norihisa Matsushita

Abstract

Most edible ectomycorrhizal (ECM) mushrooms are currently harvested from nature and many of them are high-priced. Demand for the wild mushrooms as a culinary delicacy has stimulated research that aims to understand (1) the puzzled role that the ECM fungi play in the forest ecosystem, and (2) nutritional and other requirements for fruiting, which is highly variable. In this review, we focus on understanding of the ECM fungi mediated carbon and nitrogen movement between the symbiotic partners and on the interactions with other fungi in forest ecosystems. Thereby, we better understand the diverse nitrogen requirements for edible ECM fungal growth and mushroom fruiting. We attempt to provide a theoretical basis for the future research of edible ECM mushrooms in wild and controlled conditions.

Keywords: culture, cultivation, ectomycorrhizal fungi, edible mushrooms, nitrogen uptake

1. Introduction

Forests play a crucial role in the global environment and economy. Forest-based wood products as well as non-wood forest products have offered remarkable resources and benefits for the well-being of people [1, 2]. A healthy and well-growing forest system is largely dependent on available soil nutrients and efficient nutrient cycling [3, 4], especially nitrogen (N). As we know, nitrogen is a limiting resource for plant growth in many temperate forests.

Nitrogen is necessary for plants. Most crops require N relatively high amounts, but only a small amount of available N is present in soil at a time. A large source of soil N is the atmospheric dinitrogen (N2), the major gas of air (79%) [5]. Only certain microorganisms can bind molecular nitrogen from air. All other organisms need to take up nitrogen from soil. Soil organic matter (especially humus) acts as a storage and supplier of nitrogen for plant roots and microorganisms;

almost 90–95% of soil total nitrogen originates from soil organic matter [6, 7]. Plants acquire N mostly from the inorganic forms such as ammonium and nitrate. However, plants that associate with mycorrhizal fungi are considered to have greater access to organic nitrogen pools when compared to non-mycorrhizal plants [5].

ECM fungi play an important role in the nutrient cycle of terrestrial ecosystems. Especially in a forest poor in nutrients, the growth of trees depends on the existence of mycorrhizal fungi. The value of ECM fungi is evaluated from the global framework. ECM fungi provide hidden biological fertilizers for increasing plant biomass, conventional afforestation, and ecosystem restoration practices; they also control soil pathogens [8–10].

In addition to benefits for forests, many ECM fungi produce edible mushrooms that are widely appreciated for their nutritional, medicinal, and gastronomic properties [11]. One of the major challenges of the twenty-first century is to produce sufficient food. From that perspective, wild mushrooms as non-wood forest products are getting more and more attention globally [12]. It would be convenient if these mushrooms could be cultivated. However, most edible ECM mushrooms can only be collected from nature and not cultivated artificially [11]. The main obstacle to the cultivation of edible ECM mushroom is their need to be associated with a host plant in plantations. The association is obligatory for the successful growth and fruiting of the mushrooms. The unanimous discussion of the nutritional growth requirements of ECM edible fungi is a topic of interest for scientists.

An in-depth understanding of the nutritional requirements of ECM fungi and the role of ECM fungi in nutrient cycling, particularly in ECM fungi mediating carbon and nitrogen movement within forest ecosystems will be summarized in this chapter. The nutritional requirements to successfully culture and cultivate ECM fungi will be discussed.

2. Ectomycorrhizal fungi

2.1 Ectomycorrhizal fungi

Ectomycorrhizal fungi are found in association with the roots of most forest trees throughout the world. ECM fungi form obligate symbioses with many of the dominant trees in temperate and boreal forest, as well as in some tropical forests. ECM fungi do not penetrate their host's cell walls. Instead, they form an entirely intercellular interface, known as Hartig net, consisting of highly branched hyphae that forms a latticework between epidermal and cortical cells [13]. Hartig net provides a large surface area for the two symbiotic partners and it is the site of nutrient exchange. Carbon (C) is transported to the fungus from a tree that receives limiting nutrients in exchange. The fungus can transport nutrients beyond the nutrient depletion zone surrounding the host's root system and release from immobilized sources inaccessible to the plant [13, 14]. ECM fungi are thus regarded as key elements of forest nutrient cycles and as strong drivers of forest ecosystem processes [15].

Most (86%) terrestrial plant species obtain mineral nutrients through mycorrhizal symbionts as estimated using taxonomic and ecological extrapolation methods [16]. An estimate of ECM fungal species richness is likely between 20,000 and 25,000 [16, 17]. These ECM fungi belong to more than 80 independently evolved lineages and to more than 250 genera, mainly in Basidiomycetes and Ascomycetes [18].

2.2 General roles of ectomycorrhizal fungi in forest ecosystems

Ectomycorrhizal fungi are essential contributors in forest ecosystems by forming beneficial symbiosis plants. These fungi drive forest soil processes such as soil organic matter decomposition, nutrient cycling, and carbon sequestration [19–21].

ECM fungi have the ability to provide hosts not only nitrogen but a variety of other major nutrients, including phosphorus, potassium, calcium, magnesium, sulfur, as well as micronutrients such as iron, zinc, copper, and manganese. However, they are often ignored because N is the main growth-limiting element in many forest ecosystems, particularly in the Northern Hemisphere [19, 22, 23]. In addition to nutrients, trees receive several other benefits. First, the resistance of trees against pathogens is improved due to the mycelial network [24]. Second, the ECM mycelial networks are involved in water transport [25]. Third, ECM fungi can relieve salt and heavy metal stress of the host plants [9]. The benefits that the ECM fungi offer are complicatedly regulated by the host type, ECM species, as well as climatic and environmental conditions. Recently, a study based on a climate change model predicted that the global abundance of ECM-associated trees will decline by 10% by the end of 2070, and the majority of this will take place in boreal and temperate ecotones [26]. Therefore, the conservation of ECM fungi should be taken as an important issue.

2.3 Structure of ectomycorrhizas is diverse

Fungal mycelium has been estimated as one of the largest living organisms on Earth [27]. Hyphae is composed of fungal mycelium and other structures including rhizomorphs. Rhizomorphs are structures through which fungi can spread in their environment and search for new substrates to colonize. The structure of ectomycorrhiza is diverse. Agerer [28] proposed that ECM mycelia systems influence on their patterns of differentiation and putative ecological importance. Mycorrhizal fungi have been classified into four exploitation types depending on the extent of hyphal development: contact, short-distance, medium-distance, and long-distance.

ECM fungi are characterized according to the water repellence of the mycelium. Fungi vary from extremely hydrophobic to extremely hydrophilic types [29]. All fungal growth parameters such as hyphal hydrophilicity, presence of rhizomorphs, and mat formation correspond together to how fungi interact with and exploit the environment [28, 30]. The function of extraradical mycelia of ECM fungi is the transportation of nutrients between plant and soil environment [13, 31].

Ectomycorrhizas differ in their ability to take up and transport nutrients, and thus, promote tree growth [32, 33]. The differences in ECM effectiveness are often species specific or even strain specific [34]. It is evident that the amount and differentiation of extraradical mycelium is an important ecological factor for tree performance [35–37] and soil nutrition [38].

3. Contribution of ectomycorrhizal fungi to nitrogen cycling in forest ecosystems

3.1 Forms of nitrogen in forest soil

The major N sources in the forest floor can be divided into external and internal sources. Atmospheric nitrogen deposition is an external source, and the living organisms and their decomposition products are an internal source [39, 40].

Ammonium and nitrate are the two major pools of inorganic N. Ammonium is most often the dominant inorganic N pool available to trees in coniferous ecosystems. Nitrate concentrations are usually relatively low in mature forests [41].

Most of the nitrogen in forest soils is bound to organic compounds [42]. It is well known that over 90% of N occurs in organic forms in most surface soils [7, 43]. The forms of organic N can be roughly divided into two categories. (I) Organic residues consisting of undecomposed animal and plant residues and partial decomposition products, and (ii) soil organic matter or humus. The humus is composed of non-humic, easily identifiable compounds (e.g. amino acids, carbohydrates, nucleic acids, etc.) and complex humic substances, such as high-molecular-weight amorphous and aromatic compounds, formed during the decomposition process. The importance of humus is widely recognized in maintaining and improving soil fertility [7].

The distribution of major N compounds was investigated in different climatic and geological conditions including arctic, cool, temperate, subtropical, and tropical climates early [44]. The results indicated that about 33–42% of soil N occurs as free and protein amino acids. The amino acid composition of all soils, however, was remarkably similar. The composition and concentration of amino acids has shown generally constant throughout the growing season [45], which suggests that amino acids originate from a common source or through similar biochemical processes. However, the distribution of N compounds at different regions seems to be related to decomposition process and as well as forest types [46]. Soil proteins are often not free, they are bound to humic compounds and are not soluble. These N forms cannot directly be used by plants, they need to be depolymerized by microorganisms and converted into plant available monomeric organic or mineral N forms.

3.2 Diversity in nitrogen uptake in Ectomycorrhizal Fungi

Ectomycorrhizas occur widely in forest ecosystems. Most of the terrestrial plant species are in symbiosis with mycorrhizal fungi, about 3% of them are ectomycorrhizal. The most common tree species belong to Pinaceae, Salicaceae, Betulaceae, Fagaceae and Myrtaceae [13, 47]. The general mechanism of ECM fungi to improve plant nutrition is the so called Hartig net structure that increase the surface area of roots to absorb nutrients.

Ectomycorrhizal fungi are able to take up both inorganic and organic forms of N. Ammonium is generally recognized as the most readily utilizable form for most ECM fungi when studied in mycelial cultures [48, 49] or with ECM roots in vitro and in the field [50]. Nygren and colleagues [51] demonstrated that 68 species of ECM fungi used nitrate as the sole N source in a pure culture. However, the pure culture conditions do not reflect the N preference of ECM fungi in nature [52]. *Laccaria laccata* was shown to uptake nitrate and transfer it to the host plant when in nitrate-rich conditions [53].

In other studies, ECM seedlings demonstrate a strong preference for amino acids over ammonium [54]. Already in 1953, Melin and Nilsson [55] demonstrated that ^{15}N labelled glutamate was absorbed by the mycelium of *Boletus variegatus*, and that the nitrogen was transferred to the shoots of pine seedlings that had been infected with the fungi in an aseptic culture. Many ECM fungi are able to grow with amino acids as the N source in pure culture and also in association with host trees [56–59].

The capacities of ECM fungi to mineralize organic N differ. Abuzinadah and Read [60] found that ECM fungi such as *Suillus bovinus*, *Amanita muscaria*, *Paxillus involutus*, *Cenococcum geophilum*, and *Rhizopogon roseolus* were able to use peptides and proteins as their sole N sources. In contrast, *Laccaria laccata* and *Lactarius rufus* had little ability to grow with peptides and proteins but they grew well with

ammonium. It was further demonstrated that different fungal species, even different strains had different abilities to utilize organic N and/or transfer the assimilated N to their host plants [60]. Some ECM fungi might take up the nitrogen compound completely and some break the molecules into smaller organic or inorganic forms. The difference in the ability of ECM fungi to transfer N from chitin, protein, and other organic substances in litter and humus was explained by differences in their enzyme secretion profiles [61].

ECM fungi have several functionally distinct metabolic pathways to transfer N. ECM fungal hyphal morphology, species niche (original living conditions), genetic characteristics and carbon costs to host plants may influence on their capacity to utilize and mineralize organic N.

3.2.1 Mycelium structure determines the efficiency of ECM transport nitrogen

ECM fungal hyphae morphology is diverse. Morphology seems to have a great influence on the hyphal enzymatic ability of ECM fungi. ECM species with hydrophilic ectomycorrhizal hyphae have proteolytic activities and they are adapted to N-limited conditions [62]. In contrast, other ECM fungi with hydrophobic ectomycorrhizal hyphae, similar to many saprotrophic fungi, form aggregated hyphae (rhizomorphs) for long-distance transport of elements. This is presumably an adaptation for patchily distributed resources [63].

In addition to hydrophobicity, another aspect is to consider the size of mycelia. The species that form extensive extraradical mycelia (e.g. *Cortinarius*, *Suillus*, *Tricholoma* species) have different capacity to utilize organic N than those species that form diffuse, spatially limited extraradical mycelia (e.g. *Amanita*, *Lactarius* species). These differences in mycelia are thought to be associated with different reproductive and colonization strategies [58, 62]. It is believed that extensive mycelia are established infrequently, but it is long- living. In contrary, the diffuse mycelia become more stable, usually by spores for the generation, but the mycelia do not persist. The long-living extraradical mycelia is believed to be more efficient to process N than short-living mycelia.

Studies based on the stable N isotope ratios in ECM fungal fruitbodies have provided new insights and evidence for the N sources of ECM fungi. As we know, the relative abundance of stable isotopes in food webs follows from discrimination against heavier isotopes in several biochemical processes [64]. The ratio is useful particularly in studying nitrogen cycling mediated by mycorrhizal fungi [65]. Stable N isotope ratios in ECM fungal fruitbodies showed that those having long-living mycelia exhibited higher $\delta^{15}N$ than those having short-living mycelia [58, 66, 67].

Thus, the signature of ^{15}N in ECM fruitbodies was determined by the morphological characteristics of the mycelia. Another observation revealed by the isotope studies is that ECM fungal species that can utilize organic N exhibited higher $\delta^{15}N$ in their fruitbodies than those that are restricted to mineral N sources [67, 68].

3.2.2 Nitrogen utilization of ECM fungi is related to the nitrogen status of the habitat

The form of nitrogen in the environment influences N mobilization by ECM fungi. The species common in low inorganic N soils grew well with protein, glutamine, and serine whereas species in high inorganic N soils grew well with glutamine, but poorly with protein and serine [67]. Differences among ECM fungal species in their ability to access and take up different N forms indicate that the form and abundance of N in the environment may be a defining factor for ECM fungal species niche [69]. ECM species are selected by the N form that is predominant in

their environment. Recently, an increasing number of studies showed that inorganic N enrichment in forest soils caused by pollution, fertilization or natural causes are leading to a reduction in the level of plant root colonization by ECM fungi, also shift fungal community in soils away from ECM fungi specialized in organic N acquisition to more generalist nitrophilic species and saprotrophs [70–72].

Other studies have concluded that differences in proteolytic activity between the species of ECM fungi could be explained by soil-derived selection pressures. For example, *Hebeloma crustuliniforme* expressed proteolytic activity in the presence of a readily available N source such as ammonium [73]. Ammonium has also been shown to repress the expression of amino-acid transporters and enzymes in N assimilation pathways in ECM fungi [74, 75]. The presence of inorganic N tightly down regulated soil organic matter degradation by *Paxillus involutus* as proved [76]. Such facts suggest that ECM fungal degradation activity would be controlled by environmental factors.

Different ECM species occupy different successional stages in forest development. This seems to be related to the proteolytic activity of fungi. When resource quality declines and organic matter accumulation declines during forest development, fungi with limited proteolytic activity is favored. For the cultivation of edible mushrooms, this means that we should pay attention to the natural preferences of the species for nitrogen uptake. This may concern especially the ECM species that are difficult to cultivate artificially.

3.2.3 Fungal genetic characteristics determines the efficiency of N transition

Recently, advances in genetics and molecular biological techniques have provided better understanding about nitrogen metabolism. The acquisition of inorganic N and the mineralization of organic N by ECM fungi have been proved by many molecular investigations. Ectomycorrhizal fungi encode a number of transporters to acquire nitrate and ammonium from soil, as well as a suite of enzymes and transporters necessary for utilizing organic N sources [77–79]. Ammonium importers such as AMT1, AMT2 and AMT3 have been functionally characterized in several ECM fungal species, such as, *Hebeloma cylindrosporum* [75, 80], *Tuber borchii* [81] and *Amanita muscaria* [82]. Nitrate transporters, such as LbNRT2 in *Laccaria bicolor* [83] and HcNRT2 in *H. cylindrosporum* [84], are also present in ECM genomes allowing N transport.

Ectomycorrhizal fungi have all evolved from their saprotrophic ancestors, and hence, ECM have the ability to decompose organic matter [85, 86]. The utilization of proteins by fungi requires the enzymatic degradation of proteins to peptides and amino acids before cellular uptake. Lindahl and Taylor [87] studied the genetic potential of ECM fungi to produce N-acetylhexosaminidases that hydrolyze chitin to N-acetylglucosamine. Thus, N-acetylglucosamine and amino acids replace ammonium and nitrate as the N sources [19].

Recently, the genomes of ECM fungi were found to contain the same or smaller number of copies of genes coding for secreted N and P targeting hydrolases than saprotrophs, pathogens, or ericoid mycorrhizal fungi [88]. This observation is surprising because the well-documented ability of ECM fungi to hydrolyze organic phosphate compounds and scavenge nitrogen through the degradation of proteins accumulated in litter. Miyauchi and colleagues [88] also showed that the ECM fungus *Paxillus involutus* was able, while assimilating organic N, to significantly modify organic matter with a free-radical-based mechanism similar to that of saprophytic brown-rot fungi [76]. Unlike the saprophytic fungi, *P. involutus* did not show any expression of genes encoding extracellular enzymes needed to metabolize the released C. This suggests that the degradation mechanism of this ECM fungus has evolved to assimilate organic N rather than C.

3.2.4 ECM utilizing organic N in relation to receiving C from trees

ECM fungi are able to breakdown soil organic N with differing efficiencies. It has been found that the uptake of amino acids by mycorrhizal fungi is related to the N content and carbon structure of the amino acid [89]. One hypothesis was proposed that the rate at which mycorrhizal fungi degrade large organic N polymers in soils is also controlled by the plant C resources available to the fungi to construct extracellular enzymes, as well as the bond strength and structural diversity of the target organic N compound although the direct tests of the hypothetical mechanism is still needed. Another study by Näsholm et al. [90] tested a model for C–N exchange between trees and mycorrhizal fungi. They found that ECM fungi transport smaller amounts of absorbed N to trees in N-limited than in N-rich conditions. The study found further that the greater allocation of C from trees to ECM fungi increases N retention into soil mycelium. The growth of these fungi is stimulated, and thus, N is immobilized and sequestered in soil. This mechanism was suggested to drive boreal forests towards a more severe N limitation at low N supply.

ECM fungi have diverse evolutionary origins and they use diverse decomposition mechanisms to access organic nitrogen entrapped in soil organic matter [91]. The timing and magnitude of decomposition activity seem to be controlled by the below-ground nitrogen quality and the above-ground carbon supply. Some ECM fungi might act as decomposers, not primarily to obtain C to their metabolism, but to search for organic N in the absence of readily available inorganic N [76, 92–94].

4. Challenges in establishing edible ectomycorrhizal fungal culture with fruitbody formation

More than thousand species of ECM fungi produce edible mushrooms [95]. Some of them, such as *Amanita caesarea* (Scop.) Pers. *Boletus edulis* Bull., *Cantharellus cibarius* Fr. and *Tricholoma matsutake* (S. Ito and S. Imai) Singer, have economical value on international markets. The problem is that edible ECM fungi are usually more difficult to cultivate than saprophytic fungi because of the symbiotic relationship with a host tree is needed. In the past few decades, significant progress has been made in the cultivation of some fungi, such as *Lactarius deliciosus* (L.) Gray [96–98], *Lactarius hatsudake* Nobuj. Tanaka [99], *Suillus granulatus* (L.) Roussel [96], *Rhizopogon roseolus* (Corda) Th. Fr. [100], and *Lyophyllum shimeji* (Kawam.) Hongo [101]. In controlled conditions, however, the successful fruitbody or primordium formations are limited. Most of edible ECM fungi still cannot be cultivated. The major issues that need to be understood are the trophic relationships, biotic, edaphic, and climatic requirements for each mushroom. In this review, we focus on the nitrogen acquisition of edible ECM fungi for their mycelial culture and its effect on fruitbody formation. Secondly, we take *T. matsutake* as an example and discuss in detail about its ability to acquire nitrogen, its preferences, and possible strategies. Finally, we discuss about the further challenges – to conserve proper ecological conditions for edible ECM fungi to grow.

4.1 Nitrogen sources in edible ECM fungal cultures

We summarize the nitrogen sources used in mycelium culture and the cultivation experiments of edible ECM fungi in combination with ECM fungal morphological characteristics reported from the published studies (**Table 1**). As known, most edible ECM fungi are difficult for cultivation so far. We could get some hints for the ECM cultivation from experimentally observed nitrogen preferences

ECM fungi	Mycelium growth	Mycorrhization	Fruitbody formation	Ref	Hydrophobicity	Exploration type	$\delta^{15}N$ (‰) (Mean ± SD) (n)	Ref.
Amanita					Hi	Medium-smooth	3.1 ± 0.5 (35)	[102]
A. caesarea	NH_4^+ (poor on orgN)			[103]				
Boletus					Ho	Long	5.8 ± 1.0 (17)	[102]
							8.66 (1)	[104]
B. edulis		orgN		[105]				
B. reticulatus			NH_4^+ and orgN	[106]				
Boletus sp.			NH_4^+ and orgN	[107]				
Cantharellus					Hi		4.3 ± 1.4 (8)	[102]
C. cibarius	NH_4^+ (poor on orgN)			[48]				
Cortinarius					Ho	Medium-fringe	6.8 ± 0.3 (100)	[102]
C. variecolor			orgN	[67]				
Hebeloma					Ho	Short/ medium-fringe	*2.7 ± 1.1 (7)*	[102]
H. cylindrosporum			orgN (but a variable among strains)	[59]				
			ON	[57]				
H. radicosum			NH_4^+	[108]				
Hebeloma sp.			orgN	[108]				
Hydnum					Ho	Medium-fringe	12 (1) cap	[102]
H. repandum	NO_3^- or ON (poor on NH_4^+)			[109]				
Laccaria					Hi	Short	0.5 ± 0.6 (15)	[102]

ECM fungi	Mycelium growth	Mycorrhization	Fruitbody formation	Ref	Hydrophobicity	Exploration type	$\delta^{15}N$ (‰) (Mean ± SD) (n)	Ref.
L. lacata	orgN			[110]			3.0 ± 0.4 (3) cap	[111]
	NH_4^+ (poor on orgN)			[112]				
L. bicolorNH_4^+ (poor on NO_3^-, or orgN)				[113]			1.8 (1) cap	[111]
	NH_4^+, NO_3^- (poor on amino acid, good on urea)			[114]				
	NH_4^+ (poor on orgN)			[67]				
Lactarius					Hi	Contact/ Medium-smooth	4.2 ± 0.3 (54)	[102]
							4.3 ± 0.5 (3)	[111]
L. deliciosus	NH_4^+ plus orgN			[98]				
L. rufus	orgN (a variable among strains)			[67]				
Lyophyllus								
L. shimeji			NH_4^+ and orgN	[115]				
Paxillus					Ho	Long	7.1 ± 0.7 (7)	[102]
P. involutus	orgN			[113]				
Scleroderma								
S. citrinum	NH_4^+ or orgN			[112]	Ho	Long		
Suillus					Ho	Long	8.2 ± 0.7 (17)	[102]
S. bovinus			Forest soil	[116]				

ECM fungi	Mycelium growth	Mycorrhization	Fruitbody formation	Ref	Hydrophobicity	Exploration type	$\delta^{15}N$ (‰) (Mean ± SD) (n)	Ref.
S. lutus	NH_4^+ (poor on orgN)			[112]				
S. variegatus	orgN			[113]			5.7 ± 1.1 (4) cap	[111]
Tricholoma					Ho	Medium-fringe	9.3 ± 0.6 (35)	[102]
T. imbricatum	NH_4^+ or NO_3^- or orgN (gained better grwoth in iorgN)			[117]				
T. bakamatsutake	NH_4^+ or orgN (poor on NO_3^-)			[118]				
T. matsutake	NH_4^+ pluse orgN			[119]			16.8 ± 2.3 (15)	[120]
	orgN			[121]				
		orgN (sustaining symbiotic relationship)		[122]				
T. terreum	orgN (gained better growth)			[123]				
Tuber					Hi	Short	15.1 ± 0.6 (9)	[102]
T. sinense	orgN(gained better growth)			[124]				

** NH_4^+, ammonum nitrogen; NO_3^-, nitrate nitrogen; orgN, organic nitrogen. Ho, hydrophobic; Hi, hydrophibic.*

Table 1.
Fungal growth, symbiosis and fruitbody formation observed using different nitrogen sources in edible ectomycorrhizal fungi in combination with the information of hydrophobicity, exploration type and $\delta^{15}N$ of the fruitbodies.

and mycorrhizal formation. In pure culture conditions, most of the studied fungi appeared to favor ammonium N. Some species, namely *Amanita caesarea*, *Cantharellus cibarius*, *Lactarius bicolor*, *Suillus variegatus* were not able to grow nitrate as the sole N source [48, 103, 113].

However, many of the edible ECM fungi, namely *Amanita caesarea* [103, 105], *Cantharellus cibarius* [48], *Cortinarius variecolor* [67], *Paxillus involutus*, *Suillus variegatus* [113], *Tricholoma terreum* [123], and *Tuber sinense* [124] were able to grow on the media containing organic N (protein) as the sole nitrogen. Moreover, some fungi belonging to *Lactarius* genus had limited capacity to utilize protein N [113, 114]. *Hebeloma cylindrosporum* was able to experimentally utilize a wide range of amino acids and other simple (e.g. urea) or complex (e.g. proteins) compounds [6, 59].

The studied forms of N often predominate soil solution and the culturing results might be assumed to hold true in nature. However, it is worth mentioning is that the optimal nitrogen in the mycelium culture does not necessarily reflect the nitrogen preference of the ECM fungus under natural conditions because environmental factors affect. This was shown with *H. cylindrosporum* growing in nature. Wild dikaryotic strains of *H. cylindrosporum* isolated from two different habitat types had different N preferences [6].

Cultivation of edible ECM mushrooms has been successful in cases of two truffles *Tuber melanosporum* Vittad. and *Tuber aestivum* Vittad. They are cultivated commercially around the world [125]. In addition, some success has been achieved with *Lactarius deliciosus* [126, 127] and *Boletus edulis* [128]. Regarding truffle production, it has been suggested that most soils contain enough N to maintain both fungal and tree growth [125]. Similarly, *Lactarius deliciosus* was cultivated experimentally in forest soil, which was observed to meet the demands for fruitbody formation [126]. It has also been demonstrated that the nutritional properties of soil and the forestry history the natural development of ECM mushrooms in forest ecosystems [129]. A productive and diverse ECM mushroom community resembling natural communities developed when abandoned farmland in Mediterranean dry area was forested with *Pinus* sp.

In summary, productive ECM community can grow in natural soils. However, the challenges faced in artificial cultivation has not been solved.

4.2 Nitrogen source requirements for *Tricholoma matsutake* mycelial culture and mycorrhizal synthesis

Tricholoma matsutake is among the most economically valuable mushrooms in the world. Its taxonomy, distribution, ecology, physiology, and cultivation has been studied widely [130]. Here, we summarize the key results linking matsutake ecological characteristics and nutrient requirements focusing on nitrogen.

Matsutake colonizes the roots of its host trees via an ECM association (**Figure 1a** and **b**). It develops an extraradical mycelium in the rhizosphere and in the surrounding soil area. This can be seen as a white rhizosphere area and it corresponds to the mycelium-soil aggregated zone, called a shiro [131] (**Figure 1c** and **d**). Matsutake shiro grows in the form of a concentric or horseshoe-like circle, depending on the rhizosphere conditions, around the host plant at the rate of approximately 10–15 cm per year [131, 132]. The production of matsutake mushrooms changes periodically. Based on our field observations, the part of mycorrhizal root tips is degraded prior to matsutake fruiting. The extraradical mycelium might grow towards new roots and colonizing. Such a hyphal growth strategy indicates that matsutake symbiosis may often need to be renewed and form new mycorrhizas to acquire nutrients (data not published). Among the mycorrhizal associations, such

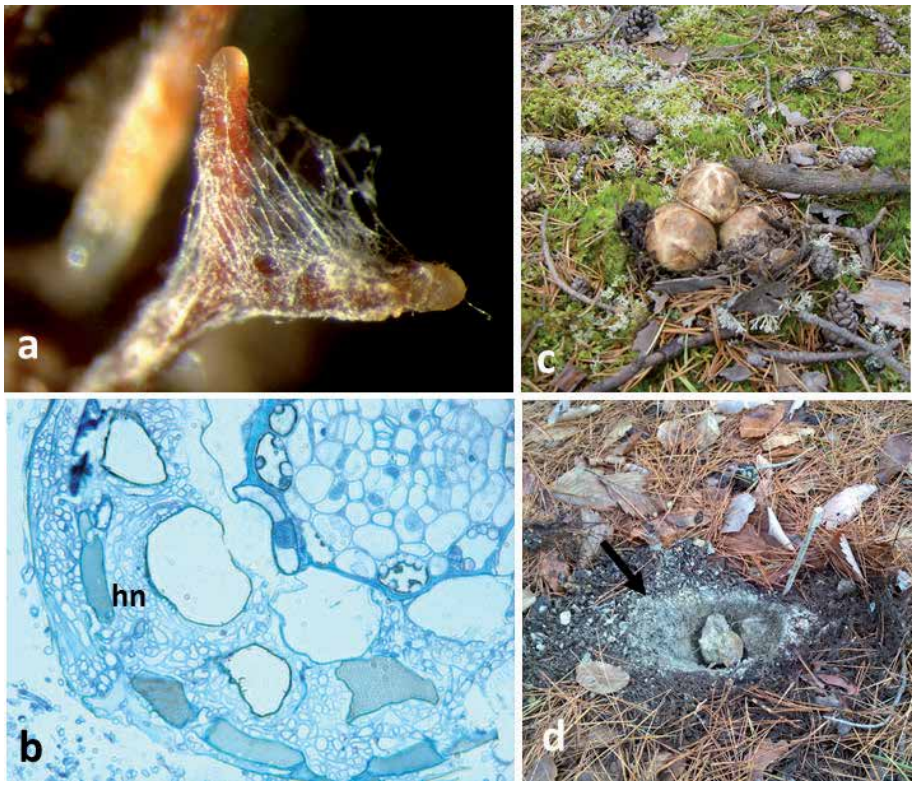

Figure 1.
The ectomycorrhizal edible mushroom—Tricholoma matsutake *(a) the root of* Pinus sylvestris *seedling is colonized by* T. matsutake *fungal mycelium, forms mycorrhizas; (b) the transverse section of ECM root showing the Hartig net (hn) development within the cortex; (c) matsutake mushrooms form in a conifer mixed forest in southern of Finland; (d) the matsutake shiro (arrow) after the mushrooms be harvested (photos were taken by Lu Min Vaario).*

a phenomenon does not seem to be rare. Hortal and colleagues [133] found that the plant had the ability to limit the root tip colonization of the least cooperative symbiont, and therefore, influence the outcome of ECM fungi competition. Such reduction in colonization did not result in a reduction in carbon allocation to the fungus providing the lowest amount of nitrogen.

It is worth noting that decayed mycorrhizal roots together with mycelium-soil aggregated zone might be important organic nutrient sources for matsutake. Recently, the natural abundance of isotopes data showed a very high $\delta^{15}N$ value in *T. matsutake* fruitbodies, which were sampled from Finland and Japan [120]. Matsutake usually grow at B layer of mineral soil [131], such taxa obtain their N could explain for high $\delta^{15}N$ values (see review [102]). More importantly, the high $\delta^{15}N$ value in matsutake is an indicator of organic N uptake from soil because the great variation of ^{15}N content observed among ECM taxa has been reported to be related to the differences in organic N utilization [111]. In addition, a literature study shows that mycorrhizal taxa with proteolytic activities generally show high $\delta^{15}N$ values [67]. Therefore, we conclude that matsutake has a greater proteolytic activity to digest chemically complex ^{15}N-enriched organic matter in soil during matsutake fruitbody development.

In addition to proteases, matsutake produces organic matter degradation enzymes such as acid proteinase [134, 135] and β-glucosidase [136]. Relatively high enzyme activities, β-glucosidase and xylosidase, were detected from matsutake cultures in vitro and in shiro soil [137, 138]. The genome of *T. matsutake* encodes

two GH7 cellobiohydrolases [88], which is in agreement with its known facultative saprotrophic activity [136, 138]. However, no further evidence of any strong saprotrophic characteristics of matsutake was found. It could be speculated that these ECM fungi produce certain levels of carbohydrase, not to fully degrade organic matter to access C but N. Kawai and Abe [121] reported that dried beer yeast, corn steep liquor, casein hydrolysate, and polypeptone were good N sources for matsutake mycelium culture whereas nitrate was not. Dry beer yeast (Ebios, Asahi Beer Inc., Tokyo, Japan), as the sole N source, showed promising matsutake mycelium growth and as well mycorrhizal formation [139] (personal communication with Dr. A. Yamada).

Several agar media such as MMN, MNC, Hamada containing both inorganic and organic N are widely used to culture the mycelium of *T. matsutake* [119, 140]. However, the question whether matsutake prefers organic nitrogen is worth of considering. Usually, more inorganic N than organic N is present in the soil top layer. Some studies suggested that increased N deposition could reduce fruitbody production [141]. Nohrstedt [142] reported a 30% decrease in sporocarp production by *Cantharellus cibarius* in a central Swedish pine forest after the application of 150 kg N ha^{-1} ammonium nitrate. The presence of nitrate ions has been shown to have negative effects on the development of some ECM fungi both in vitro and in soil [143, 144]. Removal of the litter layer has been considered an important method to improve the productivity of matsutake in many Asian countries [145]. It has also been shown that the removal of the upper organic soil layers of the forest floor can improve the sporocarp production of some other ECM fungi [146, 147]. The explanation might be that competition with other microbes diminishes. Litter and organic soil provide carbon and nutrients for microbes, especially for saprotrophic fungi that would compete with *T. matsutake* in the shiro [148].

4.3 Research prospects

Cultivation of ectomycorrhizal mushrooms is still facing many challenges. Although some species of ECM fungi can form the primordium of fruiting bodies on several media, they usually do not develop further into mature fruiting bodies. So far, the most successful efforts have been carried out with the mycorrhizal plants growing in soil. Soil nutrients and soil microbial communities together with climatic factors have shown to affect significantly the persistence of ectomycorrhizas in outplanted inoculated plants, and further, the successful fruiting. The observed suppression of many mycorrhizal mushrooms has been linked to indirect effects of air pollution, in particular to increases in nitrogen deposition accumulating into litter and humus [149, 150]. A thorough understanding of the ecological and environmental factors regulating the ECM fungal species is a prerequisite for their cultivation.

Ectomycorrhizal fungi colonize the roots of their host plants and improve plants' access to nutrients, especially nitrogen. In exchange, host plants deliver a significant portion of their photosynthesized carbon to the ECM fungi. However, we need more accurate understanding of the ECM fungi mediated C and N movement within forest ecosystems. ECM fungi may follow a similar pattern with the amount of C delivered being related to the amount of N sourced by the fungus [77, 151], although this is still controversial [133]. Production of ECM mushrooms do need a balanced nutrient either assimilating by ECM fungi or by other soil microbial.

It has been suggested that the growth of ECM fungi and the formation of mycorrhizas are promoted by certain mycorrhizosphere bacteria, termed 'mycorrhizal helper bacteria' [152]. Some mycorrhizal fungi-associated bacteria are also known to fix nitrogen [153, 154]. However, there is still no evidence that the fungus would

directly benefit from its associated bacteria. Sporocarps of *Cantharellus cibarius* contain large amount of bacteria, in particular fluorescent *Pseudomonas* [155]. Some species of bacteria such as *Streptomyces* spp., *Paenibacillus* spp. and *Bacillales* spp. were isolated from the mycorrhizal root tips and fruitbodies of *T. matsutake* as well [156–158]. Otherwise, the information about mycorrhizas-associated bacteria and their effect on the nutrient uptake of ECM fungi is limited. These studies, however, hint that the production of ectomycorrhizal mushrooms may require teamwork to obtain enough nutrients from the environment.

In conclusion, ECM fungi play an important role in the nutrient cycle of forest ecosystems, especially on mediating C and N movement. A better understanding of the nitrogen status of the habitat of ECM fungi, nutrients movements within the ecosystems, as well as the ECM fungal hyphal structures should be the first step for cultivation of ECM edible mushrooms. The methodological advances in these areas in combination with forest management may allow the successful establishment of commercial plantations and production of edible ECM mushrooms in forests.

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Chapter 11

Promotion of Nitrogen Assimilation by Plant Growth-Promoting Rhizobacteria

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Abstract

Nitrogen fertilizers are one of the highest expenses in agricultural systems and usually a limitation to the productions of many agricultural crops worldwide. The intensive use of this element in modern agriculture represents a potential environmental threat, one of the many tools for the sustainable use of this resource without losing productivity is the use of plant growth-promoting rhizobacteria, especially nitrogen-fixing bacteria. However, in considering the competitiveness of the market, studies are still needed to determine the most efficient way to use this resource and if the nitrogen mineral fertilization is indeed substitutable. As a result, this study aims to deepen the scientific knowledge of the plant-microbe interactions by addressing their main characteristics and functionalities for plant growth and development and efficiency in the use of nitrogen. For this we reviewed relevant information from scientific works that address these issues.

Keywords: biochemistry, nitrogen-fixation, growth, nitrogen fertilizers, nitrogen use efficiency

1. Introduction

Nitrogen (N) is a key component of most proteins, secondary metabolites and signaling molecules [1]. It is one of the most important macronutrients for plant development and usually one of the most limiting factor to plant production [2].

The use of N-fertilizers has produced a significant increase in food production in recent decades [3], and its consumption has grown from 11.3 Tg N $year^{-1}$ in 1961 to 107.6 Tg N $year^{-1}$ in 2013 [4]. However, less than 50% of the added N is effectively absorbed by most cultivated plants [5, 6], and even N effectively converted to biomass, eventually returns to the environment [7]. In the soil, N is available to plants in the form of nitrate (NO_3^-), ammonium (NH_4^+) and organic compounds (usually amino acids), being the NO_3, the most abundant [8]. In its ionic form, NO_3^- has a negative charge and high water solubility, being susceptible to leaching and runoff [9]. It can also be volatilized by denitrifying microorganisms [10], and lost to the atmosphere in the form of nitrous oxide (N_2O, a greenhouse gas 296-fold more potent than a unit of CO_2). Leaching of N causes eutrophication of water bodies and contamination of groundwater [11].

N can also be lost to the atmosphere in other forms such as reactive gases (NO_x; NH_3), aggravating the greenhouse effect [12], and is also related to the acidification of soils through the formation of acid rain, and depletion of exchangeable basic soil cations [13].

To minimize the loss of N in the soil, and consequently the total amount of N necessary for a high-quality production, several strategies have been used. One is the use of urease inhibitors such as N-(n-butyl)-thiophosphoric triamide (NBPT) to delay the hydrolysis of urea, thus reducing losses to the atmosphere and microbial transformations [14]. Increased N use efficiency (NUE) has also been the subject of research, through the selection of genotypes with a higher NUE [15] or through biological nitrogen fixation [16]. Biological nitrogen fixation (BNF) occurs through the conversion of atmospheric N_2 to ammonium by free-living or symbiotic diazotrophic bacteria [17]. Plant growth-promoting rhizobacteria (PGPR) can increase the N absorption capacity through BNF [18], phytohormone production [19], stimulate the production and enzymatic assimilation of NH4+ [16], as well as the transport and partition of N [20].

The study of plant nutrition related to the use of BNF as an alternative to increase efficiency and sustainability in the use of N in agriculture is essential, given the complex nature of the interactions between soil, plant, and microorganisms. Therefore, the objective of this review is to deepen the scientific knowledge of these interactions, addressing their main characteristics and functionalities for plant growth and development.

2. Mechanisms of biological nitrogen fixation

One of the largest N reservoirs is the atmosphere, second only to the lithosphere in absolute amount of N [21]. N makes up about 78% of the atmosphere [22] and is mainly in the form of molecular N (N_2). The atoms in the N_2 molecule have low-energy orbitals and the bond between the two N molecules is relatively short (1,098 Å) and stable, with a bonding energy of 930 kJ/mol [23]. This set of characteristics gives low reaction potential to the molecule. Alternatively, N_2 can be reduced to NH_3 naturally by microorganisms through the BNF process. The BNF reaction follows the following stoichiometry:

The BNF process is catalyzed exclusively by an enzyme complex called the nitrogenase complex. The nitrogenase complex is composed of dinitrogenase reductase (Iron-protein) and dinitrogenase (Molybdenum-Iron-protein) (**Figure 1a**). Dinitrogenase-reductase is a dimer of approximately 60 kDa, composed of two identical and symmetrical subunits, which coordinate a redox center 4Fe-4S (**Figure 2**). This enzyme also has sites for the binding of ATP/ADP, one in each subunit, being able to couple the hydrolysis of ATP to fuel the transfer of electrons to the dinitrogenase [26]. Dinitrogenase is a heterotetramer $\alpha_2\beta_2$ with approximately 240 kDa.

Dinitrogenase has two cofactors containing iron (**Figure 1b**), being the group P and the FeMo cofactor [27]. Group P contains a pair of 4Fe-4S centers, which share a sulfur, forming an 8Fe-7S center. The FeMo cofactor is a variation of the iron–sulfur groups, such as the P group, but differs greatly from the other metallic sites within this family. Its structure is composed of [Mo: 7Fe: 9S: C]: Homocitrate [26, 28]. Some microorganisms also have alternative forms of the MoFe cofactor, where Molybdenum (Mo) is replaced by atoms of Vanadium (V) or Iron (Fe) depending on metal availability [29].

In the N_2 reduction reaction (Eq. 1), the reduced dinitrogenase reductase couples the hydrolysis of ATP with the transfer of electrons to the dinitrogenase. The oxidized dinitrogenase reductase detaches from the dinitrogenase, only to be reduced again (by ferredoxins or flavodoxins). Again, there is the coupling between

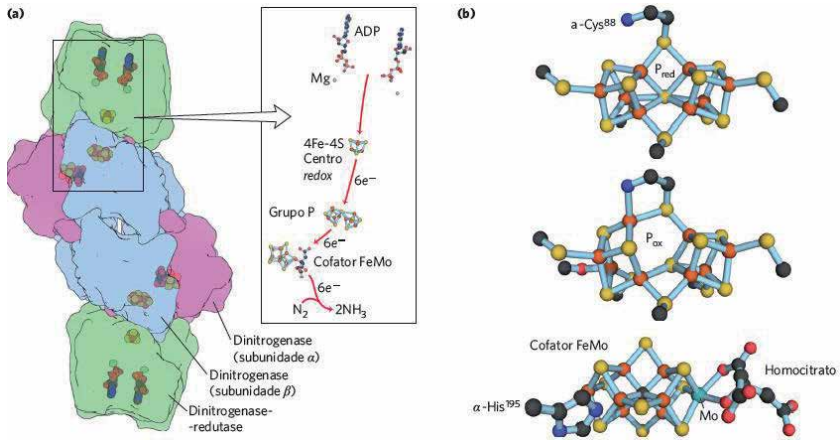

Figure 1.
Enzymes and cofactors of the nitrogenase complex. (a) The enzyme consists of two symmetrical dinitrogenase reductase molecules (in green), each with a 4Fe-4S redox center and binding sites for ATP, and two identical dinitrogenase heterodimers (in purple and blue), each one with a P group and a FeMo cofactor. (b) The cofactors for the electron transfer. A group P is shown here in its reduced (upper part) and oxidized (middle) and the cofactor FeMo is showed at the bottom. Source: Taiz et al. [24].

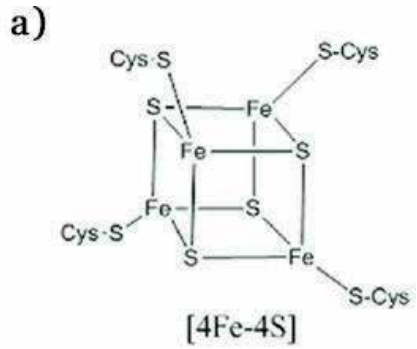

Figure 2.
Structure of 4Fe-4S clusters present in nitrogenase complex. (a) The 4Fe-4S binding site between the Dinitrogenase-reductase and Dinitrogenase contains a cubane-like structure where four iron ions and four sulfide ions are placed at the vertices. The Fe centers are typically coordinated by cysteine residuals. Source: [25].

both enzymes followed by the consumption of ATP to transfer the electrons to the dinitrogenase. This coupling-detaching cycle followed by the electron transfer is repeated until the dinitrogenase is reduced enough to reduce its substrate, which in the case of BNF, is N_2 [30].

Because it deals with large amounts of energy, enough to break the triple bond of the N_2 molecule, the nitrogenase complex is not only inactivated by the presence of oxygen (O_2) but can have its expression reduced [31]. However, diazotrophic bacteria are able to combine N fixation with their aerobic metabolism in different ways to avoid O_2 deactivation [32]. Notably, one of the most advanced means of controlling O_2 concentration is expressed by rhizobia-leguminous symbiosis.

3. Brazil: a success of BNF in legumes

Brazil is one of the best examples in the efficient research and use of the BNF in legumes [33]. The use of FBN is interesting from both an economic and an environmental point of view, since once this process is established, nitrogen fertilizers can

be dispensed with in whole or in part, thus contributing to enable reforestation and minimize possible environmental impacts resulting of use these supplies [34]. It is estimated an annual economy of more than US$ 13 billion with the total or partial substitution of nitrogen fertilizers in legumes in Brazil [33].

Rhizobium-leguminous symbiosis is the most important symbiotic system between microorganisms and plants thanks to the efficiency of the N2 fixation process, and one of the justifications is in the amplitude and geographical distribution of the hosts and the economic impact it causes in agriculture, and one of the main sources of N for the biosphere [35]. The leguminosae family comprises almost 20 thousand species, including tree, herbaceous species used as fodder, producers of raw materials or directly in human food [36].

The most successful case here in Brazil according to Hungria et al. [18], is the symbiosis of Bradyrhizobium spp. with soybean (*Glycine max* (L.) Merrill). The main leguminous species produced in Brazil does not require nitrogen-based fertilization, due to the biological nitrogen fixation which supplies the required N for crop development. To get a sense of how important this symbiotic relationship is in Brazil, in the 2018/2019 harvest, over 35 million hectares were sown. According to the National Supply Company [37], soybean production for this same harvest was 115 million tons, resulting in an average productivity of 3,208 kg ha^{-1}, with almost zero N-fertilizer input. On the other hand, the biological nitrogen fixation with other important legumes, such as beans and peanuts, and non-legumes like sugarcane cannot fully supply the demand for N like in soybeans.

Alfalfa is another plant species with a high potential for biological nitrogen fixation. As a legume, alfalfa is capable of symbiotically associating with rhizobial bacteria, with N inputs to reaching up to 470 kg of N ha-1 [38]. The main symbiotic alfalfa bacteria belong to the genus Ensifer, having Synorhizobium as a synonym, but previously classified as Rhizobium. Sinorhizobium meliloti and Sinorhizobium mediace are the main symbionts reported in several countries [39]. In Brazil, there are three strains of rhizobia that are used in commercial inoculants for alfalfa, which have been validated for more than two decades and with rare tests conducted with the same [39].

4. Growth promotion by associative and free-living diazotrophic bacteria and stimulating N metabolism

World production is dominated by the production of four grasses (FAO Stats 2019), namely: Sugarcane (*Saccharum officinarum* L. 1.95 Gt year-1), Maize (*Zea mays* L. 1.15 Gt year-1), Wheat (*Triticum aestivum* L. 0.76 Gt year-1) and Rice (*Oryza sativa* L. 0.75 Gt year-1). Scientific studies have shown that biological nitrogen fixation is not limited only to legumes, with a potentially important group of diazotrophic bacteria capable of forming associations through root colonization and the internal tissues of grasses [40]. These bacterial-grass associations also have different mechanisms of action than legumes, and in addition to fixing N, increase the absorption and assimilation of N by modulating the architecture and development of the root system through the production of phytohormones, such as indole-3-acetic acid [41] and gibberellins [42]. Cases such as diazotrophic bacteria such as those of the genus Azospirillum sp., Herbaspirillum sp. and Glucanobacter sp. are evidence of the influence of PGPR on N metabolism, inducing physiological and morphological changes that are associated with greater NUE.

Grasses are of great interest for the development of biological nitrogen fixation aiming at greater efficiency, in view of its relatively low NUE [43, 44]. The increase

in NUE is related to several characteristics, Iqbal et al., (2020) working with different cotton genotypes analyzed the biochemical and morphological responses of the accessions according to different concentrations of NO_3^- and the root architecture and efficiency of the enzymes of the assimilation of N were essential for the increase in traction related to NUE. The inoculation of diazotrophic bacteria seems to achieve the same results in different cultures of importance to the global market such as sugarcane (*Saccharum officinarum* L.), corn (*Zea mays* L.), wheat (*Triticum aestivum* L.,) and rice (*Oryza sativa* L.). In sugarcane, the effects vary from the increase in the speed of bud sprouting and the emission of roots in sugarcane stalks used for planting [45], increases in the biomass production of the thatch [46], until the increase in the number of tillers [47]. In maize, inoculation with Azospirillum brasilense increased the transcription of the genes encoding Nitrate reductase (ZmNR), Glutamina sintase (ZmGln1–3), and the intensity of assimilation of the N [48, 49]. In wheat, the inoculation of Azospirillum brasilense was able to modify the N metabolism, resulting in an increase in growth [16].

Modulation of root architecture induced by PGPR is also a morphological trait essential to the increase in NUE. The increase in the area explored by the root triggered by the increase in the volume of the roots caused by these bacteria directly influences the interception of nutrients, among them the N. Besides the influence on the morphological characteristics, the PGPR inoculation has an impact on the metabolism activity of the N. The increase in these characteristics makes these bacteria a potential solution for increasing NUE.

$$N_2 + 8H^+ + 8e^- + ATP \xrightarrow{Nitrogenase} 2NH_3 + H_2 + 16\,ADP + 16\,Pi \qquad (1)$$

5. Conclusions

Since the discovery of the Haber-bosch process in the early 20th century, the levels of N added to the biosphere continue to increase each year and its excessive use of this element is a source of numerous environmental problems.

Therefore, biological nitrogen fixation process is an essential tool in the current economic, agricultural, and environmental context of many countries, this can be seen through studies and government data that show a reduction in financial expenses in the order of millions, this technological tool. it is a reality in vegetable crops with high potentials in the agricultural network of emerging powers worldwide, in addition to contributing to the reduction of potentially harmful agents for the worsening of the greenhouse effect.

This technology has been extensively scientifically explored, aiming to expand its possibility in other promising cultures in the agricultural and forestry world, as well as other associative and free-living diazotrophic microorganisms, and their ability to promote plant growth. However, there are still major gaps in knowledge about the diversity and mechanisms of PGPR action and further research is needed to establish the use of these new bacteria as a sustainable agricultural practice.

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Appendices and nomenclature

CONAB	National Supply Company
NUE	Nitrogen use efficiency
NBPT	N-(n-butyl)-thiophosphoric triamide
PGPR	Plant growth-promoting rhizobacteria
BNF	Biologic nitrogen fixation

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Chapter 12

Mycorrhizal Fungi and Sustainable Agriculture

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Abstract

The 20thcentury witnessed an augmentation in agricultural production, mainly through the progress and use of pesticides, fertilizers containing nitrogen and phosphorus, and developments in plant breeding and genetic skills. In the naturally existing ecology, rhizospheric soils have innumerable biological living beings to favor the plant development, nutrient assimilation, stress tolerance, disease deterrence, carbon seizing and others. These organisms include mycorrhizal fungi, bacteria, actinomycetes, etc. which solubilize nutrients and assist the plants in up taking by roots. Amongst them, arbuscular mycorrhizal (AM) fungi have key importance in natural ecosystem, but high rate of chemical fertilizer in agricultural fields is diminishing its importance. The majority of the terrestrial plants form association with Vesicular Arbuscular Mycorrhiza (VAM) or Arbuscular Mycorrhizal fungi (AMF). This symbiosis confers benefits directly to the host plant's growth and development through the acquisition of Phosphorus (P) and other mineral nutrients from the soil by the AMF. They may also enhance the protection of plants against pathogens and increases the plant diversity. This is achieved by the growth of AMF mycelium within the host root (intra radical) and out into the soil (extra radical) beyond. Proper management of Arbuscular Mycorrhizal fungi has the potential to improve the profitability and sustainability of agricultural systems. AM fungi are especially important for sustainable farming systems because AM fungi are efficient when nutrient availability is low and when nutrients are bound to organic matter and soil particles.

Keywords: Actinomycetes, Bacteria, Mycorrhizal fungi, Vesicular Arbuscular Mycorrhiza

1. Introduction

The productivity of agriculture has increased steadily since the middle of the last century in temperate regions and, more recently, in tropical areas [1] mainly due to improved varieties, machinery, fertilizers, and pesticides. However, recently agricultural yields have plateaued [2], largely because of land degradation often associated with unsustainable farming practices [3, 4]. To meet future demand, food production must rise significantly through this century, utilizing less cultivable land in a sustainable manner [5]. Many factors have contributed to making this increase in crop production essential, including population increases worldwide, food shortages in developing countries, the importance of agricultural products

for trade in many countries, the competition between urbanization and agriculture for land, and the competition that is likely to develop between food and non-food production [6]. However, the mechanism available for producers to increase yields tends to increase input costs, the number of operations in the field, and the financial risks of failure. At the same time environmental degradation linked to the use of chemical inputs (i.e., water pollution from nitrates, phosphates, and pesticides) is increasingly widespread and sometimes irreversible. Moreover, secondary effects on biogenesis and soil impoverishment have weakened cropping systems to make them increasingly dependent on chemicals [7]. Thus, there is growing demand for clean agriculture, high-quality food, and more information on how food is produced are finally having an effect on decreasing the level of chemical inputs used in developed countries [8]. However, in developing countries the great need for food implies a trend towards intensification, mainly through the use of more fertilizers [9] as plant nutrition has played a key role in the dramatic increase in meeting the demand for and supply of food. The consumption of Nitrogenous (N) fertilizer has increased almost nine-fold and that of Phosphorus (P) more than four-fold. The tremendous increase of N and P fertilizers in addition to the introduction of highly productive and agricultural systems has allowed these developments to occur at relatively low costs [10]. But the increasing use of fertilizers and highly productive system have also created environmental problems such as deterioration of soil quality, surface water and ground water as well as air pollution, reduced biodiversity and suppressed ecosystem function [10, 11]. Environmental pollution resulting from greater nutrient availability can be either direct or indirect. Directly, misuse and excessive or poorly managed use of fertilizers can result in leaching, volatilization, acidification and denitrification. Indirectly, the production (use of fossil fuel in Haber-Bosch process) and transport (combustion of fossil fuel) of fertilizer result in air- borne carbon dioxide and nitrogen pollution, which will be eventually deposited into terrestrial ecosystems.

The most limiting nutrients for plant growth are N and P. Most of N is tied into soil organic matter. Even after fertilization, plants have to compete with soil microbes for easily available soluble N but problem with P are different. In acidic soils, even when phosphorus fertilizer is added in substantial quantities, it becomes non available as fertilizers P precipitates with iron or aluminum whereas in alkaline soils P precipitates as calcium phosphates [12]. Accordingly, P limitation may be a difficult problem to overcome through the addition of P-containing fertilizers. So, the recent increase in crop yields and food production in developed countries have been achieved by intensive agricultural practices. This increase, however have not come without tremendous environmental costs. In developing countries, the problems are different. The lack of fertilizers and adequate agricultural practices do not allow intensive crop production and a vast segment of the population remains undernourished. Clearly, there is an urgent need for sustainable agricultural practices on a global level. In the developed world a reduction of energy and environmental costs is necessary. In developing countries, efficient, sustainable practices are needed to allow cost efficient production of adequate nutrition for the growing populations. To overcome the ecological problems resulting from the loss of plant nutrients and to increase crop yields in the absence of resources for obtaining costly fertilizers, microscopic organisms that allow more efficient nutrient use or increase nutrient availability can provide sustainable solutions for present and future agricultural practices. Therefore, a better knowledge of the processes and factors that govern the bioavailability of soil nutrients to plants, thus including the root-soil interactions understanding of microorganisms in the rhizosphere [12, 13] is necessary. As Samuil [14] mentioned, organic farming promotes sustainable production systems, diversified and balanced crop, to prevent pollution and the environment

damages. Moreover, the importance of the mycorrhizal arbuscular fungi in organic farming and farmers' potential to increase the benefits of arbuscular mycorrhizae (AM) associations in such systems represented interesting subjects as it was synthesized by Gosling *et al.* [15]. Symbiotic soil organisms, such as mycorrhizal fungi, may be the source of many of these beneficial effects and thereby be key components of agricultural 'sustainable intensification' [5].

2. Arbuscular mycorrhizae symbiosis

The term 'Mycorrhiza' was first introduced by Frank [16] and comprises of all symbiotic associations of soil-borne fungi with roots or rhizoids of higher plants. Allen [17] described the fungal-plant interaction from a more neutral or microbially oriented aspect stating that 'Mycorrhiza is a mutualistic symbiosis between plant and fungus localized in a root or root-like structure in which energy moves primarily from plant to fungus and inorganic resources move from fungus to plant'. The group of fungi and plants, which are involved in the interaction, determines the type of mycorrhiza they form [18]. Recently, there have been significant advances in the understanding of physiological processes and taxonomy of these fungi [19, 20]. They are obligate symbionts belonging to the phylum Glomeromycota [21]. Their activity in agricultural ecosystems is well documented [22–24]. The distribution of ectomycorrhizal (ECM) fungi is also widespread, but they form associations with only 3% of terrestrial plant families [25]. ECM fungi are members of the phyla Ascomycota and Basidiomycota [26, 27]. Unlike the ECM fungi, AM fungi are dependent on plants for their carbon (C) and when a symbiosis is formed, both ECM and AM fungi can demand 20–40% of photosynthetically fixed plant C [28] (**Figure 1a** and **b**). Soil microorganisms have significant impact on soil fertility and plant health.

Microbial symbionts including arbuscular mycorrhizal (AM) fungi form an essential component of the soil microbial community playing a key role in overall plant growth and development (**Figure 1c**). Arbuscular mycorrhizal (AM) fungi form symbiotic relationships with over 80% of terrestrial plant species [32]. Arbuscular mycorrhizas are ancient and ubiquitous symbioses formed between a relatively small group of soil fungi and higher plant roots which has been traced back 460 million years [21]. During AM symbiosis, the fungal hyphae penetrate the root cortical cell walls by formation of aspersoria leading to the development of intra-radical hyphal colonization and formation of arbuscules or coils that interface with the host cytoplasm [33]. The highly branched arbuscules aid in metabolic exchanges between the plant and the fungus. AM fungi also produce vesicles, which function as storage organs [33]. It has been estimated that in natural ecosystems plants colonized with AM fungi may invest 10–20% of the photo-synthetically fixed carbon in their fungal partners [34].

AM fungi not only can promote via directs effects, but there are also a number of indirect effects such as a stimulation of soil quality and the suppression of organisms that reduce crop productivity (**Table 1**) [35]. AM fungi also interface directly with the soil by producing extra-radical hyphae that may extend several centimeters out into the soil thereby helping the host plants in uptake of nutrients especially P [36]. The extra-radical mycelium of AM fungi can also enhance mobilization of organically bound nitrogen (N) from plant litter [37]. Hyphae of AM fungi have been shown to play an important role in soil stabilization through formation of soil aggregates by secretion of glomalin [38]. Glomalin is a glycoprotein produced on hyphae of AM in the soil. It is discovered by [39] and termed as glomalin, after the source organism of phylum "Glomeromycota." It is apparently insoluble in water;

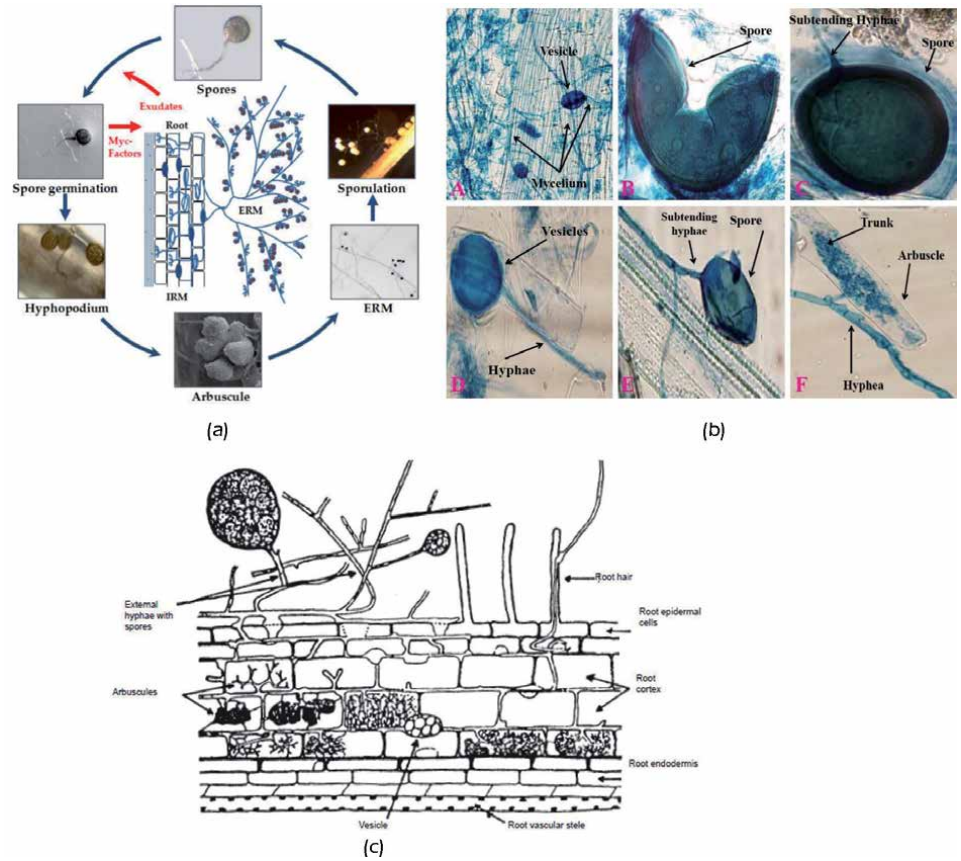

Figure 1.
(a) Life cycle of an AM fungus and the different steps during AM development (Adated from Bücking et al. [29]). (b). A-F: The structural colonization of AMF in roots [a]: Vesicle and mycelium occurrence of the AMF-colonized root sections (arrow), [B]: Spore morphology of AMF (arrow), [C]: Intact mycorrhizal spores and subtending hyphae (arrow), [D]: Vesicles and subtending hyphae of AMF (arrow), [E]: Vesicles and subtending hyphae (arrow), [F]: Trunk, arbuscle and hyphae of AMF (arrow). (adapted from Abeer [30]. (c) Diagram of a root colonized by AM fungi (adapted from Habte, M., and R.L. Fox. [31].

Direct effects on crops	*Indirect effects*
• Stimulation of plant productivity of various crops • Nutrient acquisition (P, N, Cu, Fe, Zn) • Enhanced seedling establishment • Drought resistance • Heavy metal resistance	Weed suppression Stimulation of nitrogen fixation by legumes (green manure) Stimulation of soil aggregation and soil structure Suppression of some soil pathogens Stimulation of soil biological activity Increased soil carbon storage Reduction of nutrient leaching

Table 1.
Direct and indirect effects of mycorrhizal fungi on crop productivity in organic farming systems [35].

extremely persistent glycoproteinaceous compound [40] thus remains in soil after the death of the hyphae [41]. It improves soil physical properties, carbon sequestration, mineral elements, microbes' activities, stabilizes pollutants, and ultimately helping in restoration of soil ecosystem. This kind of beneficial effect of glomalin may be through its existing impact on soil; by serving as a substrate for microbial population, a gluing mediator for aggregate formation, chelation of heavy metals

and toxic pollutants, and enhancing carbon sequestration via long-term persistence in soil [42]. The indirect or 'secondary' impacts of glomalin on the formation and stabilization of soil aggregates further improved the efficiency of the symbiotic relationship and the growth environment [43, 44].

In addition to increasing the absorptive surface area of their host plant root systems, the extra-radical hyphae of AM fungi provide an increased area for interactions with other microorganisms, and an important pathway for the translocation of energy-rich plant assimilates to the soil. The symbiosis is primarily characterized by its association with phosphorus (P) uptake by host plants and the enhancement of water uptake through the extra radical fungal hyphal networks. This symbiosis can also trigger physiological and molecular signals at subcellular levels, alter plant community structure and increase plant tolerance to various abiotic and biotic stresses. Mycorrhizal hyphal networks link plants of the same and different species below ground and are able to transfer resources between plants and release signal molecule defense-related proteins, lipochito oligosaccharides and strigolactones. Molecules like strigolactones secreted by the roots help fungi identify their host plants. Strigolactones also stimulate AM fungal growth and its branching. The fungi reciprocate to this signal by secreting a set of hypothetical factors known as Mycorrhizal Factors (Myc). These factors also play a major role in communication between AM fungi and nitrogen-fixing bacteria. The AM interactions are established further with the induction of seven genes (SYM genes) [45].

3. Application of AMF in agriculture

3.1 AMF enhances soil fertility

Microbes inhabiting in the rhizospheric region interact with the plant root system and generate a nutrient-rich situation improving crop development [46]. At the same time such microorganisms also protect the plant root from harmful chemicals either by repelling them or by diluting their impacts in rhizosphere. Symbiotic association between a particular group of fungus with the plant root system enhances the availability of essential mineral elements principally those elements which are less mobile (phosphorus, copper, zinc etc.) in soil thus allowing crop roots system to access them at ease, which are otherwise difficult to uptake because of their less mobility [47, 48]. Such association also helps crops to establish even in soil with poor fertility, as the microbes will help in uptake of essential nutrients, both major and minor [49]. Studies reported that the amount of fertilizer (particularly phosphatic) used can be decreased with the appliance of mycorrhizae [50, 51].

3.1.1 Uptake of phosphorous

P is a critical and macro essential element (**Figure 2**). It performs an important function in all biological system as it is involved in all energy transfer processes in the form of ATP. Moreover, it also acts as an indispensable constituent for various biological molecules like nucleotides, phospholipids and sugar phosphates [52]. One of the significant advantages of mycorrhizal application is the augment in the P nutrition to the crop. The normal mechanism of P uptake may be discussed in these following ways-; (i) AMF hyphae will absorb it (P) from the rhizosphere, (ii) the absorbed P will be translocated along the hyphae from outside to inner (root cortex) mycelium, (iii) ultimately the absorbed phosphorous is transferred to cortical region of the roots [53].

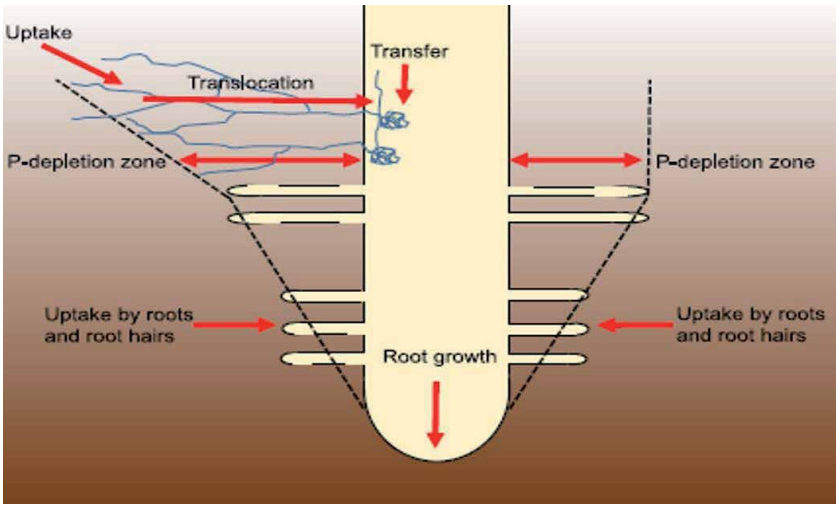

Figure 2.
Mycorrhizal and non-mycorrhizal phosphorous uptake pathways. In mycorrhizal roots, the P-depletion zone is extended by extraradical fungal hyphae beyond that of the non-mycorrhizal root and root hairs (adapted from smith and smith [52]).

A variety of techniques were anticipated in order to increase the accessibility to essential elements such as (i) enhanced assessment of soil; (ii) better movement of phosphate into roots via arbuscules; (iii) alteration of root growing region; (iv) proficient exploitation of phosphate inside crops; (v) proficient transportation of Phosphorous to crop root systems; and (vi) improved storing of captivated Phosphorous. Bhat and Kaveriappa [54] reported that the uptake of PO_4^{-3} by root systems occur more rapidly as compared to the rate at which the ions diffuse to absorbing the site the root system. Thus, it may result in a PO_4^{-3} exhaustion region in the roots surroundings. However, the vast expanded mycorrhizal hyphae lengthen into the surroundings region of plant roots scaffoldingPO_4^{-3} exhaustion region. Consequently, improves the ability of the crop to venture their growing region farther than the nutrient exhaustion region where rootlets and root hair cannot grow [55]. An enzyme known as phosphatase is responsible for converting organic Phosphorous into absorbable form and the action of this enzyme is used by mycorrhizae in order to metabolized PO_4^{-3} in the soil. This enzyme is found inside polyphosphate granules residing in the vacuoles of the fungus. The action of this enzyme will break the polyphosphate granules in fine branches of ultimately the mineralized are released in the protoplasm.

The AMF possibly will give a P uptake route; however, AMF colonization can lower the crop acquisition of PO_4^{-3} via their root PO_4^{-3} transporter [56]. If this reduction is not compensated via AMF pathway of PO_4^{-3} acquisition then it may result in lowering the ultimate cropPO_4^{-3} uptake in AMF-colonized crops [56]. Dissimilarity in the proportionate contributions of these PO_4^{-3} delivery routes can be accountable for the differences in P absorption amongst crop cultivars. In some crop families the AMF has lately been exposed to give a large enhancement on their development example *Alliaceae, Fabaceae* and the *Solanaceae* [57], however the response of other plants, particularly grass family, are ambiguous [58]. Additionally, dissimilarity in growth due to AMF colonization too occurs amongst cultivars within species [59].

3.1.2 Uptake of nitrogen

N is an essential component of amino acid and nitrogenous bases therefore, it is necessary though in direct for protein and nucleic acid bio-synthesis. AMF

colonized crops shown to have improved N content in their above ground part. Various processes may be implied for this improvement, viz. (i) enhancement of symbiotic fixing of atmospheric N; (ii) direct acquisition of combined N by mycorrhiza; (iii) crops without nodule are also benefited as a portion of N fixed by the plants with nodule is transferred towards the former; (iv) activity enzymes viz. pectinase, xyloglucanase and cellulose which are necessary for N metabolism as well as decomposing soil organic matter where enhanced [53]. AMF hyphae have the capacity to extort N from the soil and transporting it to crops. They possess the enzymes to facilitate breaking of organic nitrogen and have N reductase that transform the N present in the rhizosphere. AM not only promotes growth, nodulation and N fixing process in legume-*Rhizobium* symbiosis but also enhances acquisition of ammonia easily from soil which represent the major contribution of accessible N in numerous innate environments. Bacteria which can fix N thus distinctly have the capacity to impact AM fungi. Minerdi *et al.* [60] reported the occurrence of genes responsible for fixing of N in endosymbiotic *Burkholderia* sp., however, significant expression of this genes so that it can impact the growth of the fungal association has not been confirmed. *Rhizobium spp*. might perform collegially with AM fungi on their plant hosts. Nodulation as well as fixing of N are normally augmented in legumes subsequently after AM colonization, possibly for the reason that the fungus provides access of P to the plant and the rhizobacteria, which is necessary for the enzymes essential in the fixing of N. Supplementary fixing of N also improves growth of mycorrhizae [61]. In soils where major fraction of N is available in the form NO_3^-, AMF have a mere impact in uptake of N by legumes [62]. AMF hyphae enhance translocation of N in crop colony, as the connection of AM mycelia joins varied crops species thriving close by and helps pooling and enhancing availability of nutrients for these crops. McFarland *et al.* [63] reported that above 50% of N required by the crop is by associating with mycorrhizal.

3.2 Plant growth hormones

Fungi associated with a pants produced crop growth regulators [64]. Arbuscular mycorrhizae develop a reciprocal and advantageous symbiosis with the majority land plants as their association is not species specific. The existence of host plant at the time of beginning and during the progress of symbiosis is essential, as their symbiotic relation is obligatory in nature. However, AM fungal spores have the capacity to germinate even if the host plant is not present [65]. The fungal counterpart can sense the incidence of the host plant via some signal transduction. Amongst the signaling compounds, that can influence mycorrhizal associations are plant hormones, which might definitely or negatively influence the associations [66]. Plant hormones can manage the intensity and therefore specificity of mycorrhizal associations. Consequently, it might be probable to improve the effectiveness of mycorrhizal association during stresses by regulating the amount of stress hormones [67, 68].

3.3 Uptake of water

AMF may also have a vital position in the improving water economy. The AMF symbiosis either augments the hydraulic conductivity of the roots thereby improving water absorption by the crops or modifies the crop physiology so as to decrease the stress response to soil drought [69]. In severe arid situation, crops associated with mycorrhizae have superior endurance than those crops without the association. It was revealed that mycelial complex may lengthen beneath and broader the soil profile in explore of water and essential mineral elements. Mycorrhizal

association might also adjust the selectivity of plasma membrane to water, with enhanced P nourishment thus these AMF association could enhance the drought resistance of crops [70, 71]. During the situation of water stress, AMF apply their impact by enhancing the transpirational rate and enhancing stomatal conductance or by modifying the equilibrium of plant hormones [72]. The alteration in leaf flexibility owing to AMF association improve H_2O and turgor pressure of leaves and also enhance root growth and extension [73] thus they might affect H_2O relations and consequently, the water stress resistance of the crops. The possible basis for the improved moisture and nutrient absorption by mycorrhiza-crops association may be owed to improved allocation of absorbing hyphal system, more constructive ideotype of hyphae as compared to roots, superior absorbing area and rapid expansion rate, improved serviceable longevity, chemical modification in soil rhizosphere thus modifying microorganism inhabiting in the sphere, uptake kinetics, superior hydraulic conductivities, reduce transportation rates per unit leaf area, withdrawal of moisture from soil towards less Ψ_w and more swift revival form moisture stress.

3.4 Improvement of soil texture and structure

Interruption in ecology may influence the physical and bio-chemical activities in the soil. AMF assist in the fastening of soil particles and improving aggregation of soil and thus aiding conservation of soil [74]. Mycorrhizal fungi also enhance soil quality, because they generate glomalin subsequently when this molecule get accumulated in soil, then with fungal hyphae they produce micro aggregate and ultimately macro aggregates thereby, acting directly as a skeleton for soil aggregation and stabilization. It also produces discharge in the soil consequently improves and stabilizes soil and boosting the growth of microbes [34].

3.5 AMF in plant defense

AMF association can improve host plant endurance to pests and pathogens both above- and below-ground (**Figure 3**) [75–77]. Although the clear-cut biological and chemical reasons are uncertain, their colonization is identified to stimulate subtle modifications in plant metabolic process, like jasmonate and salicylic acid signaling routes, which are vital constituents of crop defense mechanism [78]. Mycorrhizal colonization of a plant before they are attacked by pathogen or pest may have a systemic priming effect via defense molecules/signal re-allocation [79]. So, crop defense related DNA can be expressed more rapidly and extensively than in non-colonized plants [80]. Mycorrhizal colonization may promote crop nutrients uptake and growth; however, this improvement may result in making the plant more attractive, enhance quantity and more nutritive to herbivores [81], thus promoting herbivore action [82]. In fact, phloem-feeding insect normally have enhanced performance on AMF-colonized crops than non-AM crops [81, 83]. Therefore, stabilizing these compromises ought to represent a key module of every farming management tactic associating AMF. Some plants may perhaps display a nonspecific method of defense against phloem-feeding herbivore, facilitated by AMF symbionts. Aphid-infested crops can interact signals via common mycelial networks (CMN) to an uninfected plant (**Figure 3**) [84], therefore a quick flux can be elicited for aphid-repellent volatiles compounds prior to the attack of the plant, this can check the widening or severity of invasion and consequent reduction in productivity [85].

Organic management of crop diseases is one of the important mechanisms for improving crop productivity either by repressing or killing the disease-causing microbes, thus augmenting the capability of crops to endure pathogens or by defending crops against disease causing microbes. In this context mycorrhizal

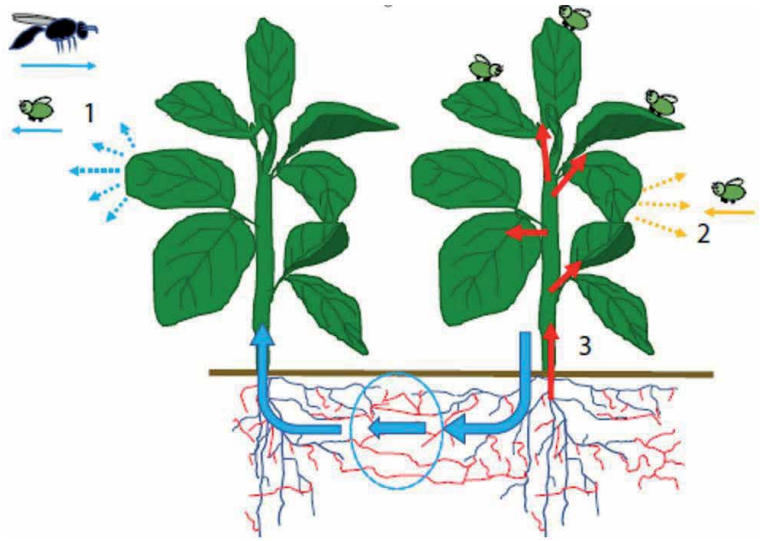

Figure 3.
Mechanisms by which arbuscular mycorrhizal fungi (AMF) may influence agroecosystem physiological ecology. (1) Common mycelia network transmits signal, altering volatile organic compounds (VOCs) in neighboring plants, repelling herbivores and attracting parasitoids (blue arrows), (2) AMF induce VOCs, repelling herbivores (yellow arrows), and (3) AMF elicit systemic plant defense priming (red arrows). (Adapted from Thirkell et al. [73]).

association proves to be the organisms which may serve as bio protectors of crops [86]. Several mechanisms might be involved in mycorrhizal symbiosis in regulating plant pathogens:

I. Generating a physical fence and obstructing the infiltration and successive expansion of disease-causing microbes [87].

II. Making the cell wall thicker via deposition of lignin and by producing many complex carbohydrates which consecutively hamper the penetration of root pathogen [88].

III. Activating host plants specially in the roots to synthesize and concentrate adequate amount of biochemical compounds (alkaloids etc.) which improve the host ability to resist against microbes invasion in their tissue [89].

IV. Promoting flavonoid wall infusions eg. *Laccaria bicolor*, subsequently preventing lesion development by the pathogen *Fusarium oxgsporum* in roots of *Douglas fir* [90].

V. Augmenting the amount of orthodihydroxy phenols in roots, which discourage the infestation of microbes [91].

VI. Synthesizing antifungal and antibacterial antibiotics and toxins function against disease causing microbes [92].

VII. Augmenting competitive advantage to host root for nutrients element in the root zone than the microbes [93].

VIII. Increasing microbial functioning and competition in the rhizosphere consequently forbidding the pathogens to acquire entrance to the roots [94].

Roots associating with VAM/AM fungi might serve as a site for actinomycetes accumulation thus opposing root pathogens [95].

IX. Balancing the trade-off in nutrient uptake mechanism of roots caused due to damage by microbes attacked [96].

X. Altering the quantity and nature of chemical produces by plant root and this would be difficulty for that pathogen which depends on specific type plant roots exudates [97].

Reduced pathogen growth in mycorrhizal and non-mycorrhizal portions of infected roots is connected with aggregation of phenolic and plant cell resistance mechanism. It has been observed that mycorrhizal infected crops were shown to be more lenient to fungal root pathogen in the presence of the root modulating symbiont viz. *Rhizobium leguminosarum*. Thus, indicating that interaction between mycorrhizae and other microorganism thriving in the rhizoplane is more effective in warding off the soil-borne pathogens than the mycorrhizae alone. Therefore, it is essential to understand of the mechanisms of plant disease resistance in mycorrhizae inoculated plants so as to get improved guidelines for developing an efficient plant production and sustainable agriculture. The compositions with mycorrhizae are extremely essential to produce novel groups of biocides which can also give a reduction in risk to both humane healthiness and the ecology [98].

4. AM fungi and sustainable agriculture

For sustainability in agriculture sector, it is essential to exploit the innate mechanisms so as to realize an adequate level of yield and food quality at the same time reducing chemicals inputs, reducing input overheads and obviate ecological contamination and its impact [27, 99]. However, it must be viable in the existing ecosystem and socially accountable. Edaphic factors have numerous contributions towards achieving sustainability in agricultural system such as controlling of pathogen in rhizosphere and augmenting the growth of soil microbes and their activeness which enhanced antagonistic and parasitic interaction in the crop root growing region [100, 101]. Plant and microbe interactions were the intriguing events contributing in agricultural sustainability. Mycorrhizal fungi are native to soil and crop rhizoplane thus making them as an essential mechanism for sustainability in agricultural. In sustainable agricultural systems specially when there are shorts supply for essential elements the mycorrhizae association turn out to play a vital role. In these conditions AM extra-radical mycelium have a vital position for mobilizing the nutrient components into usable form. Hamel and Strullu [102] stated analyzing an AM fungi have been a tough job, however nowadays they are identified as vital ingredients of edaphic environments rather than only crop root constituent. AM fungi can impact plant development, nutrient supply and production even in P-riched soils [103, 104] but the positive response of AM fungi will be more pronounced when the crop is grown in less fertile soil agro ecosystem and also resulting in reduced environmental nutrient loss.

Therefore, attention in AM fungal proliferation for sustainable agricultural system is escalating owing to its function in the encouragement of plant vigor, and enhancement in soil quality and structural stability. These fungal associations could be used efficiently for escalating productivity whilst decreasing the utilization of chemical inputs such as insecticides, fertilizers etc. To enhance crop productivity in

soil with low fertility, inorganic fertilizers have been extensively employed, organic matter is included and techniques like keeping the soil fallow or incorporation leguminous crops have been practiced to improve soil ecosystem, boost soil microbial growth and functioning and augment nutrient re-cycling in order to decrease external inputs and make the most of their utility [105]. This mechanism has been initiated for managing soil ecosystem using earthworms and micro-symbionts [106, 107]. These soil organisms might contribute above 90% of soil organic activity consequently aiding the courses like nutrient cycling, soil fertility and symbiotic association in the plant root zone. Soil fungal multiplicity and functions have not been sufficiently investigated and inferred [108]. Mycorrhizae serve as a significant faction since they have an extensive dissemination and might have significant contribution to biomass of soil microbes and to soil nutrient cycling processes in crops. For sustainable improvement in crop yield more dependence should be on natural and organic mechanism by acclimating on germplasm favorable to plants [109]. They advance absorption of essential elements, particularly phosphorous, and minor nutrients like Zn, Cu; they influence the production of growth material and may lessen both biotic and abiotic stresses [33, 71, 110].

5. Conclusion

In order to have sustainability agricultural system, administration of appropriate nutrient supply represents necessary condition and in this situation mycorrhizae involvement cannot be ignored. Native mycorrhizae spores attack the plant roots and contribute in sustainability nutrients management, moisture, soil properties and productivity [48]. With increasing concern and demand on the requirement to augment sustainability in agricultural development, mycorrhizae fungi possess a significant part to perform in decreasing the detrimental consequent of chemical inputs of agriculture viz. pesticides, synthetic nutrients for promoting plant growth and regulating numerous pathogens. It is an economic and non-detrimental way of acquiring higher yield which may leads to development of a feasible, minimum-input cropping system. It is expected that, in the coming days, limitation in crop cultivation in the globe may be bypassed by using the techniques based on organic processes like mycorrhizae fungi. In the circumstance of existing limitation associated to large population, food requirement and ecological changes, it is obvious that the recent development and progress in arbuscular mycorrhiza field research and those in the future should be considered on maximizing production, improving its quality, enhancing cost effectiveness and profit return, conserving biodiversity and protection of environment. These limitations can be attained by interacting different discipline in particular ways to generate an interdisciplinary connection and association of all researchers in the field [111]. Mattoo and Teasdale [112] stated that sustainable agriculture systems be likely to meet the common necessities in terms of, productivity efficiency and management by confined appliance of the principles as per the climate, soil and existing markets.

Presently, it is probable the beneficial effect of AMF colonization is not nutritional, through their effect on soil aggregates and activity and on plant defenses. Therefore, research in the coming days must focus on improving AM effects on nutrient acquisition so that productivity is continued at the same time as optimizing the sustainability of production. By recognizing and improving those character linked with AMF accessibility, functionality and climate resilience (e.g. water stress tolerance) in new cultivars, considerable development can be made towards acquiring food supply in coming days in more sustainable agricultural system.

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Nitrogen is the most important nutrient in agricultural practice because the availability of nitrogen from the soil is generally not enough to support crop yields. To maintain soil fertility, the application of organic matters and crop rotation have been practiced. Farmers can use convenient chemical nitrogen fertilizers to obtain high crop yields. However, the inappropriate use of nitrogen fertilizers causes environmental problems such as nitrate leaching, contamination in groundwater, and the emission of N_2O gas. This book is divided into the following four sections: "Ecology and Environmental Aspects of Nitrogen in Agriculture", "Nitrogen Fertilizers and Nitrogen Management in Agriculture", "N Utilization and Metabolism in Crops", "Plant-Microbe Interactions".

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