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TRIPARTITE INTERACTIONS OF LEGUMES WITH ARBUSCULAR
MYCORRHIZAL FUNGI AND RHIZOBIAL BACTERIA: INSIGHT INTO PLANT
GROWTH, SEED YIELD, AND RESOURCE EXCHANGE

BY

ARJUN KAFLE

A dissertation in partial fulfillment of the requirements for the

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TRIPARTITE INTERACTIONS OF LEGUMES WITH ARBUSCULAR
MYCORRHIZAL FUNGI AND RHIZOBIAL BACTERIA: INSIGHT INTO PLANT
GROWTH, SEED YIELD, AND RESOURCE EXCHANGE

ARJUN KAFLE

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Biological Sciences degree and is acceptable for meeting the dissertation requirements for his degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

AG1234: ASGROW maturity 1.2 commercial soybean cultivars

AG1636: ASGROW maturity 1.6 commercial soybean cultivars

AG1733: ASGROW maturity 1.7 commercial soybean cultivars

AM: Arbuscular mycorrhiza(l)

ANCOVA: Analysis of co-variance

ANOVA: Analysis of variance

C: Carbon

DP: Direct plant pathway

Dual: Plants inoculated with both symbionts: AM fungi and rhizobial bacteria

ERM: Extraradicle mycelium

HN: High nitrogen

HP: High phosphate

IRM: Intraradicle mycelium

LN: Low nitrogen

LP: Low phosphate

LSD: Least significance difference

Mt: *Medicago truncatula*

N: Nitrogen

N₂: Nitrogen gas

NH₃: Ammonia

NH₄⁺: Ammonium

None: Non-inoculated control (neither with AM fungi and rhizobial bacteria)

NSM: Nature Solution Mycorrhiza commercial AM inoculum

MP: Mycorrhizal pathway

MycoApply: MycoApply commercial AM inoculum

P: Phosphate

qPCR: Real time quantitative PCR

R: Rhizobial bacteria or rhizobial inoculated plants

ROC: Axenic Ri T-DNA transformed root organ culture

SUT: Sucrose uptake transporter

SWEET: Sugars will eventually be exported Transporter

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ABSTRACT

TRIPARTITE INTERACTIONS OF LEGUMES WITH ARBUSCULAR
MYCORRHIZAL FUNGI AND RHIZOBIAL BACTERIA: INSIGHT INTO PLANT
GROWTH, SEED YIELD, AND RESOURCES EXCHANGE

ARJUN KAFLE

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Under natural conditions, legumes, such as alfalfa (*Medicago*) and soybean (*Glycine max*) are colonized with arbuscular mycorrhizal (AM) fungi and rhizobial bacteria forming tripartite interactions. Legumes are important crop species due to their high nutritional and economic values. Most of the previous literatures focused on experiments with an individual symbiont: either AM fungi or rhizobial bacteria, but not with both symbionts at the same time, thus our current understanding of resource exchange in tripartite interactions is limited. It has been reported that AM fungi primarily provide phosphate (P), nitrogen (N), and other nutritional and non-nutritional benefits while rhizobial bacteria solely supply N to their host plant. In return for the nutritional benefits conferred by root symbionts, the host plant reciprocally allocates a significant proportion of its photosynthetic carbon (C) resources to its root symbionts. In tripartite interactions, AM fungi and rhizobial bacteria facilitate synergistically for plant growth and nutrient acquisition. However, how the host plant allocates its C resources to both symbionts in tripartite interactions is still poorly studied.

More attention has been paid to AM fungal benefits in terms of nutrient acquisition and growth response for soybean plants under the controlled conditions using laboratory produced AM fungal inoculum. Due to technical difficulties to produce in a large quantity of AM inoculum, it is not pragmatic to apply this lab-based AM fungi for agronomic purpose in a larger area in the field conditions. However, effects of commercially available AM fungal additives on soybean cultivars in the greenhouse and field conditions have not well reported before despite importance of AM fungi on soybean.

To address these questions, we conducted different experiments in a pot system, split root system with and without fungal access to exogenous N in a hyphal compartment. *Medicago truncatula* was kept either non-inoculated as control (none), or with only AM fungi, or with only rhizobial bacteria, or with dual symbionts (both with AM fungi and rhizobial bacteria) with different nutrient supply conditions. To track the C allocation to different symbiotic partners, we labelled/exposed the host shoot with $^{13}\text{CO}_2$. Similarly, to test how does host plant change its strategy for C allocation to symbionts if AM fungus has an exogenous source of N, we provided $^{15}\text{NH}_4\text{Cl}$ in the hyphal compartment to which only AM fungus had access not to host root. Moreover, in association with C allocation to symbiotic root halves, we examined gene expression of several plant transporters of Sucrose Uptake Transporter (SUT) and Sugars Will Eventually be Exported Transporter (SWEET) family. We also analysed P and N acquisition of host tissues in association with plant growth response.

We used four different soybean cultivars in separate experiments that usually use by farmers for the seed production in this region of Upper Midwest. These soybean

cultivars were either non-inoculated control (none), or inoculated with only commercially available AM additives, or with only rhizobial bacteria, or with both AM fungi and rhizobial bacteria (dual inoculation). Soybean plant growth response in association with plant nutrient uptake, and seed yield was compared between control and AM plants of greenhouse and field condition experiments.

Tripartite interactions favor the growth response in association with higher P and N uptake of the host plant in nutrient limited soil conditions. We found that the nutrient demand of the host, and the fungal access to nutrients are important factors that control the carbon allocation to individual root symbionts in tripartite interactions. Plant allocated more carbon to rhizobia under nitrogen demand, but more carbon to the fungal partner when exogenous nitrogen was available. The expression of genes for several SUTs and SWEETs transporters was consistent with the observed changes in carbon allocation. Exploring the full yield potential of legumes will require insights in how host plants regulate the substantial carbon costs of these interactions as host plant invest substantial amount of energy and resources to produce carbon during photosynthetic process.

We observed soybean plant growth and seed yield was significantly higher with only AM inoculation than either control or only rhizobial alone inoculation. Moreover, the difference in seed yield of AM additives plants was notably higher in limited supply of P and N both in greenhouse and field conditions. Interestingly, seed yield of AM inoculated soybean was similar with or without fertilizer application in the field conditions. Different soybean cultivars had different response to AM fungal inocula for plant growth and seed

yield. Among commercial AM fungal additives, MycoApply outperformed other two commercial inocula for plant growth and seed yield.

Taken together, tripartite interactions of legumes with AM fungi and rhizobial bacteria facilitate for the plant growth and seed yield in limited soil nutrient conditions indicating tripartite interactions may have a bigger potential role to maintain sustainable agriculture.

CHAPTER 1: LITERATURE REVIEW

Beneficial plant microbe interactions and their effect on nutrient uptake, yield and stress resistance of soybeans

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1. Introduction

The plant rhizosphere and phyllosphere is colonized by a wide range of epiphytic and endophytic microorganisms and these microorganisms can establish beneficial, neutral, or detrimental associations of varying intimacy with their host plant. Recent developments in sequencing technologies have enabled us to study the composition and function of plant microbiomes, but plant microbiomes are dynamic, and differ among different plant tissues, and in response to the environment. The plant microbiome can also be seen as “the second plant genome” or pan-genome and can consist of 10 times more genes than typical plant genomes [1]. Beneficial microorganisms that are associated with plants hold enormous potential to be developed into microbial fertilizers or microbial pesticides [2] and new biotechnological tools to increase the nutrient efficiency and stress tolerance of crops, and environmental sustainability of agroecosystems. Specific interactions between microbes and plants, such as the *Rhizobium*-legume symbioses, are

well understood, but the majority of the plant microbiome, and its contribution to the extended phenotype of the host, is not yet well defined.

Soybeans form interactions with nitrogen-fixing rhizobia and this symbiosis plays a key role for the nitrogen (N) nutrition of the plant, but also for agricultural productivity since soybean root residues provide N for other plants in crop rotations [3, 4]. Arbuscular mycorrhizal (AM) fungi colonize the root system of the majority of land plants, including soybeans, and transfer nutrients such as phosphate (P), N, potassium (K), and other nutrients to their host plants, and improve the resistance of their host plant against abiotic (e.g. drought, salinity, heavy metals), and biotic stresses [5]. In addition, soybeans are associated with endophytes that live inside their plant host for at least part of their lives, without causing apparent disease symptoms as a result of this colonization. Plant endophytes exhibit a wide range of plant growth promoting capabilities, including the production of phytohormones, an improved nitrogen (N) nutrition through biological nitrogen fixation (diazotrophic endophytes), the biosynthesis of ACC (1-aminocyclopropane-1-carboxylate) deaminase, the capability to solubilize phosphate, and also the biosynthesis and release of antimicrobial metabolites or siderophores to inhibit the growth of pathogenic microorganisms [6].

The plant microbiome is a largely unexplored resource of beneficial microorganisms with diverse properties and a hidden potential to manipulate plant growth and success in stressful environments. However, while the symbiosis of soybeans with rhizobia and with AM fungi is well characterized, the functional role of endophytes is only known for a limited number of isolates. Our functional understanding of these interactions

is mainly based on experiments with individual symbionts, but there is increasing evidence that individual symbionts can also affect the interactions of the plant with other symbionts [7-10]. We summarize here the effects of different beneficial microbes on nutrient uptake, yield and stress resistance of soybeans, and identify knowledge gaps that hinder the application of these interactions to their full potential in soybean production systems.

2. Beneficial plant microbe interactions of soybean plants

2.1 Arbuscular mycorrhizal fungi

The arbuscular mycorrhizal (AM) symbiosis is arguably the most important symbiosis on Earth and is formed by more than 65% of all known land plant species ($n > 200,000$), including all legumes and many other agronomically important crops, such as wheat, corn, and rice [11]. AM fungi are classified into the fungal subphylum Glomeromycota that consists of less than 350 fungal species [12]. AM fungi co-exist relatively morphologically unaltered with plants for more than 400 million years, and there is evidence that suggests that the AM symbiosis played a critical role for land plant evolution [13].

It is long known that AM fungi can increase the nutrient uptake of their host plant, and are able to deliver substantial amounts of P, N, K, sulfur (S), and trace elements, such as copper (Cu) and zinc (Zn) to the plant. Many AM fungi also provide non-nutritional benefits for their host that are critical for plant survival or fitness, and improve for example the resistance of plants against abiotic (e.g. drought, heavy metal, salinity) and biotic (pathogens) stresses [5]. In return for these benefits, host plants transfer up to 20-25% of their photosynthetically derived carbohydrates to the fungal symbiont [14]. It was generally

believed that carbon is transferred to the fungus in the form of hexoses [15], but recent evidence suggests that also fatty acids can move across the mycorrhizal interface to the fungal partner (Figure 1) [16-18].

AM fungi are ubiquitous in soils and can account for up to 50 % of the microbial biomass in soils [19]. AM fungi form extensive hyphal networks in soils, and the extraradical mycelium (ERM) of the fungus acts as an extension of the root system and increases the nutrient absorbing surface of the root. The ERM with its mycorrhizosphere (interface between fungal hyphae and the soil) acts as an important conduit between microbial communities and the host plant [20] and can provide soil microbial communities with plant-derived carbon (C) inputs in large distance from the root. The mycorrhizosphere represents in soils an important ecological niche for diverse microbial communities that are specifically adapted to this mycorrhizosphere. According to estimates, the bacterial density in the mycorrhizosphere is 4 to 5 times higher than in the plant rhizosphere [21]. However, the presence of AM fungal mycelia does not only lead to quantitative, but also to qualitative changes in the microbial community composition in soils [22]. The presence of AM fungal hyphae plays an important role in the bacterial community assembly during decomposition [22] and affects the access of members of these microbial communities to C sources during decomposition [23].

Within the host root, the fungus can spread intercellularly, but also penetrates the root cortex intracellularly, and forms here highly branched specialized structures, called arbuscules that are separated from the plant symplast by the plant periarbuscular membrane [24]. Some AM fungal species also form vesicles, thick-walled, lipid containing storage

organs in the roots. Arbuscules are the site of nutrient exchange between the plant and the fungus, and both the fungal cell membrane and the plant periarbuscular membrane are characterized by the presence of specific transport proteins that play a critical role for the resource exchange between both partners (Figure 1) [15, 25, 26].

The colonization of host roots by AM fungi is based on a molecular dialog between both partners that facilitates partner recognition and triggers responses in both partners that are critical for the establishment of the symbiosis [27]. After fungal spore germination, an extensive hyphal branching in close proximity to host roots can be observed that is triggered by strigolactones and other compounds in root exudates [28]. After attachment to the host root surface and the differentiation of a fungal hyphopodium, the fungus penetrates the root, and spreads with the help of a prepenetration apparatus [29], and forms arbuscules in the cells of the root cortex. Initiated is this process by the release of lipochitooligosaccharides, or Myc factors by the fungus, that are perceived by specific receptors on the host root surface and trigger a cascade of molecular responses in the host root. The pathway is called the common symbiotic signaling pathway (CSSP), since similar responses can be observed after the perception of rhizobial Nod factors [27, 30]. A key role for the perception of fungal Myc or Nod factors by the rhizodermis plays the membrane-bound receptor-like kinase SYMRK that activates the mevalonate (MVA) biosynthetic enzyme HMGR1(3-hydroxy-3-methylglutaryl CoA reductase 1). A second set of CSSP proteins is located in the nuclear pore complex and includes the three nucleoporins NUP133, NUP85, and NENA, the ATP-powered Ca^{2+} pump MCA8, and cation channels encoded by CASTOR and POLLUX involved in the strong Ca^{2+} oscillations in the nucleus of rhizodermal cells that can be observed shortly after Myc factor perception. Another set

of proteins is located in the nucleoplasm and decodes these Ca^{2+} signals [30, 31]. A Ca^{2+} /calmodulin-dependent protein kinase (CCaMK) phosphorylates with the help of calmodulin CYCLOPS, which then regulates gene expression either directly, or through GRAS transcription factors such as NSP1, NSP2, and RAM1 [30-32]. The elucidation of the CSSP is mainly based on studies in the model legumes *Medicago truncatula* or *Lotus japonicus*, but the fact that the proteins of the CSSP are highly evolutionary conserved, and even present in plants that are unable to form AM interactions, suggest that this pathway is also established in soybeans.

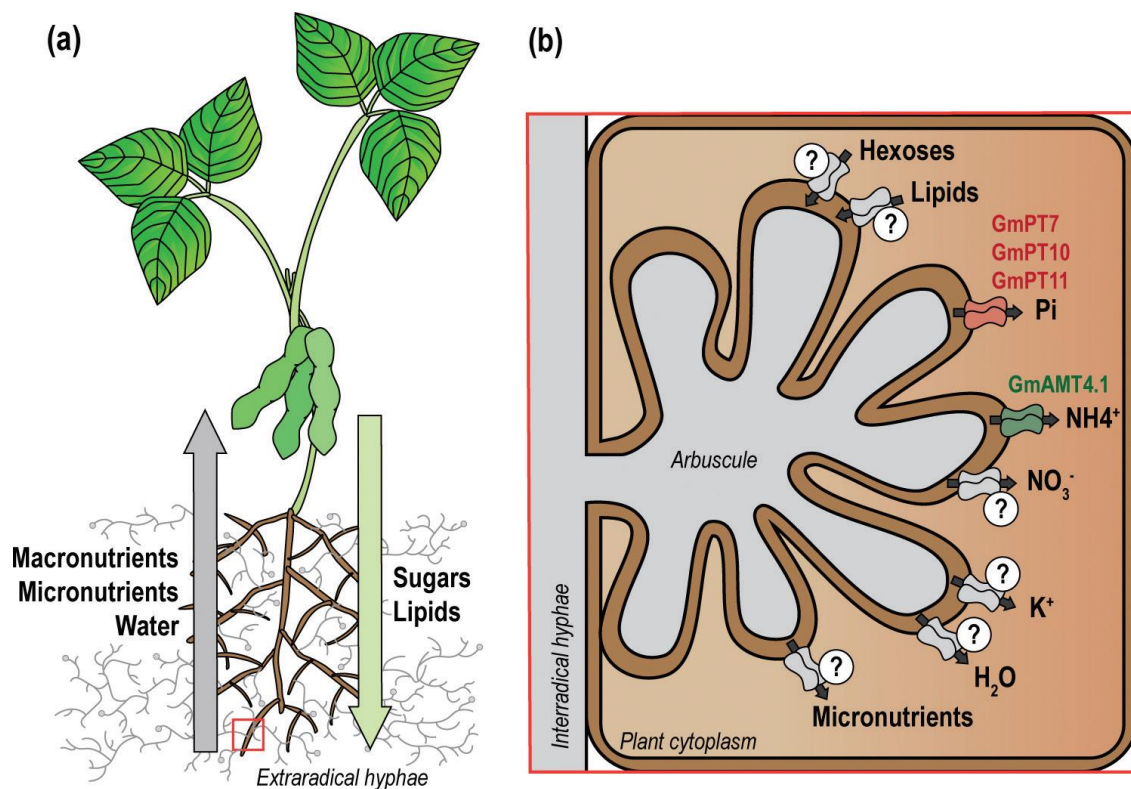


Figure 1.1. Overview of the mycorrhizal nutrient uptake pathways in AM roots of soybean plants via the extraradical mycelium of the fungus (a), and the mycorrhizal interface consisting of the fungal arbuscule in root cortical cells surrounded by the periarbuscular membrane of the host (b). Both, fungal cell membrane and plant periarbuscular membrane are characterized by the presence of mycorrhiza specific transporter that play a critical role

for the nutrient exchange across the mycorrhizal interface of soybean plants (e.g. GmPT7 or GmAMT4.1, see also below).

Mycorrhizal plants have two pathways that are involved in the nutrient uptake from the soil: the ‘plant pathway’ via high- and low-affinity transporters in root epidermis and root hairs or the ‘mycorrhizal pathway’ that first involves the uptake of nutrients via the ERM of the fungus, transport to the arbuscules, and then the uptake by the plant from the interfacial apoplast through specialized transporters in the periarbuscular membrane. In response to the colonization with AM fungi, transporters that are involved in the plant pathway are often down-regulated, while mycorrhiza-specific transporters in the periarbuscular membrane are induced [33], indicating that there is a shift in the nutrient acquisition strategy, and that the mycorrhizal pathway can become the dominant pathway for nutrient uptake [34, 35].

2.1.1 Importance of arbuscular mycorrhizal fungi for yield and nutrient uptake of soybeans

Under both greenhouse and field conditions, increases in nutrient content, yield and overall fitness of soybeans in response to an AM colonization have been reported [36, 37], and soybean yields were found to be significantly correlated to the colonization of the roots with AM fungi [38]. Many reports clearly demonstrate the positive effects of AM fungi on the nutrient uptake of soybeans, and here particularly on the uptake of phosphorus (P) and of nitrogen (N) [39-41]. However, the effects can differ greatly among AM fungi. Our own studies demonstrated for example that while the AM fungus *Rhizophagus irregularis* can increase the P nutrition of soybeans with low or high P acquisition efficiency, *Glomus*

custos had no effect and *Glomus aggregatum* even led to slight growth depressions under medium P supply conditions [39].

Some of the observed differences among these AM fungi seem to be related to the impact of the AM fungus on plant P transporter expression. Fourteen genes of the Pht1 family have been identified in soybeans [42], and three of these transporters show high expression levels in mycorrhizal roots [43]. While the colonization of the roots with *R. irregularis* led to the down-regulation of *GmPt4*, a high affinity P uptake transporter that is presumably involved in the uptake of P from the soil, was the expression of *GmPt9*, and *GmPt10* up-regulated in AM roots. *GmPt9* and *GmPt10* cluster with the mycorrhiza-inducible P transporters *OsPt11* of *Oryza sativa* (rice) and *MtPt4* of *Medicago truncatula* that play a critical role for the P uptake from the mycorrhizal interface [26, 44]. *GmPt9* was up-regulated by *G. aggregatum* and *R. irregularis*, but *GmPt10* was only upregulated by *R. irregularis*, indicating that this transporter is involved in the P uptake from the interface, and that *GmPt10* expression can serve as an indicator for mycorrhizal P benefits in soybean plants. *GmPt7*, another soybean P transporter, shows a high expression in cells with mature and active arbuscules, but is not expressed in cells with collapsed and degenerated arbuscules, suggesting that this transporter may also play a role for the P transport across the AM interface. However, *GmPt7* is not a mycorrhiza specific transporter, and is also expressed in columella cells of root caps and in lateral root primordia of non-mycorrhizal roots [45]. Similarly, out of the 16 ammonium (NH_4^+) transporters of soybean, five transporters are mycorrhiza-inducible, and one of them, *GmAMT4.1* is specifically expressed in arbusculated cells (Figure 1), indicating that this transporter could be involved in the NH_4^+ transport across the AM interface [46].

There is evidence from the model legume *Medicago truncatula*, that AM fungi can also improve the acquisition of other macronutrients such as potassium (K) or sulfur [47, 48]. K deficiency is a common problem in soybeans and can lead to yellowing of the leaves, stunted growth and reduced yields and can become particularly severe under drought stress. Although transcriptional and physiological responses to K deprivation have been studied in other legumes [49], whether AM fungi also play a role in the K acquisition of soybean plants is not yet known.

2.1.2. Importance of arbuscular mycorrhizal fungi for the stress resistance of soybean

AM fungi can also increase the resistance of soybeans against other abiotic stresses such as drought, salinity or soil contaminations. It is known for several decades that the AM colonization can improve the tolerance of soybeans against drought [50]. AM fungi can influence leaf water potential, solute accumulation, and oxidative stress of soybeans under drought stress [51], and delay nodule senescence triggered by water deprivation [52]. In AM soybeans, plasma membrane aquaporins were down-regulated in response to drought stress, and this could reduce the permeability of membranes for water and contribute to water conservation [53]. In addition, both fungal and plant mitogen-activated protein kinases (MAPKs) are up-regulated in AM soybean plants under drought stress. MAPK cascades are known to regulate many cellular processes in response to various stimuli, including abiotic and biotic stresses [54]. AM fungi also improve the tolerance of soybeans against salinity. AM plants had a higher biomass, and proline concentrations in roots, but reduced proline and Na concentrations in the shoot under salt stress. When the fungus was pre-treated with NaCl, the alleviating effects were even stronger, indicating

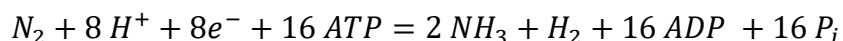
that the acclimation of the fungus to salinity may play a role for the stress response [55]. AM fungi can also improve the tolerance of soybeans against arsenic [56] and aluminum [57] by reducing the uptake of these toxic metals.

Soybean yield and productivity is also threatened by many fungal or bacterial diseases, and soil inhabiting nematodes. Soybean cyst nematodes (SCN, *Heterodera glycines*), brown spot (*Septoria glycines*), charcoal rot (*Macrophomina phaseolina*), rot and stem rot (*Phytophthora sojae*), and soybean rust (*Phakopsora pachyrhizi* and *P. meibomia*) are among the most important pathogens of soybeans and cause substantial yield losses in the U.S. [58]. SCN are often responsible for hidden yield losses, since soil infestations remain often undetected since they become severe. SCN can spread easily from field to field via soil movements with machinery, wind, or by humans, and can now be detected in 90% of the soybean producing states in the U.S. [59]. SCN infestations can lead to yield losses of more than 30% and are responsible for about \$ 1.5 billion in soybean crop damage each year in the U.S. AM fungi can protect soybeans against a wide range of pathogens, including fungi, bacteria, nematodes or insects [60], and reduce the SCN egg population in soils by 70% [61]. The positive impact of AM fungi on biotic stresses has been attributed to the overall positive effect on nutrient uptake and a damage compensation effect, the competition for root space and soil nutrients, induced systemic resistance (ISR) and altered rhizosphere interactions. In addition, AM fungi form extensive hyphal networks in soils and can connect plants of the same or of different plant species by common mycelial networks (CMNs). CMNs play an important role in the plant-to-plant communication and can transfer infochemicals and warning signals from infested plants to uninfested plants and stimulate defence reactions in these plants [62].

2.2. Nitrogen fixing symbiosis with rhizobia

Most Legume plants are able to interact with N-fixing bacteria, called rhizobia that are able to reduce atmospheric dinitrogen (N_2) into ammonia (NH_3) in specialized root nodules. The symbiosis evolved in legumes between 25 and 50 million years ago [63, 64], and plays an important role for plant nitrogen (N) nutrition. Rhizobia can contribute with up to 70% to the total N nutrition, and grain legumes can gain up to 300 Kg N, and legume trees (e.g. *Acacia* sp.) up to 600 Kg N per ha and year from these interactions [4, 65]. Free living rhizobia produce Nod factors that are perceived by plant roots and act as triggers for the common symbiotic signaling pathway (CSSP; see above). Nod factors are also lipochitooligosaccharides that are composed of chitin chains with various lipid modifications. Chitin is the main constituent of fungal but not of bacterial cell walls, and the functional and structural similarities between Nod and Myc factors has led to the assumption that rhizobia adopted the evolutionary far more ancient (~ 450 million years) CSSP to establish this endosymbiotic interaction with legumes [66]. Nod factors stimulate the curling of root hairs, and entrapped bacteria within these curls are transported within infection threads, to the inner zone of developing root nodules. Inside of cortical cells, the rhizobia divide and multiply, and are released into vesicles, called symbiosomes, in which they differentiate to fully functional bacteroids. One or more differentiated bacteroids are surrounded by the plant symbiosome membrane, that represents a barrier by which the host plant can control the movement of solutes to the bacteroids through specialized transporters or channels [67].

Bacteroids express the nitrogenase complex that consists of six protein subunits (two each of NifH, NifD, and NifK) and two [4Fe–4S] and two (Fe₈S₇) iron–sulfur clusters and two iron–molybdenum cofactors (Fe₇MoS₉N) called FeMoco, which catalyze the N₂ reduction to NH₃ [68]. The nitrogenase metallo-centres are all oxygen-labile and must operate in an environment with a low level of free oxygen, and nodules provide their bacterial symbionts with this oxygen reduced environment for optimum N fixation [69]. N fixation by bacteroids is a highly energy consuming process, and rapid respiration in the bacteroids is necessary to produce the 16 ATP required for the conversion of each atmospheric N₂ into two NH₃.



The product of biological N fixation (BNF) is ammonia, which diffuses out of the bacteroids into the acidic symbiosome space and is here protonated to ammonium. The symbiosome membrane is energized by an H⁺-ATPase, that pumps protons into the symbiosome space and thereby promotes the uptake of NH₃/NH₄⁺ into the plant cytosol, where NH₄⁺ is rapidly assimilated into amino acids, and the ureides allantoin and allantoic acid [69]. A candidate for the uptake of NH₄⁺ from the symbiosome space is *NOD26*, that was first identified in soybeans [70]. *NOD26* belongs to the major intrinsic protein/aquaporin (MIP/AQP) channel family, and is exclusively localized in the symbiosome membrane [67]. The ureides allantoin and allantoic acid serve as the dominant long-distance transport of N from the root nodules to the shoots [71, 72]. Cortex cells and the vascular endodermis of nodules express *GmUPS1*—1 and *GmUPS1*-2, which play a role for the transport of allantoin and allantoic acid out of the root nodules to the sink

organs. RNAi knockouts of these proteins accumulate ureides in the root nodules, and show a reduced N transport to the shoots [73].

BNF is an energy expensive process, which requires 16 ATP to fuel the reduction of one N_2 . Plants allocate up to 30% of their photosynthetically fixed C to rhizobia [74], which is oxidized in the bacteroids to ATP. The N_2 fixation rate of rhizobia is higher when the nodules receive more C, suggesting that the allocation of C to nodules is a limiting factor for BNF. Transgenic *Medicago sativa* plants that over-express a sucrose phosphate synthase, a key enzyme for sucrose biosynthesis in plants, show higher C contents in nodules, more and larger nodules per plant and an enhanced nitrogenase activity of the root nodules [75]. Free living rhizobia can grow on a variety of different sugars, including mono- and disaccharides, but the absence of transporters for these sugars in bacteroids suggests that rhizobia in symbiosis take up dicarboxylates, and here particularly malate from the symbiosome space. The C4-dicarboxylate transport system that is localized in the inner bacteroid membrane is encoded by the *dctA* gene, has a high mobility for malate, and is essential for symbiotic nitrogen fixation [76]. Although the mechanisms of N fixation and assimilation are well documented, key steps are still unknown. For example, little is known about the C metabolism inside nodules, the regulatory steps that control the C export to rhizobia, and the proteins involved in the C and N transport between partners. Recent evidence in the model legumes *M. truncatula* and *Lotus japonicus* suggest that sucrose transporters from the Sugar Will Eventually be Exported Transporter (SWEET) family could be involved in the sucrose efflux from the phloem towards nodulated cells [77].

2.2.1. Significance of rhizobia for soybean agriculture

According to estimates, soybeans with their rhizobia populations fix around 20 million tons of N each year, and this has an enormous influence on agricultural productivity, not only on soybeans, but also for other crops in crop rotation systems [3, 4]. Soybean residues in the soil enrich the soil with N, improve soil organic matter and can lead to yield increases in non-legume crops that follow soybeans. Crop rotations or intercropping systems of cereals with legumes can result in higher crop yields without fertilizer additions [78]. However, conventional agricultural management practices and other anthropogenic factors can have a negative impact on rhizobial function. In addition, excessive tillage, applications of higher N fertilizer dosages, extended fallow periods can also have detrimental effects on rhizobia populations in soils. As a consequence, integrating this symbiosis more efficiently in modern agricultural practices is crucial to limit the amount of fertilizers used and to make agriculture more environmentally sustainable. Exploring ecologically best fitted ecoregions for soybeans and best adapted soybean cultivars will help farmers to produce more yield with reduced inputs. Rhizobial strains differ in their efficacy in symbiosis with different soybean cultivars, and the input of N into agricultural systems can be increased by the inoculation of legumes with optimized rhizobia for different environments [65]. The development of better inoculation strategies, and specifically adapted rhizobia for different soybean cultivars could reduce the dependency of farmers on agrochemicals and enhance food security [65].

2.3. Tripartite symbiosis with arbuscular mycorrhizal fungi and rhizobia

In natural environments, legume roots form tripartite interactions, and are simultaneously colonized by both AM fungi and rhizobia [7, 79]. Tripartite interactions have been shown to improve plant productivity, seed yield, P and N acquisition, and photosynthetic rates [10, 80, 81]. The rhizobial nitrogenase complex requires at least 16 ATP to reduce one N₂ molecule into two NH₃. Consequently, nodules act as strong P sinks in legume root systems to provide sufficient P resources to the bacteroids for optimum BNF [79, 82]. Since AM fungi are able to improve the P nutrition of legume plants, AM fungi can increase the BNF by root nodules by at least 50% [10]. Non-mycorrhizal soybean plants have lower nodule numbers and weights, and particularly under low P supply lower N fixation rates [7, 83]. AM fungi can also provide their hosts with microelements that are essential for N₂ fixation, including zinc, iron, manganese and molybdenum [84, 85].

AM fungi and rhizobial bacteria can act synergistically and can improve plant productivity, seed yield, and grain quality [7, 10, 81]. However, the prior inoculation by either rhizobia or AM fungi can also reduce the subsequent colonization by the other symbiont [86]. Plants control the extent of root colonization by both symbionts by an autoregulatory mechanism, possibly to limit the high C costs associated with these interactions [83, 87]. Whether AM fungi and rhizobia interact antagonistically or synergistically depends on the environmental context [81], and the compatibility between symbiotic partners [10, 88]. For example, the rhizobial strain STM 7183 is more compatible with the AM fungus *Rhizophagus clarus*, and leads to higher nodulation rates, nitrogenase activities, and plant growth responses than STM 7282 [10]. Similarly, plant

productivity and seed yields of nodulated soybeans were higher when the plants were co-inoculated with the AM fungus *Rhizophagus irregularis*, than with *Acaulospora tuberculata* or *Gigaspora gigantea* [88]. Soybean cultivars also differ in their ability to benefit from their microbial communities [89]. Consequently, the symbiotic efficiency should be integrated into soybean breeding programs, and AM fungi and N-fixing bacteria with high compatibility should be identified to improve the productivity and stress resistance of soybeans and other legumes.

Both interactions are costly, and the host plant allocates up to 20% of its photosynthetically fixed C to its fungal [14, 90], and up to 30% to its N-fixing symbionts (Figure 2) [74]. C acts as an important trigger for symbiotic functioning, and a reduction in the C fluxes to the symbionts decreases BNF by rhizobia [91], and P and N uptake and transport by AM fungi [92-94]. Considering the high C costs of these symbioses for the host, plants are under a selective pressure to strongly regulate the C fluxes to both root symbionts, but these control mechanisms are currently poorly understood. Resource exchange between host and AM fungi are controlled by a reciprocal reward mechanism that is driven by biological market dynamics [95]. Our own results recently demonstrated that similar mechanisms may also control the resource to C exchange in tripartite interactions, and that *Medicago* plants allocate C to the different root symbionts in tripartite interactions in response to nutrient demand conditions, and that the AM fungus becomes a stronger competitor for C resources from the host, when the fungal partner has access to N

79].

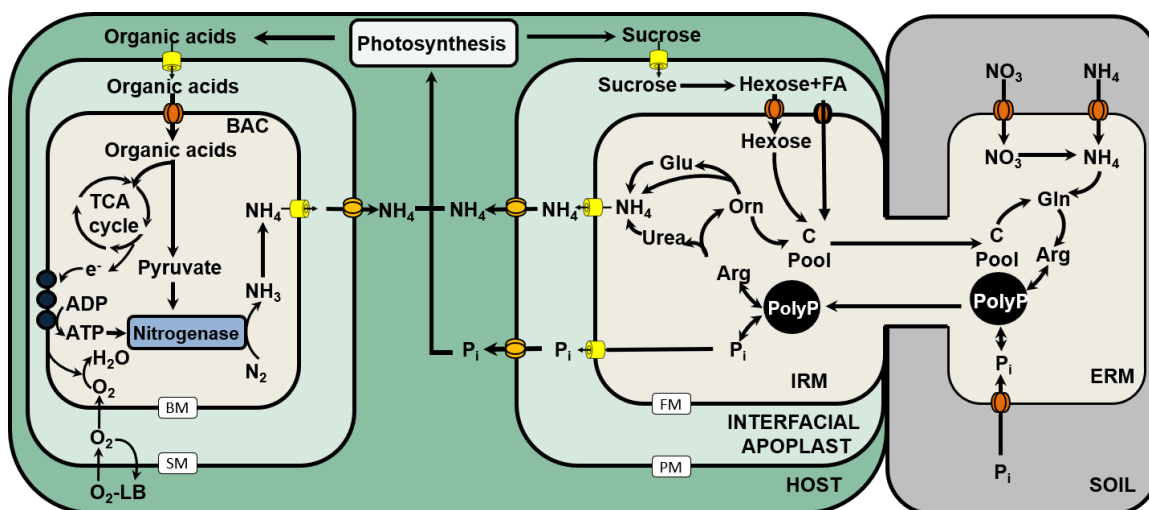


Figure 1.2 Transport and nutrient exchange pathways in the symbiosis with N-fixing bacteria (BAC) and AM fungi (IRM and ERM). Abbreviations: BAC-N-fixing bacteroids; BM-bacteroid membrane; ERM-extraradicle mycelium; FA-fatty acid; FM-fungal plasma membrane; IRM-intraradical mycelium; PM-periarbuscular membrane; SM-symbiosome membrane.

AM fungi have stronger effects on plant gene expression than rhizobia [96], but our current understanding of the molecular mechanisms involved in the C allocation to individual root symbionts is limiting. An overexpression of a leaf sucrose phosphate synthase of *M. truncatula* increases starch production, allowing the plant to allocate more photosynthates to root nodules and consequently improved nitrogenase activity and overall plant growth [75]. There is evidence that suggests that sucrose transporters (SUT) could be involved in the regulation of beneficial C fluxes towards the fungal symbiont [97], and the expression of *MtSUT2* and *MtSUT4-1* has been shown to be positively correlated to the C allocation to different symbiotic partners in tripartite interactions [79]. *MtSWEET1b* and *MtSWEET6* of the Sugars Will Eventually be Exported Transporter family (SWEET) are highly expressed in AM roots, and preferentially transport hexoses such as glucose, and could be involved in the transport of hexoses or fatty acids across the mycorrhizal interface

to the fungal partner [79, 98]. *MtSWEET11* is specifically expressed in root nodules, and could be involved in the sugar distribution within root nodules, but loss-of-function mutants indicate that *MtSWEET11* is not essential for BNF [99]. A better understanding of these processes is critical, because it may be key to improve the resource exchange between plants and symbionts, and ultimately to enhance productivity of agronomically important legumes.

2.4 Symbiosis with endophytic bacteria or fungi

Endophytes are defined as organisms that live inside plant hosts for at least part of their lives, without causing apparent disease symptoms in the host as a result of this colonization [100]. Fungal and bacterial endophytes are nearly ubiquitous across all groups of vascular plants [101], but there is a large biological diversity among endophytes, and it is not rare for some plant species to host hundreds of different endophytic species [102]. Fungal endophytes have been shown to enhance growth and seed production or protect against environmental stresses such as drought or P deficiency or provide defense against herbivory through the synthesis of various biologically active metabolites, such as alkaloids. In soybeans diverse communities of fungal endophytes can be found, and several of these endophytes have plant growth promoting capabilities, and enhance for example soybean growth in nickel or copper contaminated soils by reducing the levels of stress-related phytohormones such as abscisic acid and jasmonic acid [103], and increase glutathione activities and thereby reduce oxidative stress [104]. The inoculation of soybean plants with fungal endophytes can also lead to higher shoot biomasses, chlorophyll

contents, and photosynthetic rates compared to non-inoculated soybeans under salt stress and decrease the abundances of SCN in soils [105].

Soybeans host also a diverse group of bacterial endophytes, and many endophytic bacteria have plant growth promoting capabilities [106], such as the ability to produce plant growth hormones, or ACC (1-aminocyclopropane-1-carboxylate) deaminase, to solubilize phosphate, or to release antimicrobial metabolites or siderophores that can inhibit the growth of pathogenic microorganisms. ACC deaminase reduces the levels of ethylene, an important stress hormone in plants. Several endophytic bacteria are also diazotrophs, and have like rhizobia bacteria the ability to fix N. Bacterial endophytes also interact with rhizobia bacteria, and can enhance root nodulation, and activity, and as a consequence the N content of soybean plants [107]. The dual inoculation with rhizobia and a salt tolerant bacterial endophyte led to synergistic responses and promoted the fitness of soybean plants under salt stress [108].

3. Important research gaps and future challenges

Beneficial plant microbe interactions with AM fungi, rhizobia, or bacterial and fungal endophytes have enormous potential to improve plant growth and nutrient uptake in stressful environments and to increase the environmental sustainability of soybean agriculture. However, while the beneficial effects of AM fungi and rhizobia on soybean productivity are long known, the effect of only a small number of endophytes is currently known. The plant microbiome is a still unexplored resource of microorganisms with a so far hidden potential to promote plant growth, and success under abiotic or biotic stress conditions, and with unknown effects on the plant phenotype.

The obligate lifestyle of AM fungi, has made for a long time the production of fungal inoculum in large quantities difficult, but the development of sterile transgenic root organ cultures has led to an increased commercialization of AM fungal inocula for the utilization in agroecosystems [109]. Although increases in yield and biomass have been reported in different crops after inoculation with these inocula [36, 110], in other studies inconsistent or neutral effects were observed [111]. AM fungi differ in the benefit that they provide for their host plant [112], and mycorrhizal growth responses are highly context-dependent. Several factors can alter the success of AM fungal inoculation in agroecosystems, including plant/fungal compatibility, degree of competition with the native microbial population, or timing of inoculation [113]. All these aspects need to be taken into consideration to find the most adapted and specific conditions for an efficient use of AM fungal inocula in a given field, or for a certain crop. Our current understanding of the effect of beneficial plant microbes on soybeans is mainly based on studies with single symbionts, but plant productivity and stress resistance in agroecosystems depends on diverse microbial communities, and the interactions among the different microorganisms in these communities.

Identifying and characterizing the molecular mechanisms responsible for the functioning of different plant microbe interactions is crucial to harness these symbiotic microorganisms in agroecosystems. Currently, most knowledge is gathered on model legumes, such as *Medicago truncatula*, but the information about soybeans is limited. However, the accumulation of genomic and transcriptomic data, along with the development of molecular tools such as stable transformations [e.g. 114], CRISPR-Cas9

system [115], or mutant populations will provide us with a better understanding of these interactions in soybeans.

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Conflict of Interest

The authors have no conflict of interest.

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CHAPTER 2: NUTRIENT DEMAND AND FUNGAL ACCESS TO RESOURCES
CONTROL THE CARBON ALLOCATION TO THE SYMBIOTIC PARTNERS IN
TRIPARTITE INTERACTIONS OF *MEDICAGO TRUNCATULA*

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2.1 Abstract

Legumes form tripartite interactions with arbuscular mycorrhizal (AM) fungi and rhizobia, and both root symbionts exchange nutrients against carbon from their host. The carbon costs of these interactions are substantial, but our current understanding of how the host controls its carbon allocation to individual root symbionts is limited. We examined nutrient uptake and carbon allocation in tripartite interactions of *Medicago truncatula* under different nutrient supply conditions, and when the fungal partner had access to nitrogen, and followed the gene expression of several plant transporters of the SUT and SWEET family. Tripartite interactions led to synergistic growth responses and stimulated the phosphate and nitrogen uptake of the plant. Plant nutrient demand but also fungal access to nutrients played an important role for the carbon transport to different root symbionts, and the plant allocated more carbon to rhizobia under nitrogen demand, but more carbon to the fungal partner when nitrogen was available. These changes in carbon allocation were consistent with changes in the SUT and SWEET expression. Our study provides important insights into how the host plant controls its carbon allocation under different nutrient supply conditions and changes its carbon allocation to different root symbionts to maximize its symbiotic benefits.

Keywords: arbuscular mycorrhizal symbiosis, carbon transport, *Ensifer meliloti*, legumes, nitrogen uptake, *Rhizophagus irregularis*, rhizobia, sucrose transport, Sucrose Uptake Transporter (SUT), Sugars Will Eventually be Exported Transporter (SWEET).

2.2 Introduction

Legumes, such as soybean, cowpea and *Medicago*, are among the most important crop species worldwide. They account for 27% of the world's primary crop production, for 33% of the dietary nitrogen (N) needs of humans (Vance, 2001), and play a significant role in crop rotations and in the soil nitrogen cycle. The majority of legumes form tripartite interactions and are simultaneously colonized with N-fixing bacteria and arbuscular mycorrhizal (AM) fungi. It is well known that these interactions can substantially contribute to the nutrient efficiency of legumes and increase the fitness of both the host and the different root symbionts (Mortimer, Pérez-Fernández & Valentine, 2009; Ossler, Zielinski & Heath, 2015).

N-fixing rhizobia bacteria reside within specialized root nodules that provide them with an oxygen-reduced environment for biological N₂-fixation (BNF). Within nodules, rhizobia differentiate into bacteroids that are able to convert atmospheric N₂ to NH₃ through their nitrogenase complex. NH₃ is exported together with amino acids through the bacteroid membrane towards the host cells (Udvardi & Poole, 2013), and can contribute with up to 99% to the total N uptake of the plant under low N supply conditions (Burchill *et al.*, 2014). AM fungi, on the other hand, form an extensive extraradical mycelium in the soil that takes up water and nutrients, such as phosphate (P), nitrogen (N) and potassium, and transfers them to the host via specialized fungal structures in root cortical cells, called arbuscules (Smith & Read, 2008; Bücking & Kafle, 2015; Garcia, Chasman, Roy & Ané, 2017). In addition, AM fungi improve the resistance of their host plant against abiotic (e.g. drought, salinity) and biotic stresses (pathogens) (Smith & Read, 2008).

It is well established that a synergy of benefits can occur, and that the host plant can gain more from tripartite interactions than from single inoculations with either symbiont (Antunes, de Varennes, Zhang & Goss, 2006; Yasmeen, Hameed, Tariq & Ali, 2012; Meng *et al.*, 2015; Bournaud *et al.*, 2017). The N-fixing capability of rhizobia is often limited by the P availability and AM fungi can stimulate root nodulation, nitrogenase activity and BNF through their positive effect on plant P nutrition (Owino-Gerroh, Gascho & Phatak, 2005; Vesterager, Nielsen & Høgh-Jensen, 2006; Ding *et al.*, 2012; Püschel *et al.*, 2017). Plants can simultaneously benefit from N that is provided by both root symbionts, and nodulated legumes colonized by AM fungi with access to an external NH_4^+ source became less reliant on BNF (Mortimer, Perez-Fernandez & Valentine, 2012). Similarly, Nod factors produced by rhizobia have been shown to enhance AM colonization (Xie *et al.*, 1995; Xie, Muller, Wiemken, Broughton & Boller, 1998) and both symbiotic interactions share parts of a common signal transduction pathway (Kistner *et al.*, 2005; Zhu, Riely, Burns & Ané, 2006; Delaux, Séjalon-Delmas, Bécard & Ané, 2013). However, negative effects have also been observed, and the prior inoculation by either rhizobia or AM fungi can limit the subsequent colonization by either symbiont (Catford, Staehelin, Lerat, Piché & Vierheilig, 2003; Catford, Staehelin, Larose, Piché & Vierheilig, 2006; Valentine, Mortimer, Kleinert, Kang & Benedito, 2013). It has been suggested that plants control the extent of root colonization by both symbionts by an auto-regulatory mechanism, possibly to limit the high carbon (C) costs associated with these interactions (Mortimer, Pérez-Fernández & Valentine, 2008; Reid, Ferguson, Hayashi, Lin & Gresshoff, 2011b; Kassaw, Jr. & Frugoli, 2015). Both interactions are costly, and AM fungi can receive up to 20% (Snellgrove, Splittstoesser, Stribley & Tinker, 1982; Jakobsen & Rosendahl, 1990;

Wright, Read & Scholes, 1998), and rhizobia up to 30% of the host photosynthates (Provorov & Tikhonovich, 2003).

In plants, sucrose is the main carbohydrate for long-distance transport, and is loaded in the leaves into the phloem, and then transferred to the sink tissues. In root nodules, sucrose is mainly converted to malate, which is considered to be the primary C source transferred across the symbiosome membrane to the bacteroids (Oldroyd, Murray, Poole & Downie, 2011; Udvardi & Poole, 2013). Hexoses were long seen as the major C form that is transferred across the interface to the AM fungus (Helber *et al.*, 2011), but recent reports revealed that fatty acids can also be exported out of the root cell and transported to the fungal symbiont (Bravo, Brands, Wewer, Dörmann & Harrison, 2017; Jiang *et al.*, 2017; Keymer *et al.*, 2017; Luginbuehl *et al.*, 2017; Rich, Nouri, Courty & Reinhardt, 2017). This C supply plays a critical role for symbiont function (Kiers, Rousseau, West & Denison, 2003; Kiers *et al.*, 2011; Fellbaum *et al.*, 2014), but how C is partitioned and directed to different symbiotic partners is still unknown. It has been suggested that Sucrose Uptake Transporters (SUT) could be involved in the regulation of beneficial C fluxes towards the fungal symbiont (Doidy *et al.*, 2012; Garcia, Doidy, Zimmermann, Wipf & Courty, 2016), and recently, Sugars Will Eventually be Exported Transporters (SWEET) have been identified in AM and nodulated roots (Kryvoruchko *et al.*, 2016; Manck-Götzenberger & Requena, 2016; Sugiyama *et al.*, 2017). The SWEET family mediates the influx and efflux of sugar molecules from cells and plays a role in phloem loading and unloading (Lemoine *et al.*, 2013). In *Medicago*, *MtSWEET11* is specifically expressed in root nodules, but loss-of-function mutants were not compromised in BNF, indicating that

this transporter could be involved in sugar distribution within root nodules, but may not be a critical component for BNF (Kryvoruchko *et al.*, 2016).

Despite their importance for nutrient uptake and crop yield, nutrient to C exchange dynamics in tripartite interactions are only poorly understood. Exploiting the full yield potential of legumes will require a better understanding of these interactions, but functional insights into these interactions are currently mainly derived from experiments of plants associated with a single symbiont. The goal of our study is to contribute to a better understanding on how host plants regulate their C allocation to their different root symbionts in tripartite interactions, since this knowledge is critical to improve the nutrient efficiency and symbiotic benefits in agriculturally important legumes.

2.3 Material and methods

2.3.1 Plant, fungal, and bacterial material

Medicago truncatula (A17) seeds were scarified with concentrated H₂SO₄, and surface sterilized with 8% bleach for two minutes. The plants were pre-germinated on moist filter paper in Petri dishes for 3 days in the dark, followed by 7 days under light. To facilitate lateral root development, we cut the primary roots of the germinated seedlings before transferring them for 20 days into a hydroponic solution containing 0.05 mM KH₂PO₄, 0.125 mM NH₄NO₃, 0.30 mM KCl, 0.5 mM CaCl₂ x 2H₂O, 0.312 mM MgSO₄ x 2H₂O, 6.8 μM Fe-EDTA, 1.50 μM MnCl₂ x 2H₂O, 8.08 μM H₃BO₃, 0.05 μM Zn-EDTA, 0.14 μM CuCl₂ x 2H₂O, 0.01 μM Na₂MoO₄ x 2H₂O (Ingestad, 1960). Twice daily, the solution was stirred, and replaced once after 10 days.

We then transferred the seedlings into custom-made multi-compartment systems (12 cm x 8 cm x 8 cm, L x H x W) with three compartments, two root compartments (RC) and one hyphal compartment (HC) (Figure S2.1). All compartments were filled with 200 mL soil substrate consisting of 60 % turf (Profile Products LLC, IL, USA), 30 % sand, and 10 % organic soil (13.05 mg/l nitrate, 2.28 mg/l ammonium, and 24.19 mg/l available phosphate, Olsen's extraction) (experiment 1) or 80 % sand, 10 % perlite, and 10 % organic soil (14.77 mg/l nitrate, 9.03 mg/l ammonium, and 20.77 mg/l available phosphate, Olsen's extraction) (experiment 2). Both RCs were separated by a 0.1 cm thick plastic sheet that was sealed at all sides by silicone (Aqueon, Franklin, WI, USA) to prevent any cross-contamination between the RCs. The HC was separated from the RC by a plastic sheet with a hole (~3.12 cm diameter) that was closed on both sides with a 50 μm nylon mesh. In between the two fine mesh layers, we placed a coarse nylon mesh with a pore size of 1000 μm to form an air gap and to prevent mass flow from the HC to the RC. The mesh layers prevented the crossover of roots from the RC to the HC, but allowed in the AM colonized growth systems the crossover of the fungal mycelium into the HC. We divided the root system of the plants equally into two root halves, and each root half was transferred into an independent RC. After transplanting, the plants were grown in a controlled-environment chamber with a 25°C/20°C day and night cycle, 30% humidity, and a photosynthetic active radiation of 225 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

We produced the fungal inoculum of *Rhizophagus irregularis* Schenck & Smith (DAOM 197198) in axenic Ri T-DNA transformed carrot (*Daucus carota* clone DCI) root organ cultures in Petri dishes filled with mineral medium (St-Arnaud, Hamel, Vimard, Caron & Fortin, 1996). After approximately eight weeks of growth, the spores were

isolated by blending the medium in 10 mM citrate buffer (pH 6.0). The bacterial inoculum was produced by growing *Ensifer meliloti* Dangeard (1021; previously *Sinorhizobium meliloti*) in tryptone yeast media on a rotatory shaker at 250 rpm at 28°C for 20 h. Before the inoculation, the bacteria were centrifuged and resuspended in autoclaved tap water. Fungal and bacterial inocula were added into a hole in the soil close to the root approximately 5 cm below the soil surface.

2.3.2 Experimental design

We conducted two experiments and examined the C allocation to different root symbionts depending on whether the fungal partner had access to an exogenous nitrogen supply (experiment 1), and depending on the nutrient demand conditions of the host (experiment 2) (Figure S2.1). In experiment 1, we studied the C allocation in four different systems with: (1) two non-inoculated root halves (\emptyset/\emptyset), (2) one non-inoculated root half and one inoculated with *Rhizophagus irregularis* (\emptyset/AM), (3) one non-inoculated root half and one inoculated with *Ensifer meliloti* (R/\emptyset), and (4) two inoculated root halves, one inoculated with *Ensifer meliloti* and one inoculated with *Rhizophagus irregularis* (R/AM) (Figure S2.1). The AM root halves were inoculated with 500 spores at transplanting and the rhizobia root halves three weeks after transplanting. Since the root system of one sacrificed plant did not show clear signs of AM inoculation after three weeks, we repeated the AM inoculation with 100 spores four and seven weeks post transplanting. To induce nutrient demand, the plants were fertilized three times with relatively low P and N concentrations (125 μM N as NH_4NO_3 , and 50 μM KH_2PO_4 in the soil) in a modified Ingestad nutrient solution (Ingestad, 1960). To test whether the access of N for the AM

fungus has an effect on the C allocation, we added 4 mM of $^{15}\text{NH}_4\text{Cl}$ (Sigma Aldrich, St. Louis, USA) (+ N) in a modified nutrient solution (Ingestad, 1960; no other P or N source) to the HC of half of the systems 12 weeks after transplanting. The controls (- N) received the same nutrient solution but without ^{15}N . To control for any leakage or any mass flow from the HC to the RC, we also added $^{15}\text{NH}_4\text{Cl}$ to the control treatments (\emptyset/\emptyset), and to systems that were only inoculated with *E. meliloti* (R/ \emptyset). Since none of these systems showed any ^{15}N labeling, we later considered them as - N. Four weeks later (16 weeks post transplanting), the plants were labeled with $^{13}\text{CO}_2$ as described below.

In experiment 2, we examined the C allocation to both root symbionts in tripartite interactions under different nutrient demand conditions for the host. We inoculated one of the RCs at transplanting with ~1000 spores of *R. irregularis*, and the other RC three weeks later with 1 mL (O.D. of 0.28) of a bacterial suspension with *E. meliloti*. Until the final nutrient treatment, we fertilized each RC every week with a modified Ingestad (1960) nutrient solution containing 250 μM NH_4NO_3 and 50 μM KH_2PO_4 . The nutrient concentrations were relatively low to induce P and N demand conditions, and to stimulate the AM and rhizobial colonization of the root systems. Ten weeks after transplanting, the nutrient demand conditions of the plants were varied by adding a modified Ingestad (1960) nutrient solution with combinations of low (L) or high (H) P or N concentrations to both RCs (LPLN, LPHN, HPLN, HPHN). The nutrient levels in the soil were 50 μM or 650 μM KH_2PO_4 (LP or HP), or 0.25 mM or 1.8 mM NH_4NO_3 (LN or HN), respectively. Three weeks later (13 weeks post transplanting), the plants were labeled with $^{13}\text{CO}_2$.

For the labeling with $^{13}\text{CO}_2$, we covered the soil in the growth chambers with a transparent plastic foil, transferred all plants into an air tight chamber (76 x 61 x 15.6 cm), in which $118 \mu\text{L mL}^{-1} \text{ }^{13}\text{CO}_2$ was released for two hours. A fan ensured a homogenous distribution of $^{13}\text{CO}_2$ within the chamber during the labeling. The plants were harvested 24 h after labeling and were analyzed for their biomass characteristics, fungal and bacterial colonization rates, nutrient contents, ^{13}C -labeling and gene expression.

2.3.3 Biomass characteristics, and quantification of rhizobial and AM root colonization

After harvest, each root half was weighed and divided into three aliquots; one aliquot was flash frozen in liquid nitrogen, and stored at -80°C for gene expression analysis, one aliquot was stored in 50 % ethanol (v:v) to determine the fungal and bacterial root colonization, and one aliquot and the plant shoots were dried in an oven at 70°C for 48 h. Based on the fresh to dry weight ratio of this root aliquot, the total root biomass was determined. Root nodules were removed, counted and dried in an oven at 70°C for 48 h. To determine the AM colonization, the roots were cleared with 10 % KOH solution at 80°C for 30 min, rinsed, and stained with 5% ink at 80°C for 15 min (Vierheilig, Coughlan, Wyss & Piché, 1998). We analyzed a minimum of 150 root segments to determine the percentage of AM root colonization by the gridline intersection method (McGonigle, Miller, Evans, Fairchild & Swan, 1990).

2.3.4 Measurements of nitrogenase activity

To measure the nitrogenase activity of the root nodules, we carefully removed an aliquot of the nodulated root half at plant harvest, loosely wrapped it in moist filter paper

and transferred the samples into airtight 30 mL tubes sealed with rubber cork. We injected 10 % (3 mL) acetylene gas and measured the production of ethylene after 24 h using an Agilent Technologies 7890A Gas Chromatography System (Santa Clara, CA, USA). Sample peak areas were fitted to a calibration curve and the ethylene production was normalized to the nodule number of the root aliquots.

2.3.5 Quantification of P, ^{13}C and ^{15}N in plant tissues

Aliquots of shoot and root tissues were first pulverized with a tissue homogenizer (Precellys 24, Cayman Chemical Company, Ann Arbor, MI, USA). We digested the plant tissues with 2N HCl for 2 h at 95°C, and determined the P content spectrophotometrically at 436 nm after adding ammonium molybdate vanadate solution (Fisher Scientific, Pittsburgh, USA). ^{13}C in the shoot and root tissues was quantified using a Costech 4010 and Carlo Erba 1110 Elemental Analyzer coupled to a Thermo Delta Plus XP IRMS at the stable isotope facility of the University of Wyoming (Laramie, WY, USA). The conversion of $\delta^{13}\text{C}$ into the C contents in plant biomass was conducted according to Ruehr et al. (2009). For the ^{15}N analysis by quantitative NMR spectroscopy, we first digested 10-15 mg aliquots of homogenized and oven-dried root and shoot material in 750 μL concentrated H_2SO_4 . Samples were then heated for 2 h at 225°C followed by an addition of 36 drops of 30% H_2O_2 (three drops at a time every 30 sec) as previously described (Fellbaum *et al.*, 2012). The solution was then heated for an additional 3 h at 225°C to remove any traces of water and allowed to cool. Forty μL of the resulting clear solution of $(\text{NH}_4)_2\text{SO}_4$ in H_2SO_4 was dissolved into 600 μL of 99.9 % d_6 DMSO containing 0.05% (v:v) TMS reference (Norell Scientific, Vineland, NJ). The ^1H spectrum was obtained in a 5 mm tube placed in

a z-axis pulsed field gradients probe on a 14.1 Tesla Agilent NMR spectrometer (Santa Clara, CA, USA) operating at 600 MHz. The spectra were acquired using ~1400 transients with a 90° (10.8 µsec) pulse width, spectral width of 12 ppm, pulse delay of 2.0 seconds, acquisition time of 1.7 seconds at 25°C. The percentage of total N labeled with ¹⁵N in the tissue was determined by dividing the integrated area of the ¹H-¹⁵N doublet resonances by the sum of the integrated doublet and triplet resonance areas.

2.3.6 Gene expression analysis

We determined the transcript levels in the roots of two AM-inducible plant genes, the P transporter *MtPT4* (Chiou, Liu & Harrison, 2001; Harrison, Dewbre & Liu, 2002; Javot, Penmetsa, Terzaghi, Cook & Harrison, 2007) and the ammonium transporter *MtAMT2;3* (Straub, Ludewig & Neuhäuser, 2014; Breuillin-Sessoms *et al.*, 2015). In addition, we analyzed the expression levels of three plant sucrose transporters from the SUT family, *MtSUT1-1*, *MtSUT2* and *MtSUT4-1* (Doidy *et al.*, 2012), and seven transporters of the SWEET family, *MtSWEET1b*, *MtSWEET6*, *MtSWEET9*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d*. Since *MtSWEET9* showed only low and very inconsistent levels of expression in our experiments, the results of this transporter are not shown. All steps were performed according to the manufacturer's instructions unless stated otherwise. We homogenized the root samples with a mortar and pestle cooled with liquid nitrogen, and extracted total RNA using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNAs were treated with TURBO™ DNase (Thermo Fisher Scientific) and quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNAs were synthesized from 400 or 600

ng of DNase-treated RNAs using the RNA Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermo Fisher Scientific) and diluted with RNase-free water to a final concentration of 20 ng μl^{-1} if needed. qPCRs were performed using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 1 μl of 20 ng μl^{-1} cDNAs, and 5 μM of forward and reverse primers (Table S1) for each gene in a 20 μl reaction mix using a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). The PCR conditions were as follows: 50°C for 2 min; 95°C for 15 min; 40 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s; dissociation at 95°C for 15 s; 60°C for 15 s; and 95°C for 15 s. We used *MtTefl α* as a reference gene (Gomez et al., 2009) and the expression coefficients were calculated using the $2^{-\Delta\text{Ct}}$ method. The results are based on three to four biological replicates and three technical replicates.

2.3.7 Statistical analysis

The data of experiment 1 are based on three to seven biological replicates (plants that showed any sign of a cross contamination between the two root compartments were removed, on average 5 biological replicates), and the data of experiment 2 are based on three biological replicates. We used one-way ANOVA ($p \leq 0.05$) with colonization type or nutrient treatment as fixed factor followed by the Least Significance Difference (LSD) or the Student's t-test when the data passed Leven's test for homogeneity of variance and the Shapiro-Wilk normality test. If the data set failed these tests, the data set was log-transformed prior to the analysis. An ANCOVA was used to confirm the results of the ANOVA analysis and to account for the effects of the covariate (biomass) in experiment 1 on the statistical evaluation of the nutritional benefits. To identify statistical significant

differences between the means, the statistical software Statistix 9 Analytical Software (Tallahassee, Florida, USA) or R was used. The regression analysis of the SUT transporter expression and ^{13}C allocation was conducted by R using one standard deviation from the mean for the analysis. The results of the statistical analysis are provided in Table S2 and S3 (supplementary information).

2.4 Results

2.4.1 Tripartite interactions can act synergistically on biomass and nutrient uptake of plants

In experiment 1, we examined plants that were colonized with different symbiotic partner combinations (\emptyset/\emptyset , R/ \emptyset , \emptyset /AM, R/AM), and in which the fungal partner of half of the systems had access to $^{15}\text{N-NH}_4\text{Cl}$ via the HC (+N). The plant biomass data demonstrate that the host plants were under N demand (Figure 2.1). The plants that were inoculated with *E. meliloti* (R/ \emptyset , R/AM) had a significantly higher shoot and root biomass than control plants (\emptyset/\emptyset), or plants that were only inoculated with *R. irregularis* (\emptyset /AM). However, plants that were co-inoculated (R/AM) had a higher shoot and root biomass than plants that were inoculated with *E. meliloti* alone (R/ \emptyset). Colonized root halves were larger than non-colonized root halves, and rhizobial root halves were larger than AM root halves (Figure 2.1b). Fungal access to N led to an increase in shoot biomass, but only in plants that were inoculated with *R. irregularis* alone (\emptyset /AM) (Figure 2.1a). In contrast, plants that were co-inoculated with both symbionts (R/AM) showed a slight decrease in shoot biomass when the fungus had access to N.

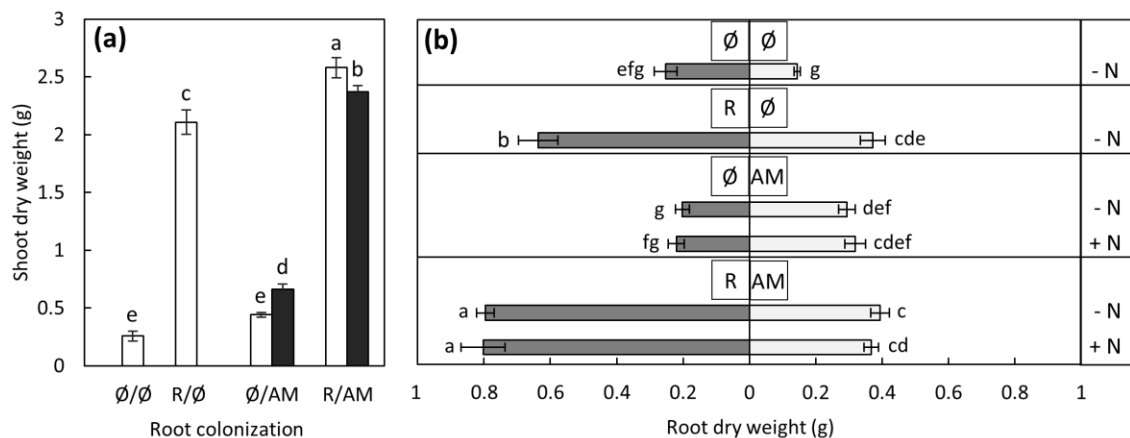


Figure 2.1. Shoot (a) and root (b) dry weights of *Medicago truncatula* depending on the colonization with different root symbionts and under different N supply conditions for the fungal partner (+ N, black bars in (a) – addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment; - N, white bars in (a) – no addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment) (Experiment 1). Root colonization abbreviations: Ø/Ø – controls, both root halves non-inoculated; R/Ø – one root half colonized by *Ensifer meliloti*, one root half non-inoculated; Ø/AM – one root half colonized by *Rhizophagus irregularis*, one root half non-inoculated; R/AM – one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti*. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($p \leq 0.05$). ANOVA results are shown in Table S2.2.

We compared the root colonization and the activity of N-fixing root nodules in single (Ø/AM or R/Ø) or dual inoculated systems (R/AM), and found that the AM colonization in dual inoculated systems was significantly lower than in Ø/AM systems (Figure S2.2a). In contrast, the total nodule number per root system was not affected (not shown), but the dry weight of individual root nodules and the N fixing activity of these nodules were significantly higher in dual inoculated systems than in R/Ø systems (Figure S2.2b, c). The addition of ^{15}N to the hyphal compartment did not have an effect on the root colonization patterns, or the N fixing activity of the root nodules.

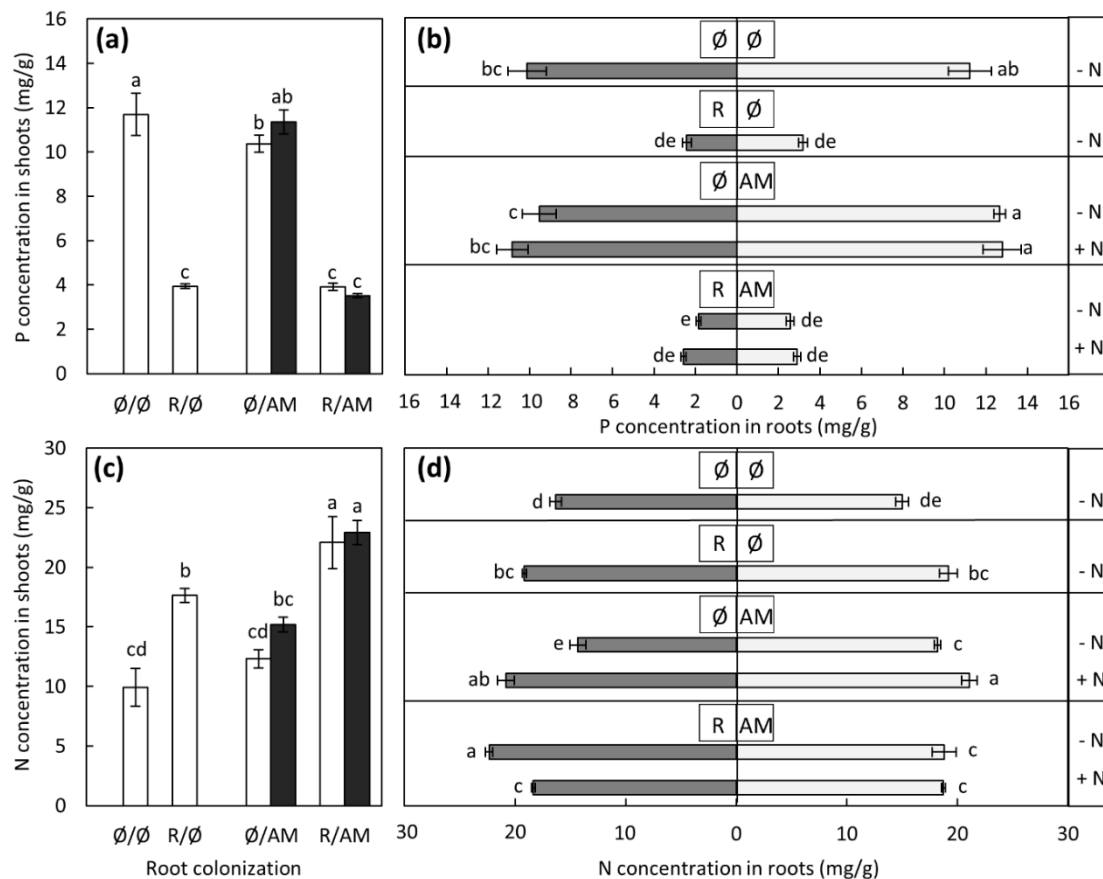


Figure 2.2. Phosphate (a, b) and nitrogen (c, d) tissue concentrations in the shoots (a, c) or roots (b, d) of *Medicago truncatula* plants depending on the colonization with different root symbionts and under different N supply conditions for the fungal partner (+ N, black bars in (a) and (c) – addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment; - N, white bars in (a) and (c) – no addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment) (Experiment 1). Root colonization abbreviations: ∅/∅ – controls, both root halves non-inoculated; R/∅ - one root half colonized by *Ensifer meliloti*, one root half non-inoculated; ∅/AM - one root half colonized by *Rhizophagus irregularis*, one root half non-inoculated; R/AM – one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti*. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($p \leq 0.05$). ANOVA results are shown in Table S2.2.

Control plants (∅/∅) and plants that were only inoculated with *R. irregularis* (∅/AM) showed higher levels of P in their tissue than plants that were dual-inoculated (R/AM) or inoculated with *E. meliloti* alone (R/∅) (Figure 2.2a, b). This is likely the result

of a dilution effect caused by the higher biomass in *E. meliloti* inoculated systems and suggests that N was a more limiting factor than P for plant growth during the experiment. The AM symbiosis increased the P contents in the mycorrhizal root halves of \emptyset /AM systems, and in the shoots of dual-inoculated plants (R/AM) compared to all the other plant systems (Figure S2.3a, b). Consistent with the higher N fixing activity of the nodules in tripartite interactions, we found an increase in the N concentrations in the shoots and the N contents of shoots and rhizobial root halves of dual inoculated plants (R/AM) compared to plants that were only inoculated with *E. meliloti* (R/ \emptyset) (Figure 2.2c, d and S2.3c, d). However, single or dual inoculated systems with *E. meliloti* (R/ \emptyset , R/AM) had higher N root and shoot concentrations and contents than control plants (\emptyset / \emptyset) or systems that were only AM inoculated (\emptyset /AM). Fungal access to N increased the root and shoot N concentrations in single inoculated (\emptyset /AM), but not in dual inoculated systems (R/AM) (Fig. 2.2c, d).

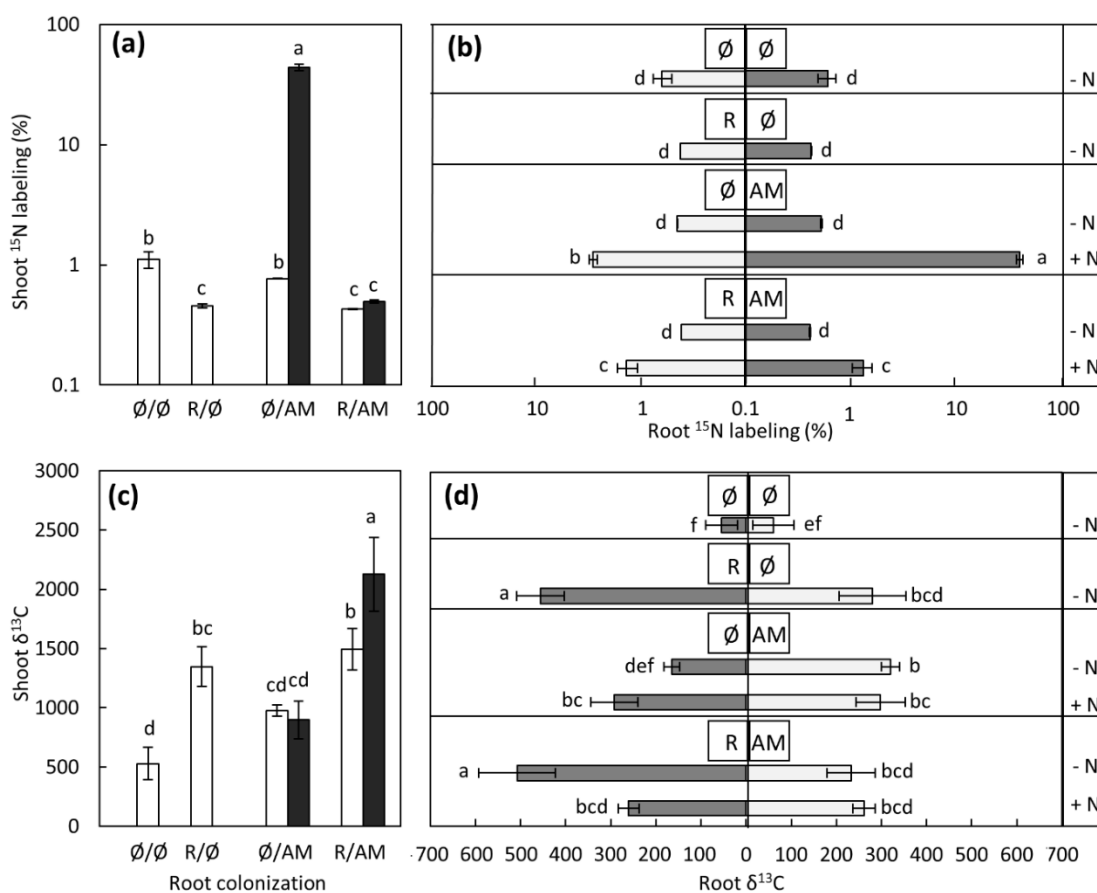


Figure 2.3. Labeling with ^{15}N (a, b) or ^{13}C (c, d) in the shoots (a, c) or roots (b, d) of *Medicago truncatula* plants depending on the colonization with different root symbionts and under different N supply conditions for the fungal partner (+ N, black bars in (a) and (c) – addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment; - N, white bars in (a) and (c) – no addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment) (Experiment 1). Root colonization abbreviations: \emptyset/\emptyset – controls, both root halves non-inoculated; R/ \emptyset – one root half colonized by *Ensifer meliloti*, one root half non-inoculated; \emptyset/AM – one root half colonized by *Rhizophagus irregularis*, one root half non-inoculated; R/AM – one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti*. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($p \leq 0.05$). ANOVA results are shown in Table S2.2.

2.4.2 Carbon allocation to root symbionts depends on the pathway for symbiotic nitrogen uptake

We added labelled $^{15}\text{N-NH}_4\text{Cl}$ to the hyphal compartment and found labeling exclusively in the roots and shoots of plants that were colonized with AM fungi (Figure 2.3a, b). The labeling in the shoots and in the roots with ^{15}N , however, was significantly higher in single inoculated (\emptyset/AM) than in dual inoculated (R/AM) systems. The relatively low labeling with ^{15}N in the R/AM systems can be explained by a dilution effect caused by the strong increase in biomass, and the relatively low root colonization of these plants (Figure 2.3 and S2.2a). The transport of ^{15}N through the extraradical mycelium to the host, led also to a higher labeling in the second root half (non-mycorrhizal root half in \emptyset/AM systems or nodulated root half in R/AM systems). Consistent with an ^{15}N -isotope dilution effect through the BNF activity of root nodules, the ^{15}N labeling in the control roots of non-inoculated systems (\emptyset/\emptyset) was slightly higher than in systems inoculated with *E. meliloti* (significant according to the non-parametric Wilcoxon Mann Whitney's Rank Sum test). None of the control plants (\emptyset/\emptyset), or plants that were only inoculated with rhizobia (R/ \emptyset), showed any ^{15}N labeling above natural abundance in roots or shoots, indicating that there was no mass flow from the HC to the RCs. Therefore, these systems are considered as – N treatments.

Plants that were inoculated with *E. meliloti* had significantly higher $\delta^{13}\text{C}$ levels in their shoots than non-inoculated (\emptyset/\emptyset) or AM inoculated (\emptyset/AM) systems (Figure 2.3c). Nodulated root halves acted as strong C sinks and showed a significantly higher $\delta^{13}\text{C}$ labeling than the non-inoculated root halves in R/ \emptyset systems, or the mycorrhizal root halves

in R/AM systems (Figure 2.3d). When the fungus had no access to N, plants allocated more C to the nodulated root half and the $\delta^{13}\text{C}$ labeling in the AM root half was significantly lower. The C allocation to the nodulated root half, however, was significantly lower when the fungus had access to an exogenously supplied N source and did not differ from the C allocation into the AM root halves. Expressed on a percentage base, plants that were colonized with rhizobia (R/Ø; R/AM) allocated only 19.7% of the assimilated C to their root system, while AM plants invested 38.9 %, and control plants 52.5 % of their assimilated C into their root systems.

2.4.3 Fungal access to nitrogen affects the expression of sucrose transporters in the roots of tripartite interactions

In order to identify the molecular mechanisms that control the C allocation to AM or nodulated roots, we evaluated the expression levels of the three sucrose transporters, *MtSUT1-1*, *MtSUT2*, and *MtSUT4-1*, and of seven SWEETs from *M. truncatula*, *MtSWEET1b*, *MtSWEET6*, *MtSWEET9*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d*. Since *MtSWEET9* showed only low and inconsistent expression levels in our experiments, we did not further consider this transporter in the analysis. With the exception of *MtSWEET11* that was exclusively expressed in rhizobial roots, all other transporters were expressed in non-inoculated, mycorrhizal, and in nodulated roots, but their transcript levels were dependent on the root colonization and on the nutrient availability for the fungal partner. Compared to control roots (Ø/Ø), rhizobial roots in single inoculated systems (R/Ø) showed higher transcript levels of *MtSUT2* and *MtSUT4-1* (Figure S2.4b,c). The rhizobial and AM root half of single inoculated systems showed

higher expression levels of *MtSWEET1b* and *MtSWEET6* than the non-inoculated root halves (Figure S2.5), but in dual inoculated systems the transcript levels were down-regulated in the AM root halves (Figure 2.4).

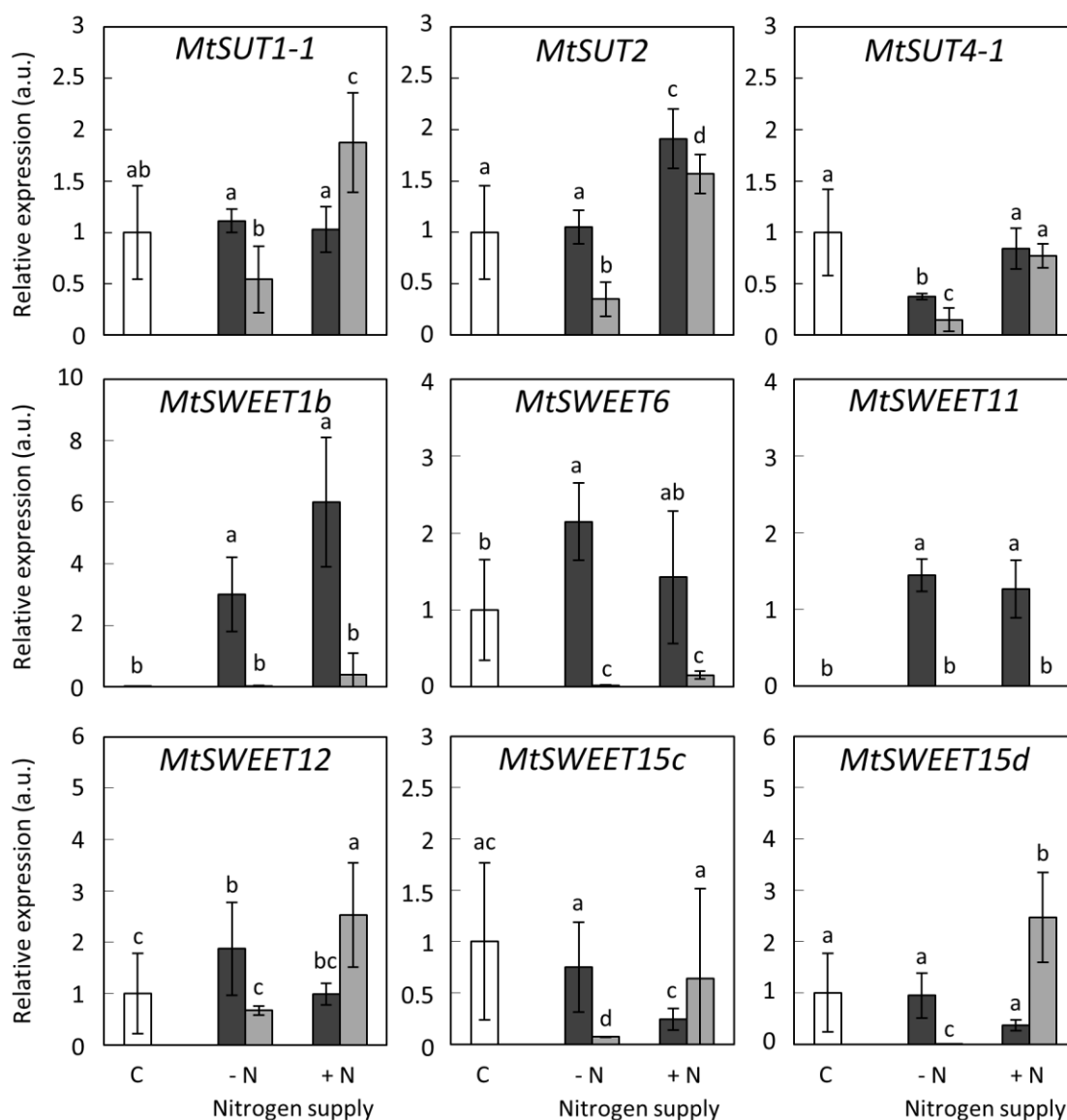


Figure 2.4. Relative expression of three sucrose transporters (*MtSUT1-1*, *MtSUT2* and *MtSUT4-1*) and of six *SWEETs* (*MtSWEET1b*, *MtSWEET6*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, *MtSWEET15d*) in *Medicago truncatula* roots depending on the colonization with different root symbionts and under different N supply conditions for the fungal partner (+N – addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment; -N – no addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment) (Experiment 1). Shown is the expression of R/AM systems (rhizobial

root halves – black bars; AM root halves – grey bars) compared to control roots of \emptyset/\emptyset systems (C). Data (means \pm SEM) are expressed in arbitrary units (a.u.). Independent statistical analyses were performed for each split-root system compared to the control, with letters indicating statistically significant differences (LSD-test, $P < 0.05$). ANOVA results are shown in Table S2.

The transcript levels of several transporters were consistent with the observed changes in C allocation to the AM or rhizobial root halves in tripartite interactions (Figure 2.4). *MtSUT1-1*, *MtSUT2*, *MtSUT4-1*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* were significantly up-regulated in the AM roots of dual-inoculated systems when the fungus had access to N. When the fungus was unable to provide N, nodulated root halves showed higher transcript levels, but when the fungus had access to an exogenous N supply, the transcript levels of all transporters increased in the AM root halves. The transcript levels of the AM root halves were now higher than in the rhizobial root halves (*MtSUT1-1*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d*), comparable to the rhizobial root halves (*MtSUT4-1*), or only slightly lower than in rhizobial root halves (*MtSUT2*) (Figure 2.4). *MtSUT2* and *MtSUT4-1* were also up-regulated in the nodulated root halves of R/ \emptyset systems, indicating that these transporters do not only play a role in the C allocation to AM colonized roots, but also to the roots colonized with the N-fixing symbiont (Figure 2.4).

Significant transcript levels of the AM-specific phosphate transporter *MtPT4* and the NH_4^+ transporter *MtAMT2;3* were only detected in the mycorrhizal root halves of the \emptyset/AM systems, independent on whether the fungus had access to ^{15}N or not. The low expression levels of both transporters in the mycorrhizal root halves of dual-inoculated systems (R/AM), are consistent with the strong reduction of the AM colonization in these systems (Figure S2.6a, b).

2.4.4 *Plants allocate carbon resources to their symbiotic partners depending on their nutrient demand*

We examined the effect of different nutrient demand conditions on the C allocation to different root symbionts in tripartite interactions of *M. truncatula*. Three weeks before the plants were labeled with $^{13}\text{CO}_2$, we changed the nutrient demand conditions of the host plant by adding low (L) or high (H) P or N concentrations to both RCs (LPLN, LPHN, HPLN, and HPHN). The nutrient treatments did not have a significant effect on shoot biomass, AM colonization ($76.2 \pm 4.2\%$, mean \pm SEM) or nodule dry weights of the roots (51 ± 2.4 mg, mean \pm SEM) (Fig. S7). We only observed that the biomass of the AM root half was smaller than the rhizobial root half under LPLN conditions (Figure S2.8).

The P and N concentrations of the shoots were not significantly affected by the different nutrient treatments (Figure 2.5a, c). There were, however, indications for an increase in the P and N shoot contents with higher nutrient availabilities (significant according to the non-parametric Wilcoxon Mann Whitney's Rank Sum test, Figure S2.9a, c). Root nodules acted as strong P sinks, and the tissue concentration of P and N in the root nodules was higher than in the AM roots or in the rest of the rhizobial root halves (Figure 2.5b, d). The AM root halves had, however, higher P concentrations and contents than the rhizobial root halves (Figure 2.5b, Figure S2.9b). While the P and N tissue concentrations and contents in the rhizobial root half and the root nodules were generally not affected by the nutrient treatments, the N tissue concentration and the N and P contents of the AM root halves increased when the plants were supplied with higher P and N concentrations (Figure

2.5d and S2.9b, d). We found, however, a lower N tissue concentration in the root nodules at LPLN, indicating reduced N fixation rates of the nodules at low P supply conditions (Figure 2.5d).

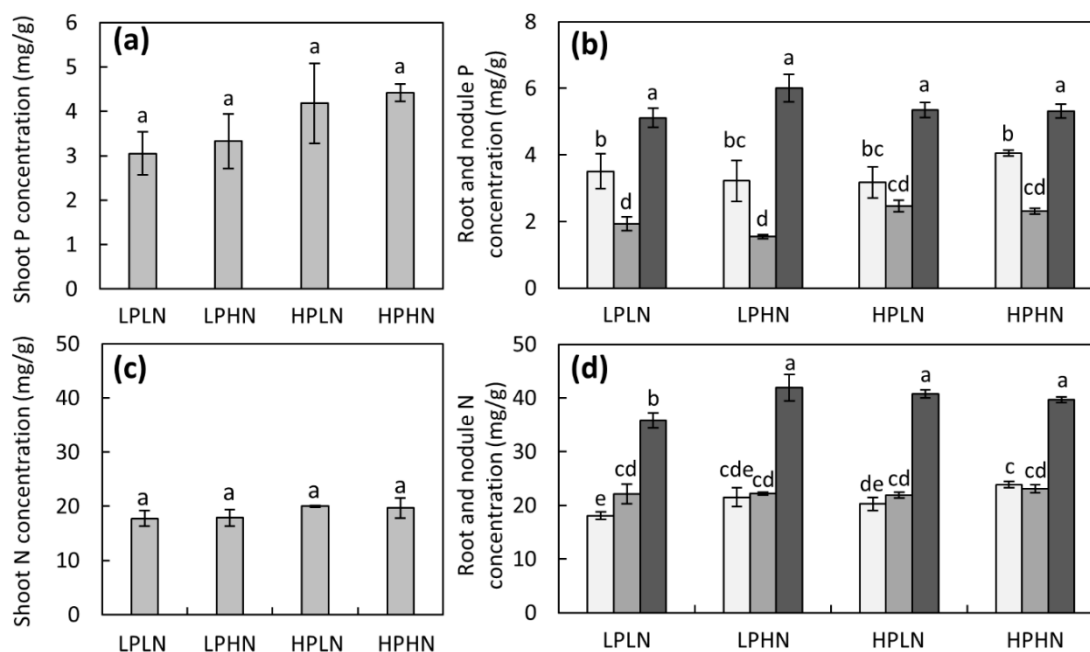


Figure 2.5. Phosphate (a, b) and nitrogen tissue concentration (c, d) in shoots (a, c) and different root fractions (b, d) of *Medicago truncatula* in symbiosis with the AM fungus *Rhizophagus irregularis* and the nitrogen-fixing diazotroph *Ensifer meliloti* under different nutrient supply conditions (low – L, or high – H, phosphate – P, nitrogen - N) (Experiment 2). Root fractions in b, d: AM root halves – light grey, rhizobial root halves – middle grey, root nodules – dark grey. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($P \leq 0.05$, $n = 3$). ANOVA results are shown in Table S2.3.

The different nutrient demand conditions had a clear effect on the C allocation in tripartite interactions of *M. truncatula*. While under low N supply conditions (LPLN and HPLN) significantly more assimilated ^{13}C could be recovered from the rhizobial root half, the ^{13}C contents in the AM root halves increased under high N supply conditions (LPHN and HPHN) (Figure 2.6b). When the N supply for the plants was low, only 19.7 ± 5.0 %

(LPLN) or 23.3 ± 4.3 % (HPLN) of the total ^{13}C that was allocated to the root system, was transferred to the AM root halves, but under high N supply conditions, this percentage increased to 29.9 ± 5.3 % (LPHN) or 35.4 ± 2 % (HHPN), respectively (data not shown).

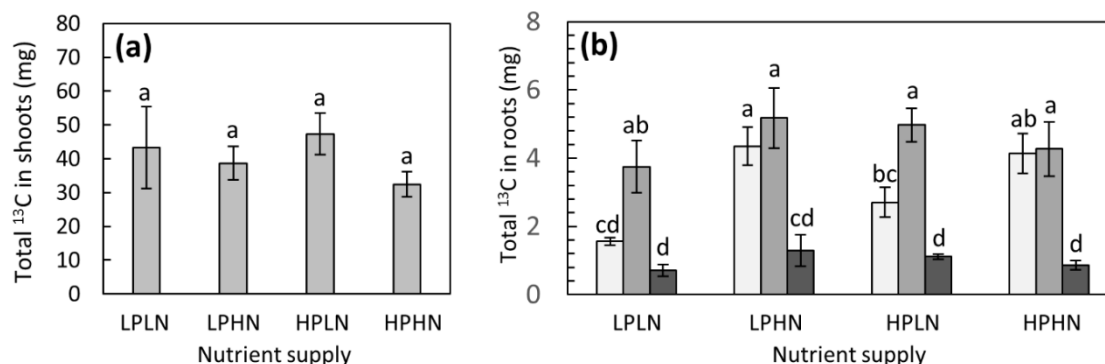


Figure 2.6. Recovered ^{13}C contents in shoots (a) and different root fractions (b) of *Medicago truncatula* in symbiosis with the AM fungus *Rhizophagus irregularis* and the nitrogen-fixing diazotroph *Ensifer meliloti* under different nutrient supply conditions (low – L, or high – H, phosphate – P, nitrogen – N) (Experiment 2). Root fractions in b: AM root halves – light grey, rhizobial root halves – middle grey, root nodules – dark grey. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($P \leq 0.05$, $n = 3$). ANOVA results are shown in Table S2.3.

2.4.5 The expression of plant *SUT* and *SWEET* transporters is consistent with the observed differences in carbon allocation under different nutrient demand conditions for the host

The observed changes in the C allocation to both root symbionts in response to different nutrient supply conditions, are consistent with changes in the plant sucrose transporter expression. The transcript levels of *MtSUT1-1* in the AM root halves were significantly higher than in the nodulated root halves under all nutrient supply conditions, indicating that this transporter may play a role for the C transport to AM roots (Figure 2.7a). The expression of *MtSUT1-1* in AM roots was particularly high under LPHN

conditions. However, the transcript levels of *MtSUT1-1* were not correlated to the measured C allocation. By contrast, changes in the expression levels of *MtSUT2* and *MtSUT4-1* were clearly correlated to the amount of C that was allocated into the different root halves. While nodulated roots had significantly higher transcript levels of *MtSUT2* than AM roots under low N supply conditions (LPLN and HPLN), there were no significant differences under high N supply conditions (LPHN and HPHN) (Figure 2.7b). In contrast, the expression levels of *MtSUT4-1* were not affected by different nutrient supply conditions, and also did not differ between AM and nodulated root halves (Fig. 2.7c).

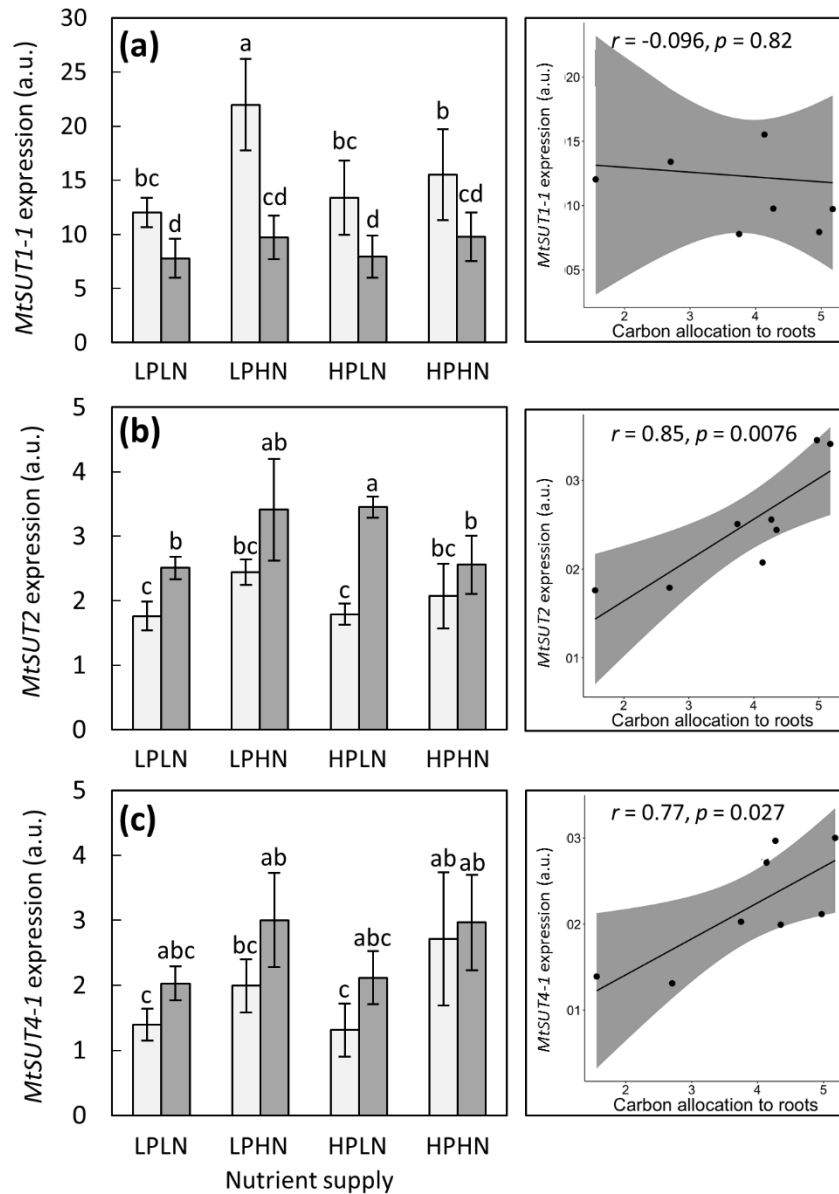


Figure 2.7. Relative expression of *MtSUT1-1* (a), *MtSUT2* (b) and *MtSUT4-1* (c) in the roots of *Medicago truncatula* in symbiosis with the AM fungus *Rhizophagus irregularis* (light grey bars) and the nitrogen-fixing diazotroph *Ensifer meliloti* (middle grey bars) under different nutrient supply conditions (low – L, or high – H, phosphate – P, nitrogen – N) (Experiment 2). Data are expressed in arbitrary units (a.u.). Figures on the right show the correlation between the means in the expression level of each transporter and the measured carbon allocation into the root halves. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($P \leq 0.05$, $n = 3$). ANOVA results are shown in Table S3.

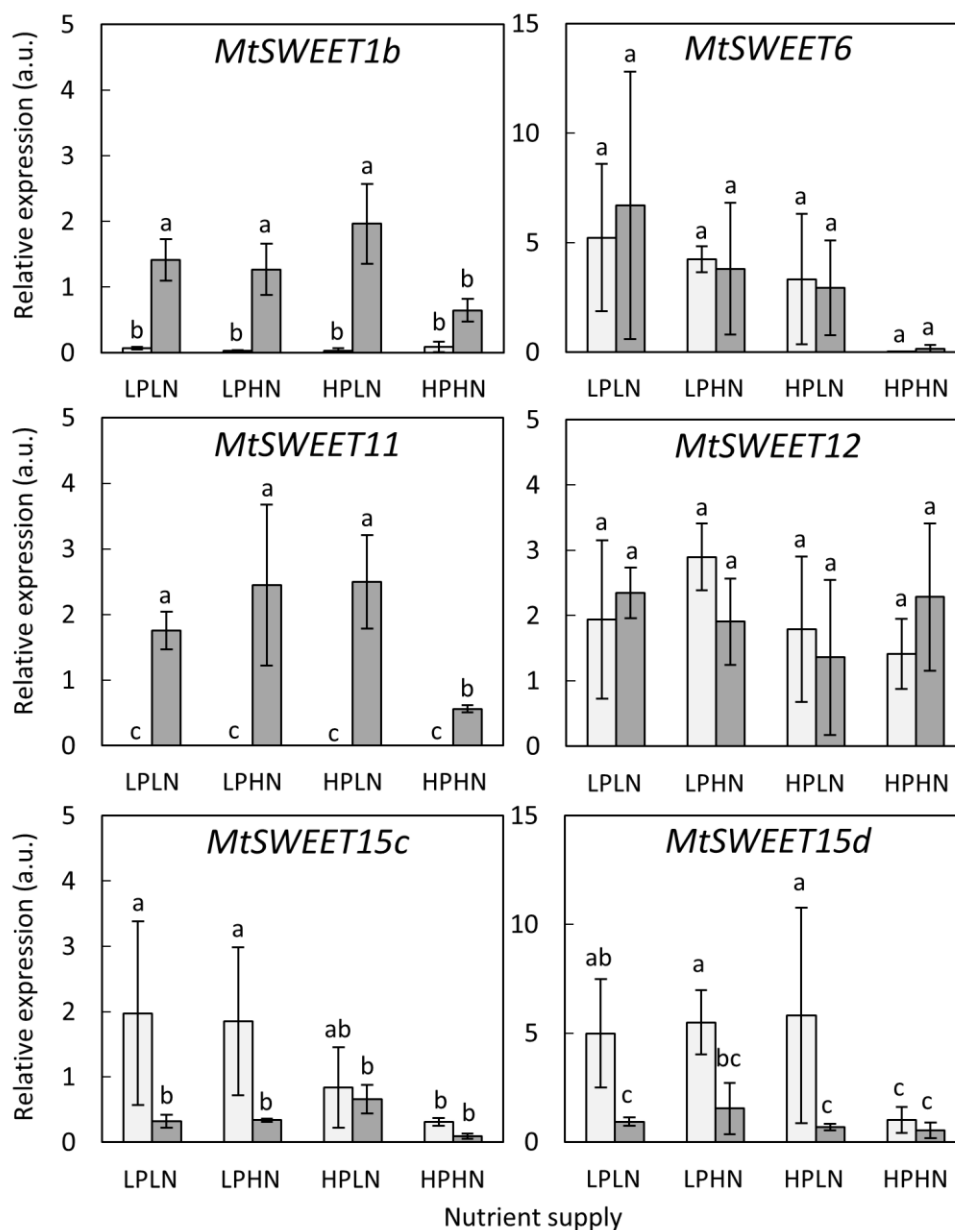


Figure 2.8. Relative expression of *MtSWEET1b*, *MtSWEET6*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* in the roots of *Medicago truncatula* in symbiosis with the AM fungus *Rhizophagus irregularis* (light grey bars) and the nitrogen-fixing diazotroph *Ensifer meliloti* (dark grey bars), under different nutrient supply conditions (low – L, or high – H, phosphate – P, nitrogen - N) (Experiment 2). Data are expressed in arbitrary units (a.u.). Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($P \leq 0.05$, $n = 3$). ANOVA results are shown in Table S3.

The transcript levels of *MtSWEET1b*, *MtSWEET15c*, *MtSWEET15d*, and also of the rhizobial specific transporter *MtSWEET11* were down-regulated in the AM or rhizobial root halves under high nutrient supply conditions for the host (Figure 2.8). *MtSWEET1b* showed high expression levels in the rhizobial root halves, and *MtSWEET15c* and *MtSWEET15d* in the AM root halves. In contrast, *MtSWEET6* and *MtSWEET12* showed similar transcript levels in both root halves, and the expression levels were not affected by the nutrient demand conditions of the host. We also examined the expression of the AM-inducible P transporter *MtPT4* and NH_4^+ transporter *MtAMT2;3*. We found an expression of these transporters only in the roots colonized by *R. irregularis*, but not in nodulated roots (Figure S2.10a, b). The exclusive expression of *MtPT4* and *MtAMT2;3* in the AM root halves, and of *MtSWEET11* in the rhizobial root halves (Figure 2.4 and 2.8) indicates that there was no cross-contamination between both RCs.

2.5 Discussion

Legumes form tripartite interactions with AM fungi and rhizobia, and both symbionts play a key role for the nutrient efficiency of this agronomically important group of plants. Both symbionts affect the interactions of the plant with the other partner (Xie *et al.*, 1998; Larimer, Clay & Bever, 2014), but our functional understanding of these complex interactions is mainly based on experiments with individual symbionts, either AM fungi or rhizobia. We analyzed nutrient transport, C allocation, and plant gene expression in different interactions when the fungal partner had access to an exogenous N supply, and in tripartite interactions under different nutrient demand conditions for the host, to better understand how host plants control the C costs of these interactions to maximize their symbiotic benefits.

Tripartite interactions can have a synergistic effect on plant biomass particularly under low N conditions. We found that plants in tripartite interactions had a significantly higher root and shoot biomass, N tissue concentrations and contents, and P contents than plants that were only colonized by rhizobia or AM fungi (Figure 2.1a, b; Figure 2.2c, d; S2.3a, c). Synergistic responses in tripartite interactions have also been described by other authors especially under low P and N supply conditions (Larimer *et al.*, 2014; Bournaud *et al.*, 2017). The dual inoculation with rhizobia and AM fungi can lead to higher photosynthetic rates and improves the harvest index (proportion of seed yields in relation to the total plant biomass) of legumes (Kaschuk, Kuyper, Leffelaar, Hungria & Giller, 2009). In our study, the positive impact of tripartite interactions on plant growth was mainly the result of a higher BNF activity of the nodules and the improved plant N nutrition

(Figure S2.2b, c). Higher BNF rates in tripartite interactions have mainly been attributed to an improved P supply by the colonization with AM fungi (Kucey & Paul, 1982; Mortimer *et al.*, 2009; Püschel *et al.*, 2017). Root nodules act as very strong P sinks (Figure 2.5b), and P deficiency can cause lower BNF rates of root nodules and inhibit nodule growth (Kleinert, Venter, Kossmann & Valentine, 2014).

While the positive effect of the AM symbiosis on P nutrition is long known, the contribution of AM fungi to N nutrition of their host plant is still under debate (Smith & Smith, 2011). However, there is increasing evidence that AM fungi can deliver substantial amounts of N to their host plant, even if the percentage contribution to total N nutrition of the host can vary considerably, and is context dependent (Ngwene, Gabriel & George, 2013). We found that when the fungus had access to an exogenous ^{15}N source, ^{15}N was delivered to the host, and the shoot biomass and N concentrations in the roots increased (Figure 2.1a, Figure 2.2d and Figure 2.3a, b). The capability of some AM fungi to deliver N can even lead in legumes, such as *Medicago sativa*, to strong growth responses (Mensah *et al.*, 2015). There is evidence suggesting that fungal N uptake and transport to the host make legumes less reliant on BNF and can inhibit the development of nodules (Mortimer *et al.*, 2008; Mortimer *et al.*, 2009).

In our experiments, we found no evidence for a suppression of root nodulation or nodule growth in the presence of AM fungi. However, we found a suppression of AM colonization in the dual inoculated systems of experiment 1 (Figure S2.2a). This reduced AM colonization was likely the reason why the ^{15}N transport in these systems was much lower than in the \emptyset /AM systems (Figure 2.3a, b). The reduced AM root colonization likely

caused a reduced exploration of the hyphal compartment to which the ^{15}N was supplied, and the low expression of *MtPT4* and *MtAMT2;3* (that are specifically expressed in arbusculated cells) suggest that less arbuscules were formed in the R/AM systems (Figure S2.6). However, the C allocation to different root halves and gene expression data indicate that N was transferred across the AM interface in R/AM systems, and that the ^{15}N labeling is partly hidden by a dilution effect due to the strong increase in biomass (Figure 2.1a, b). The transport of ^{15}N labelled ammonium across the AM interface, despite the low expression of *MtAMT2;3* in the roots of R/AM systems, could be due to the functional redundancy of mycorrhiza inducible AMT transporters in mycorrhizal roots. Although only in knock out mutants of *MtAMT2;3* a premature degeneration of arbuscules was observed, *MtAMT2;4* and *MtAMT2;5* were also up-regulated in mycorrhizal roots, and *MtAMT2;4* was able to complement NH_4^+ uptake of yeast mutants (in contrast to *MtAMT2;3*) (Breuillin-Sessoms *et al.*, 2015).

A suppression of the other root symbiont by a prior colonization of the root system by AM fungi or rhizobia has also been reported by other authors (Catford *et al.*, 2003; Catford *et al.*, 2006; Mortimer *et al.*, 2013; Sakamoto, Ogiwara & Kaji, 2013). It is well established that a prior exposure to rhizobia can limit the subsequent formation of root nodules on the root system (Ferguson *et al.*, 2010; Foo, Heynen & Reid, 2016). This process is known as autoregulation of nodulation (AON) and involves a root-derived signal that is perceived by a CLAVATA1-like leucine rich repeat receptor kinase (*MtSUNN* in *Medicago*) and triggers the production of a shoot-derived inhibitor that suppresses further nodule development (Reid, Ferguson & Gresshoff, 2011a; Reid *et al.*, 2011b). Loss-of-function mutations in these genes lead to a “supernodulation” phenotype with increased

nodulation, and their overexpression prevents nodulation (Reid *et al.*, 2011a). However, since this supernodulation phenotype is dependent on the nitrate supply levels, it has been suggested that an additional regulatory pathway exists in *M. truncatula*, and that the transport of N or of a N derivative or changes in C partitioning could also be involved in AON (Schnabel *et al.*, 2011; Kassaw *et al.*, 2015). Whether this autoregulatory pathway is also active in the regulation of AM colonization in tripartite interactions is not well understood, but mutants defective in elements of this pathway also showed elevated levels of AM root colonization (Stahelin, Xie, Illana & Vierheilig, 2011). We observed a suppression of the AM colonization in nodulated root systems only in experiment 1, but not in experiment 2. This discrepancy could be due to different time points of colonization by both root symbionts or could be caused by differences in the P demand conditions of the plants. There is reason to believe that in experiment 1 the AM colonization of the plant was delayed, and the earlier colonization with N fixing bacteria could have suppressed the subsequent colonization with AM fungi more strongly (Catford *et al.*, 2003; Catford *et al.*, 2006). However, the high P tissue concentrations of the non-inoculated control plants compared to the nodulated plants also indicate that plant growth in experiment 1 was primarily limited by the N supply (Figure 2.2a, b). By contrast, the increase of the P contents of the dual inoculated plants under high nutrient supply conditions, suggests that the plants in experiment 2 were also limited by the P supply (Figure S2.9a, b).

Root symbionts compete with their nutrient resources for host plant C. Our results demonstrate that the nutrient demand of the host plays a significant role in the C allocation to AM fungi or rhizobia in tripartite interactions. Plants under N demand preferentially allocated C to their nodulated root system, while plants that were supplied with N allocated

proportionally more C to their AM root system (Figure 2.6d). Given the large C investment entailed in symbiotic associations, with estimates of up to 20% of the assimilated C for the AM symbiosis (Snellgrove *et al.*, 1982; Jakobsen & Rosendahl, 1990; Wright *et al.*, 1998), and up to 30% for N-fixing root nodules (Provorov & Tikhonovich, 2003), plants have to strictly control the extent of microbial colonization to limit their C investment into these interactions. Our current understanding of how the host plant controls its C supply in tripartite interactions is limited. It has, however, been shown that N demand is a driver for C partitioning in plants. Legumes preferentially expand root nodules of efficient N-fixing rhizobia, and selectively transfer more C to active than to inactive root nodules (Singleton & van Kessel, 1987; Laguerre *et al.*, 2012). Host plants penalize rhizobia that fail to fix N₂ inside their root nodules (Kiers *et al.*, 2003), and arbuscules of AM fungi that are unable to provide P for the host plant are prematurely degenerated (Javot *et al.*, 2007; Javot *et al.*, 2011). Carbon acts as an important trigger for symbiotic functioning, and a reduction in the C supply reduces BNF by rhizobia (Kleinert *et al.*, 2014), and P and N uptake and transport by AM fungi (Fellbaum *et al.*, 2012; Fellbaum *et al.*, 2014; Konvalinková & Jansa, 2016). It has been shown that resource exchange between host and AM fungi are controlled by a reciprocal reward mechanism that is driven by biological market dynamics (Kiers *et al.*, 2011). Our results demonstrate that similar mechanisms may also control the resource to C exchange in tripartite interactions. In agreement, we observed that the fungus became a stronger competitor for host plant C when the fungus had access to an exogenous N source (Figure 2.3d). This is consistent with a biological market model, since N derived from AM symbionts is less costly for the host than N from BNF (Mortimer *et al.*, 2009).

To unravel the molecular mechanisms by which the C transport to different symbiotic partners is controlled, we analyzed the expression of three sucrose transporters of *M. truncatula* and found that the expression levels of *MtSUT2* and *MtSUT4-1* were positively correlated to the C allocation to different symbiotic partners (Figure 2.7). These transporters are not symbiosis-specific transporters, and are expressed in non-inoculated roots, and in AM and nodulated roots. *MtSUT1-1* encodes a H⁺-sucrose symporter and is putatively involved in phloem loading and unloading (Doidy *et al.*, 2012). The high transcript levels of *MtSUT1-1* particularly in AM roots (Figure 2.7a), and its upregulation in AM roots when the fungus had access to an exogenous N source (Figure 2.4) supports a possible role of this transporter in phloem unloading towards AM-colonized sink roots (Doidy *et al.*, 2012). *MtSUT4-1* shows similarities with the sucrose transporter of *Lotus japonicus* *LjSUT4*, that is involved in the transport of glucosides from the vacuole into the cytoplasm (Reinders, Sivitz, Starker, Gantt & Ward, 2008). Therefore, this transporter could play a role in the release of stored C sources from the vacuole towards symbiotic root sinks (Doidy *et al.*, 2012). *MtSUT4-1* shows a higher expression in cortical cells adjacent to arbusculated cells (Gaude, Bortfeld, Duensing, Lohse & Krajinski, 2012), and the high correlation of its transcript levels with the observed carbon allocation pattern (Figure 2.7c) clearly suggests a role of this transporter in symbiotic carbon flux to both root symbionts. Consistent with a role of *MtSUT4-1* in the remobilization of C from vacuolar C storage pools, we found that *MtSUT4-1* was down-regulated in AM roots, when the fungus was unable to provide N (Figure 2.4c). The functional role of *MtSUT2* on the other hand has not yet been deciphered (Doidy *et al.*, 2012). In our experiments, *MtSUT2* shows a higher expression in nodulated roots under N stress, as well as in AM roots in

response to an exogenous supply of N (Figure 2.4 and 2.7b). This suggests that also this transporter might play a role in the C transport towards both beneficial root symbionts.

We also determined the expression of six SWEETs in the roots of *Medicago truncatula* after colonization with different root symbionts. SWEETs can not only catalyze the efflux of carbohydrates but also their uptake (Chen, 2014), and it has recently been suggested that members of the SWEET family could be involved in the symbiotic C flux (Kryvoruchko *et al.*, 2016; Manck-Götzenberger & Requena, 2016; Sugiyama *et al.*, 2017). In contrast to *MtSWEET11* that is specifically expressed in root nodules, none of the other *SWEETs* we tested showed a mycorrhiza-restricted induction, but three of the *SWEETs*, *MtSWEET1b*, *MtSWEET6*, and *MtSWEET15d*, were upregulated in AM roots compared to control roots (Figure S2.5). According to the *Medicago truncatula* gene expression atlas (MtGEA; <http://mtgea.noble.org/v3/>), *MtSWEET1b* and *MtSWEET6* are highly expressed in arbusculated cells, and their putative orthologs *StSWEET1a*, *StSWEET1b* and *StSWEET7a* from potato also show high transcript levels in mycorrhizal roots (Manck-Götzenberger & Requena, 2016). Although *MtSWEET1b* and *MtSWEET6* are also highly expressed in rhizobial roots, the downregulation of both transporters in the tripartite interactions of experiment 1 (Figure 2.4), in which a suppression of the AM colonization by rhizobia was observed (Figure S2.2a), is an agreement with a potential role of both transporters for the sugar transport in arbusculated cells. Both *MtSWEET1b* and *MtSWEET6* belong to the SWEET clade I and II, and preferentially transport hexoses, mainly glucose (Chen, 2014), what is consistent with an induction of a monosaccharide transporter MST2 with a high affinity for glucose in the fungal membrane of arbuscules (Helber *et al.*, 2011). The significance of hexoses for C transport to the AM fungus has recently been questioned by

reports revealing that fatty acids can also be exported out of the root cell and transported to the fungal symbiont. However, the transport of hexoses to the arbusculated cells, will also provide the host cells with the precursor of the biosynthesis of fatty acyl groups that can be translocated to the fungal partner (Bravo *et al.*, 2017; Keymer *et al.*, 2017; Luginbuehl *et al.*, 2017).

MtSWEET11, *MtSWEET12*, *MtSWEET15c* and *MtSWEET15d* are clustered in clade III, which presumably encodes primarily sucrose transporters, and play an important role for sucrose translocation from source to sink tissues. *MtSWEET11* is exclusively expressed in nodulated roots, and its expression is down-regulated in response to high nutrient supply conditions (Figure 2.8), which is consistent with a reduced carbon transport from the host to the root nodules under high nutrient supply conditions. Similar to the changes in the gene expression patterns that were observed for the SUTs, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* were upregulated in the mycorrhizal roots of tripartite interactions when the fungus had access to N (Figure 2.4) or were down-regulated in AM roots when the host plant itself had access to nutrients (Figure 2.8). This is in agreement with the observed changes in the C allocation to AM or rhizobial roots (Figure 2.3d, 2.6b), and suggests that these transporters play an important role for the sucrose transport to symbiotic sink tissues. The fact, however, that all *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* show similar changes in their expression patterns, also indicates some level of redundancy in the function of these transporters. This redundancy in the SWEET family has also been discussed as the reason, why loss of function mutants of *MtSWEET11* and *LjSWEET3* that are highly expressed in the nodules of wild-type roots, did not show an impairment in nodular function (Kryvoruchko *et al.*, 2016; Sugiyama *et al.*, 2017).

We used in this study recovered ^{13}C from roots as an indicator for the C allocation to different symbiotic partners but did not consider the ^{13}C that was integrated into the fungal or rhizobial biomass or respired by the symbiotic partners from the soil (Kucey & Paul, 1982). To exactly measure the fungal or bacterial biomass in colonized roots is challenging, but the alignment of the recovered ^{13}C from individual root halves with the observed shifts in plant gene expression suggests that in the recovered ^{13}C in the root halves was a sufficient indicator for the carbon allocation to different symbiotic partners. Further biochemical, spatial, molecular and physiological analyses will be required to profile the role of all transporters for symbiotic functioning, and to identify the shared and specific mechanisms for C allocation towards AM fungi and N-fixing bacteria. A better understanding of these processes may prove critical in maximizing the benefits of symbionts for agricultural legumes.

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AUTHOR CONTRIBUTIONS

H.B. and X.W. designed the research; A.K., X.W., K.G., P.E.P. and G.D.S. performed the experiments; A.K., K.G., and H.B. collected, analyzed, and interpreted the data; H.B., K.G. and A.K. wrote the manuscript.

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2.7 Supporting information

Article title: Nutrient demand and the availability of resources control the carbon allocation to the symbiotic partners in tripartite interactions of *Medicago truncatula*.

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The following Supporting Information is available for this article:

Figure S2.1. Schematic model of the growth chamber systems.

Figure S2.2. Root colonization of *Medicago truncatula* by AM fungi and rhizobia in different growth chamber systems, and activity of root nodules (Experiment 1)

Figure S2.3. Phosphate and nitrogen contents in shoots and roots of *Medicago truncatula* plants depending on the colonization with different root symbionts (Experiment 1).

Figure S2.4. Relative expression of *MtSUT1-1*, *MtSUT2*, and *MtSUT4-1* in the roots of *Medicago truncatula* plants depending on the colonization with different root symbionts (Experiment 1).

Figure S2.5. Relative expression of *MtSWEET1b*, *MtSWEET6*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* in the roots of *Medicago truncatula* plants depending on the colonization with different root symbionts (Experiment 1).

Figure S2.6. Relative expression of *MtPt4* and *MtAMT2;3* in the roots of *Medicago truncatula* plants depending on the colonization with different root symbionts (Experiment 1).

Figure S2.7. Root colonization and nodule dry weight of *Medicago truncatula* plants under different nutrient supply conditions (Experiment 2).

Figure S2.8. Root and shoot biomass of *Medicago truncatula* under different nutrient supply conditions (Experiment 2).

Figure S2.9. Phosphate and nitrogen contents in shoots and roots of *Medicago truncatula* plants under different nutrient supply conditions (Experiment 2).

Figure S2.10. Relative expression of *MtPt4* and *MtAMT2;3* in the roots of *Medicago truncatula* plants under different nutrient supply conditions (Experiment 2).

Table S2.1 Primer sequences.

Table S2.2 ANOVA results of Experiment 1.

Table S2.3 ANOVA results of Experiment 2.

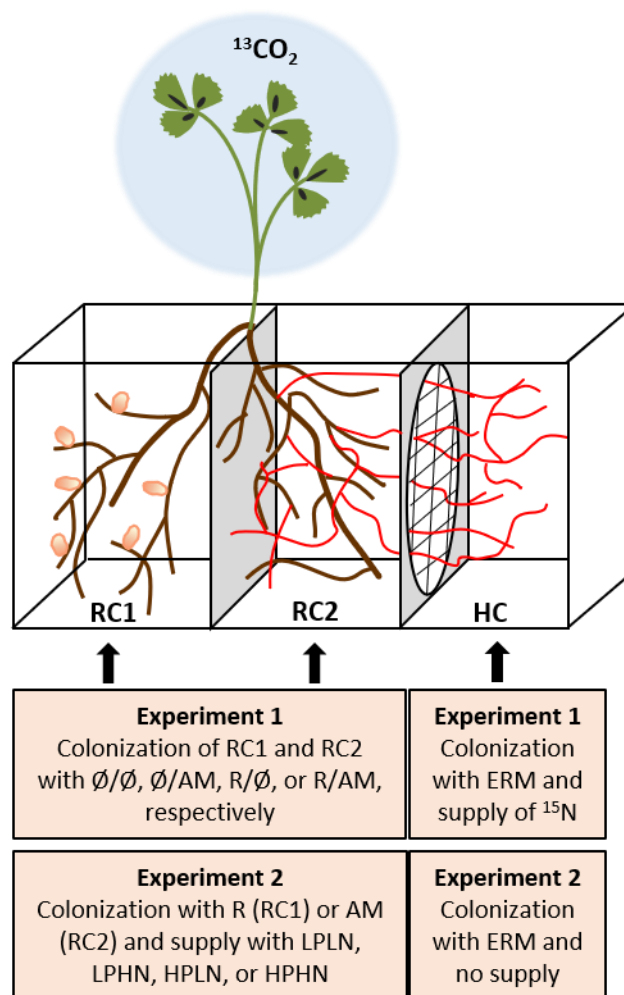


Figure S2.1. Schematic model of the growth chamber systems and the design of experiment 1 and 2. Abbreviations of the root chamber systems used in experiment 1: \emptyset – non-inoculated root half, AM – root half inoculated with *Rhizophagus irregularis*, R – root half inoculated with *Ensifer meliloti*. Abbreviations of the different nutrient supply conditions in experiment 2: LP – low phosphate, HP – high phosphate, LN – low nitrogen, and HN – high nitrogen.

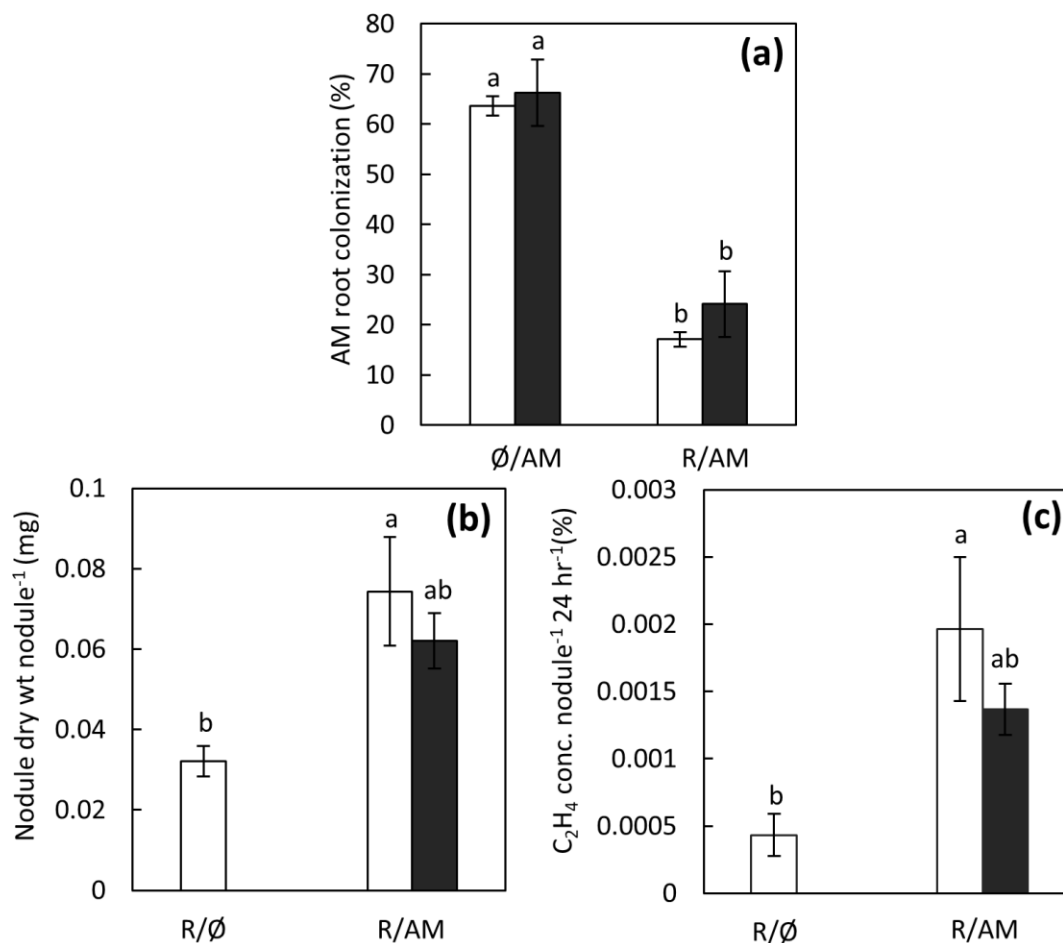


Figure S2.2. Root colonization of *Medicago truncatula* by the AM fungus *Rhizophagus irregularis* (a) or nodule dry weight (b) and BNF rate of root nodules (c) depending on the colonization with root symbionts and under different N supply conditions for the AM fungus (Experiment 1) (white bars – without ¹⁵NH₄Cl supply to the hyphal compartment, black bars – with ¹⁵NH₄Cl supply to the hyphal compartment). Growth chamber system abbreviations: Ø/AM - one root half inoculated with *R. irregularis*; Ø/R - one root half inoculated with *Ensifer meliloti*; R/AM - both compartments inoculated by either *R. irregularis* or *E. meliloti*. Different letters on the bars (means ± SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($P \leq 0.05$, $n = 4$ to 7).

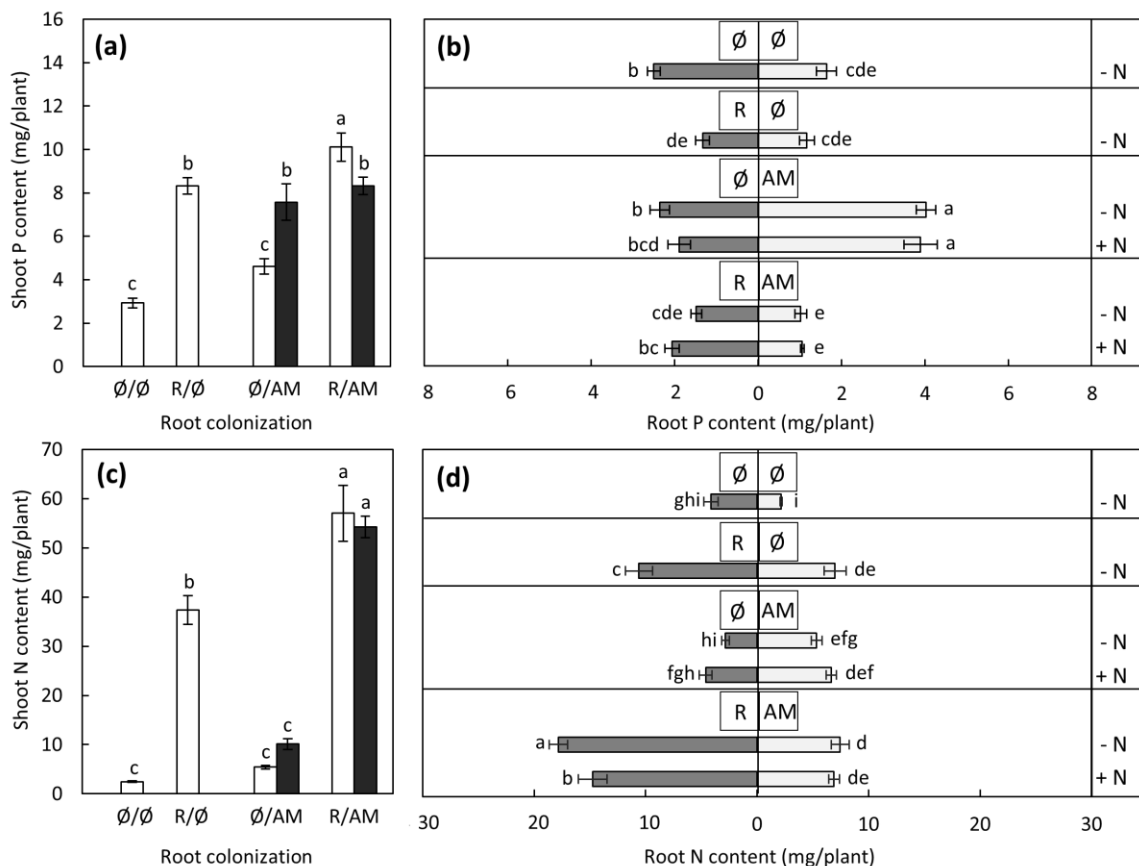


Figure S2.3. Phosphate (a, b) or nitrogen (c, d) contents in shoots (a, c) and roots (b, d) of *Medicago truncatula* depending on the colonization with different root symbionts and under different N supply conditions for the AM fungus (Experiment 1). In (a) and (c): white bars – without $^{15}\text{NH}_4\text{Cl}$ supply to the hyphal compartment, black bars – with $^{15}\text{NH}_4\text{Cl}$ supply to the hyphal compartment). Growth chamber abbreviations: \emptyset/\emptyset – both root halves non-inoculated, \emptyset /R - one root half inoculated with *Ensifer meliloti*; \emptyset /AM - one root half inoculated with *R. irregularis*; R/AM: both compartments inoculated by either *R. irregularis* or *E. meliloti*. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($p \leq 0.05$, $n = 3$ to 7). ANOVA results shown in Table S2.2.

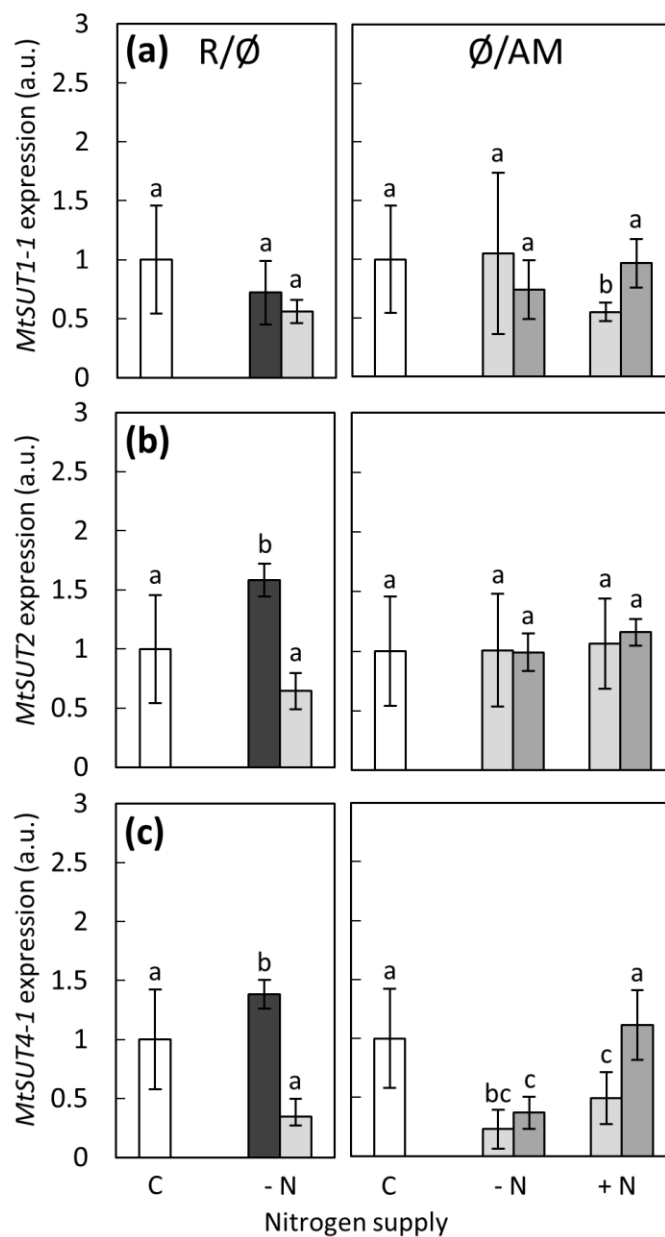


Figure S2.4. Relative expression of *MtSUT1-1* (a), *MtSUT2* (b) and *MtSUT4-1* (c) in *Medicago truncatula* roots depending on the colonization with different root symbionts and under different nitrogen supply conditions for the fungus (+N – $^{15}\text{NH}_4\text{Cl}$ addition to the hyphal compartment, -N – no addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment) (Experiment 1). Shown is the expression in R/∅ systems (rhizobial root half – black bars; non-inoculated root half – light grey bars) and ∅/AM systems (non-inoculated root half – light grey bars, AM inoculated root half – middle grey bars) compared to control roots of ∅/∅ systems (C). Data (means \pm SEM) are expressed in arbitrary units (a.u.). Independent statistical analyses were performed for each split-root system compared to the control, with letters indicating statistically significant differences (Student's t-test, $p \leq 0.05$). ANOVA results are shown in Table S2.

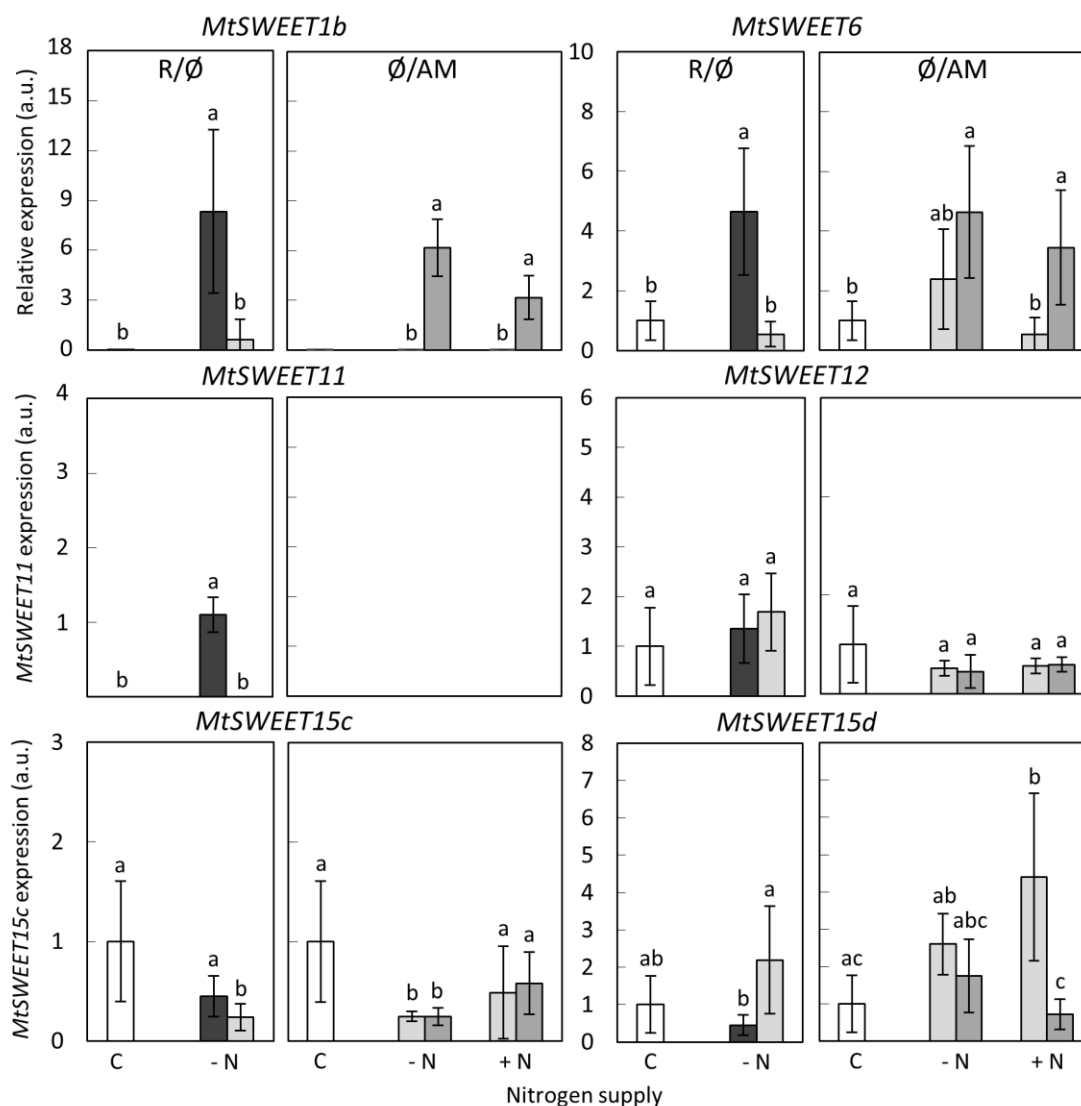


Figure S2.5. Relative expression of *MtSWEET1b*, *MtSWEET6*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* in *Medicago truncatula* roots depending on the colonization with different root symbionts and under different nitrogen supply conditions for the fungus (+N – $^{15}\text{NH}_4\text{Cl}$ addition to the hyphal compartment, -N – no addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment) (Experiment 1). Shown is the expression of R/∅ systems (rhizobial root half – black bars; non-inoculated root half – light grey bars) and ∅/AM systems (non-inoculated root half – light grey bars, AM inoculated root half – middle grey bars) compared to control roots of ∅/∅ systems (C). Data (means ± SEM) are expressed in arbitrary units (a.u.). Independent statistical analyses were performed for each split-root system compared to the control, with letters indicating statistically significant differences (Student's t-test, $p \leq 0.05$). ANOVA results are shown in Table S2.2.

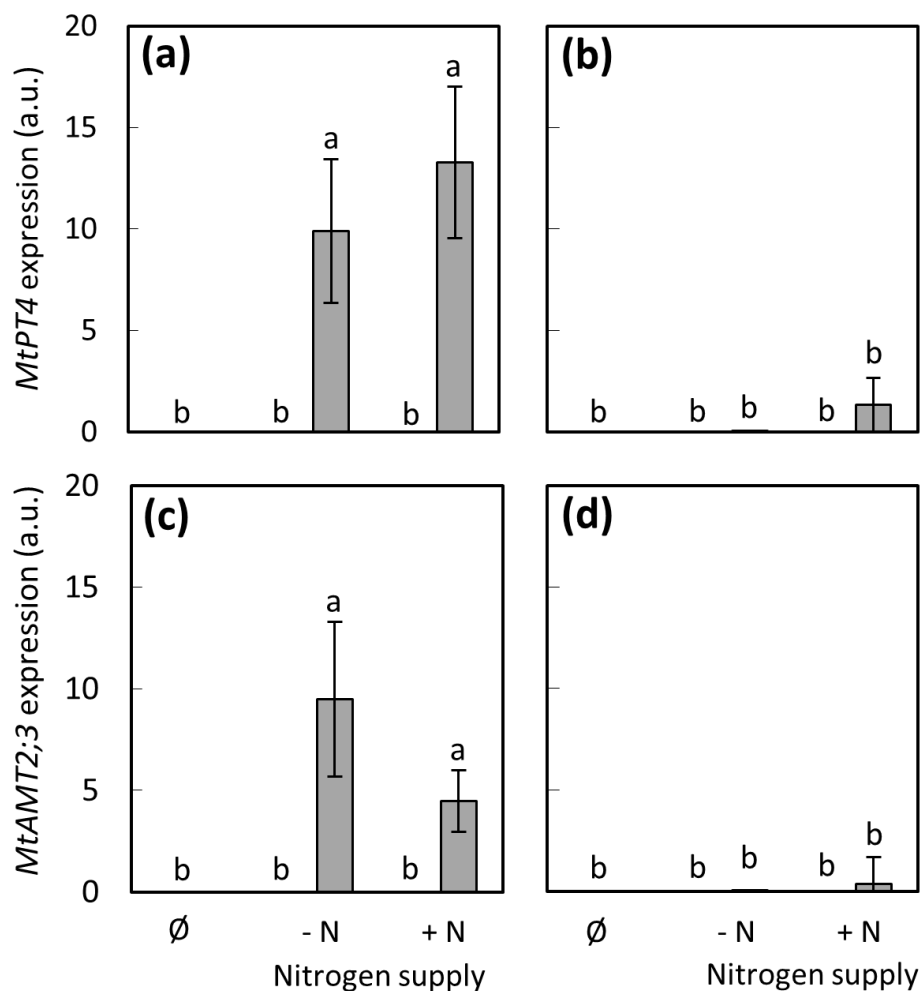


Figure S2.6. Relative expression of *MtPT4* (a, c) and *MtAMT2;3* (b, d) in *Medicago truncatula* roots depending on the colonization with different root symbionts and under different nitrogen supply conditions for the fungus (+N – $^{15}\text{NH}_4\text{Cl}$ addition to the hyphal compartment, -N – no addition of $^{15}\text{NH}_4\text{Cl}$ to the hyphal compartment) (Experiment 1). The left panels (a, c) show the expression levels of control roots (\emptyset) and of both root halves of AM/ \emptyset systems (one compartment inoculated with *Rhizophagus irregularis*, one compartment non-inoculated); the right panels (b,d) show the expression levels of control roots (\emptyset) and of both root halves of R/AM systems (one compartment inoculated with *R. irregularis*, one compartment inoculated with *Ensifer meliloti*). A gene expression above the threshold was only found in the AM root halves (grey bars). Data (means \pm SEM) are expressed in arbitrary units (a.u.). Independent statistical analyses were performed for each split-root system compared to the control, with different letters indicating statistically significant differences to the control (LSD test, $p \leq 0.05$, $n = 3$ to 4). ANOVA results are shown in Table S2.2.

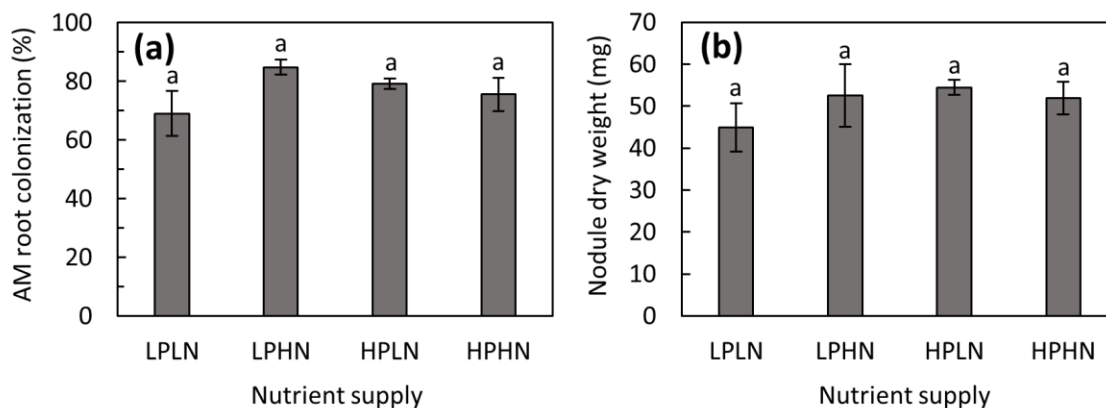


Figure S2.7. Root colonization of *Medicago truncatula* by the AM fungus *Rhizophagus irregularis* (a) and the nitrogen-fixing diazotroph *Ensifer meliloti* (b) under different nutrient supply conditions (Experiment 2). Abbreviations of the different nutrient supply conditions: LP – low phosphate, HP – high phosphate, LN – low nitrogen, and HN – high nitrogen. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($p \leq 0.05$, $n = 3$). ANOVA results are shown in Table S2.3.

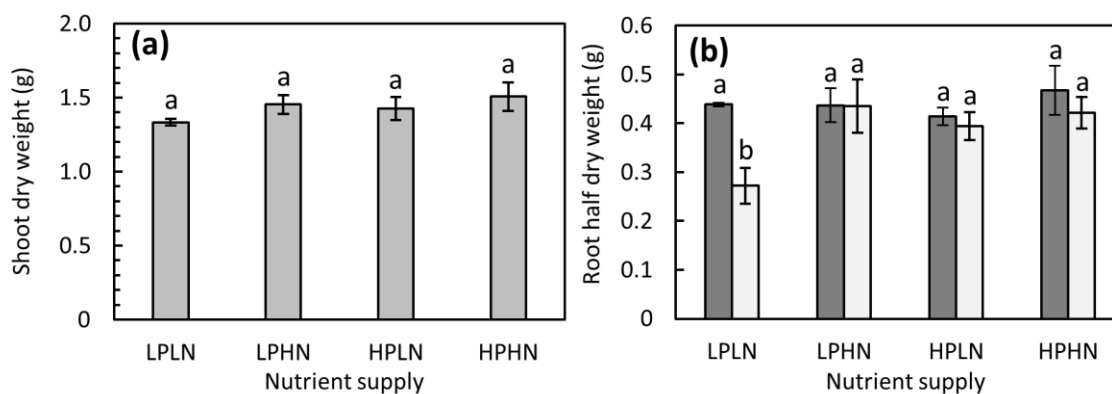


Figure S2.8. Shoot (a) and root (b) biomass of *Medicago truncatula* in tripartite interactions under different nutrient supply conditions (Experiment 2). In (b): nodulated root half in dark grey, and AM root half in light grey. Abbreviations of the different nutrient supply conditions: LP – low phosphate, HP – high phosphate, LN – low nitrogen, and HN – high nitrogen. Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($p \leq 0.05$, $n = 3$). ANOVA results are shown in Table S2.3.

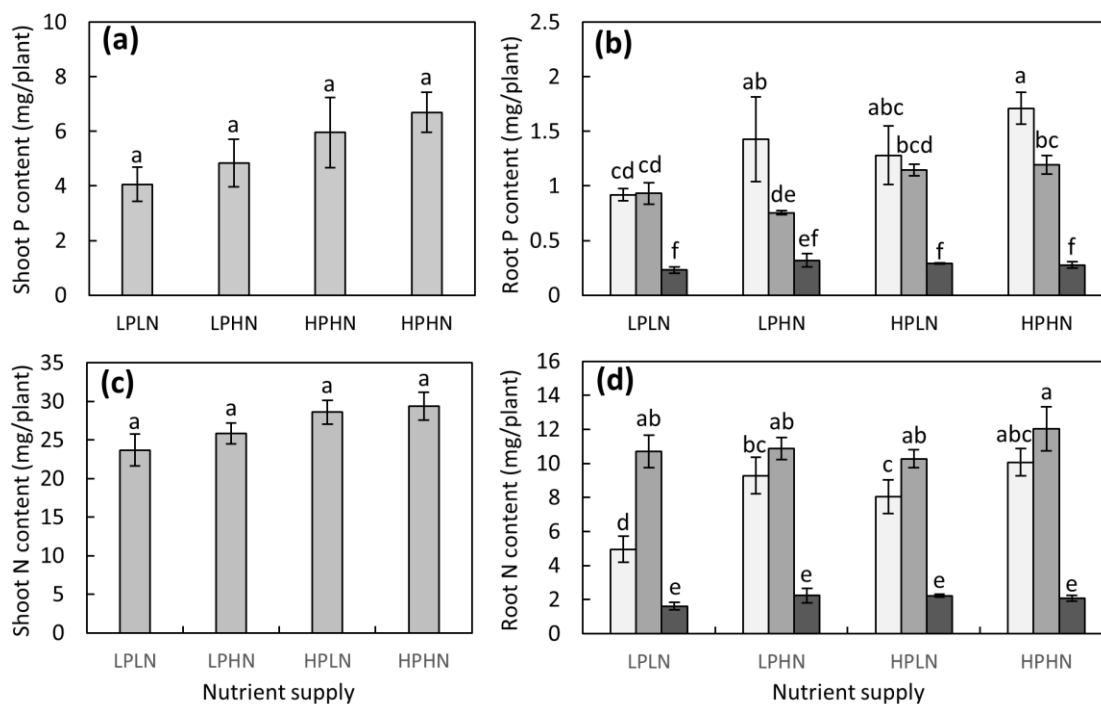


Figure S2.9. Phosphate (a, b) and nitrogen (c, d) contents in the shoots (a, c) and different root fractions (b, d) of *Medicago truncatula* plants in tripartite symbiosis with the AM fungus *Rhizophagus irregularis* and the nitrogen-fixing diazotroph *Ensifer meliloti* under different nutrient supply conditions (Experiment 2). The different root fractions in (b), and (d) represent the AM colonized root halves (light grey), the rhizobia colonized root halves (middle grey), and root nodules (dark grey). Different letters on the bars (means \pm SEM) indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($p \leq 0.05$, $n = 3$). ANOVA results are shown in Table S2.3.

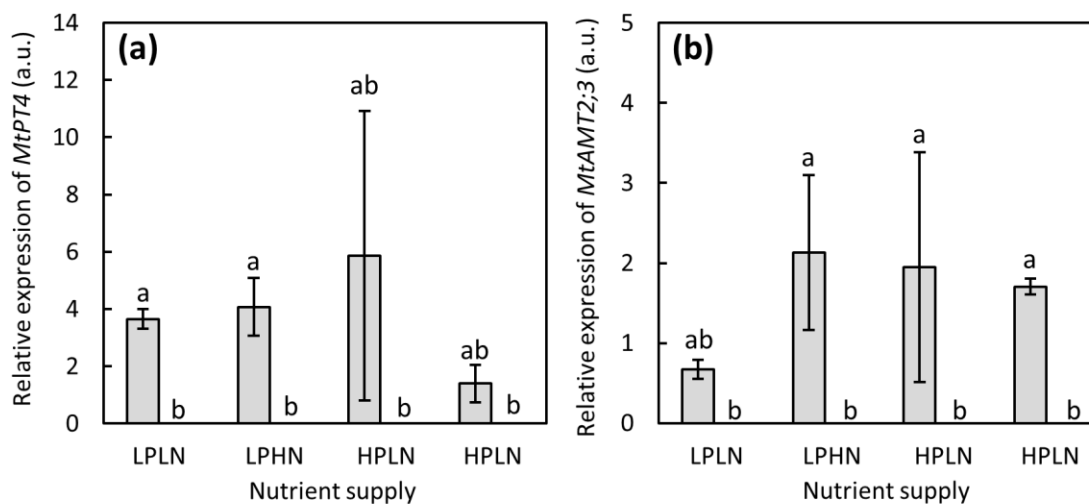


Figure S2.10. Relative expression of *MtPT4* (a) and *MtAMT2;3* (b) in mycorrhizal (light grey bars) and nodulated root halves of *Medicago truncatula* under different nutrient supply conditions (Experiment 2). Only the AM root halves showed a gene expression higher than the detection limit of these mycorrhiza-inducible transporters. Data (means \pm SEM) are expressed in arbitrary units (a.u.). Different letters on the bars indicate statistically significant differences within each graph according to the least significant difference (LSD) test ($p \leq 0.05$, $n = 3$). ANOVA results are shown in Table S2.3.

Table S2.1 List of primers used for the gene expression analysis by RT-qPCR. Publication where these sequences were taken is mentioned in the reference column.

Gene	Gene Bank ID	Primer sequence	Reference
<i>MtSUT1-1 F</i> <i>MtSUT1-1 R</i>	JN255789	AGTGGCATATTATCCGTAGTC TGAAGAAAATGTTCCCACTG	Doidy <i>et al.</i> (2012)
<i>MtSUT2 F</i> <i>MtSUT2 R</i>	JN255792	AACTGCCAAACCTTTCTAGC CACAATCAACGTGCCTACTC	
<i>MtSUT4-1 F</i> <i>MtSUT4-1 R</i>	JN255793	GCAGATTGTGGTATCACTG TAAGTGCCAAAAGAAAACAGC	
<i>MtPT 4 F</i> <i>MtPT4 R</i>	AY116211.1	GACACGAGGCGCTTTCATAGCAGC GTCATCGCAGCTGGAACAGCACCG	
<i>MtAMT2;3 F</i> <i>MtAMT2;3 R</i>	XM_003629175	TGTCCGGTTCAATTCCATGG TGGCAAACACACCAGAAAGG	Breuilin- Sessoms <i>et al.</i> (2015)
<i>MtSWEET1b F</i> <i>MtSWEET1b R</i>	XM_013605920.1	GTGTTTCTTTGCGGCAGTTC ACCATTAGGTACAGCAACAAATAGG	Kryvoruchk o <i>et al.</i> (2016)
<i>MtSWEET6 F</i> <i>MtSWEET6 R</i>	XM_003601416.1	ACCCAAGAACAGATAGCAGCA TCTTCTTGTTGTCTTGTACAGTAGC	
<i>MtSWEET9b F</i> <i>MtSWEET9b R</i>	XM_013592024.1	GTAGAAACTAATTCAACTGAGGAGCA CATTCCCCCACCCTACAG	
<i>MtSWEET11 F</i> <i>MtSWEET11 R</i>	XM_003602732.2	TATCGACGACTTTTATATCGCTATACC GTTGACTCTAGTGGAATGGCATC	
<i>MtSWEET12 F</i> <i>MtSWEET12 R</i>	XM_013591502.1	GTGGTGGTCATATAATTGATGTTGTG CTACCAGCACCTCCACCTG	
<i>MtSWEET15c F</i> <i>MtSWEET15c R</i>	XM_013592326.1	CGTATTGGGGTACTTCAGATGC TTTGGGTGGCTCAATTGGTG	
<i>MtSWEET15d F</i> <i>MtSWEET15d R</i>	XM_013592325.1	ACAGAAATGGTGATAAGAAGAAGGC TCTTCTTCTCCATCATTTTTCTCGAC	
<i>MtTef1a F</i> <i>MtTef1a R</i>	TC106485	ACTGTGCAGTAGTACTTGGTG AAGCTAGGAGGTATTGACAAG	Doidy <i>et al.</i> (2012)

Table S2.2. ANOVA and ANCOVA results of Experiment 1. Analysis of covariance (ANCOVA) was conducted by including tissue biomass as covariable (effect of shoot biomass on P and N content in the shoot tissues, effect of AM root biomass on P and N concentration and content in AM roots, effect of rhizobial root biomass on P and N concentration and content).

Plant characteristic	F	P
Shoot biomass (g) (Figure. 1a), ANOVA	F _{5,26} = 287.26	0.001
Root biomass (g) (Fig. 1b), ANOVA	F _{11,53} = 40.15	≤ 0.001
AM colonization (%) (Fig. S2a), ANOVA	F _{3,19} = 33.44	≤ 0.001
Nodule dry weight nodule ⁻¹ (mg) (Fig. S2b), ANOVA	F _{2,12} = 4.65	0.037
C ₂ H ₄ production by nodules (%) (Fig. S2c), ANOVA	F _{2,12} = 4.08	0.050
Shoot P concentration (mg/g) (Fig. 2a), ANOVA	F _{5,24} = 97.36	≤ 0.001
Shoot P concentration (mg/g) (Fig. 2a), ANCOVA	F _{5,24} = 5.70	0.002
Root P concentration (mg/g) (Fig. 2b): ANOVA	F _{11,49} = 73.57	≤ 0.001
Root P concentration (mg/g) (Fig. 2b): ANCOVA	F _{11,49} = 51.65	≤ 0.001
Shoot N concentration (mg/g) (Fig. 2c), ANOVA	F _{5,26} = 15.99	≤ 0.001
Shoot N concentration (mg/g) (Fig. 2c), ANCOVA	F _{5,26} = 1.54	0.221
Root N concentration (mg/g) (Fig. 2d): ANOVA	F _{11,51} = 14.63	≤ 0.001
Root N concentration (mg/g) (Fig. 2d): ANCOVA	F _{11,51} = 10.47	≤ 0.001
Shoot P content (mg/plant)(Fig. S3a), ANOVA	F _{5,24} = 23.06	≤ 0.001
Shoot P content (mg/plant) (Fig. S3a), ANCOVA	F _{5,24} = 13.02	≤ 0.001
Root P content (mg/plant) (Fig. S3b): ANOVA	F _{11,49} = 20.80	≤ 0.001
Root P content (mg/plant) (Fig. S3b): ANCOVA	F _{11,49} = 43.07	≤ 0.001
Shoot N content (mg/plant)(Fig. S3c), ANOVA	F _{5,26} = 72.67	≤ 0.001
Shoot N content (mg/plant)(Fig. S3c), ANCOVA	F _{5,26} = 1.69	0.182
Root N content (mg/plant) (Fig. S3d): ANOVA	F _{11,51} = 39.82	≤ 0.001
Root N content (mg/plant) (Fig. S3d): ANCOVA	F _{11,51} = 13.49	≤ 0.001
Shoot N content (% of total N) (Fig. 3a)	F _{5,26} = 253.26	≤ 0.001
Root ¹⁵ N (% of total N) (Fig. 3b)	F _{11,53} = 205.19	≤ 0.001
Shoot δ ¹³ C (Fig. 3c)	F _{5,26} = 11.58	≤ 0.001
Root δ ¹³ C (Fig. 3d)	F _{11,53} = 7.05	≤ 0.001
<i>MtSUT1-1</i> (Fig. 4a)	F _{3,31} = 8.995	≤ 0.001
<i>MtSUT2</i> (Fig. 4b)	F _{3,31} = 5.202	0.0127
<i>MtSUT4-1</i> (Fig. 4c)	F _{3,31} = 17.633	≤ 0.001
<i>MtSWEET1b</i> (Fig. 4e, f)	F _{3,31} = 17.761	≤ 0.001
<i>MtSWEET6</i> (Fig. 4e, f)	F _{3,31} = 5.204	≤ 0.001
<i>MtSWEET11</i> (Fig. 4e, f)	F _{3,31} = 14.86	≤ 0.001
<i>MtSWEET12</i> (Fig. 4e, f)	F _{3,31} = 6.264	≤ 0.001
<i>MtSWEET15c</i> (Fig. 4e, f)	F _{3,31} = 9.601	≤ 0.001
<i>MtSWEET15d</i> (Fig. 4e, f)	F _{3,31} = 2.476	0.049
<i>MtPT4</i> (Fig. S4a, b)	F _{3,31} = 24.794	≤ 0.001
<i>MtAMT2;3</i> (Fig. S4c, d)	F _{3,31} = 1.079	0.0387

Table S2.3. ANOVA results of Experiment 2.

Plant trait	F	P
AM colonization (%) (Fig. S6a)	$F_{3,11} = 0.74$	0.554
Nodule dry weight (g) (Fig. S6b)	$F_{3,11} = 0.65$	0.605
Shoot dry weight (g) (Fig. S7a)	$F_{3,11} = 1.08$	0.411
Root dry weight (g) (Fig. S7b)	$F_{7,23} = 2.75$	0.044
Shoot P concentration (mg/g) (Fig. 5a)	$F_{3,11} = 1.17$	0.378
Root P concentration (mg/g) (Fig. 5b)	$F_{3,35} = 20.37$	≤ 0.001
Shoot N concentration (mg/g) (Fig. 5c)	$F_{3,11} = 0.7$	0.579
Root N concentration (mg/g) (Fig. 5d)	$F_{3,35} = 54.15$	≤ 0.001
Shoot ^{13}C content (mg) (Fig. 6a)	$F_{3,11} = 0.73$	0.563
Root ^{13}C content (mg) (Fig. 6b)	$F_{11,35} = 10.51$	≤ 0.001
Shoot P content (mg/plant) (Fig. S8a)	$F_{3,11} = 1.65$	0.254
Root P content (mg/plant) (Fig. S8b)	$F_{11,35} = 11.28$	≤ 0.001
Shoot N content (mg/plant) (Fig. S8c)	$F_{3,11} = 2.33$	0.150
Root N content (mg/plant) (Fig. S8d)	$F_{11,35} = 29.10$	≤ 0.001
<i>MtSUT1-1</i> (Fig. 7a)	$F_{3,31} = 8.259$	≤ 0.001
<i>MtSUT2</i> (Fig. 7b)	$F_{3,31} = 7.466$	≤ 0.001
<i>MtSUT4-1</i> (Fig. 7c)	$F_{3,31} = 3.857$	0.012
<i>MtSWEET1b</i> (Fig. S9c)	$F_{3,31} = 21.2$	≤ 0.001
<i>MtSWEET6</i> (Fig. S9c)	$F_{3,31} = 1.851$	0.182
<i>MtSWEET11</i> (Fig. S9c)	$F_{3,31} = 7.777$	≤ 0.001
<i>MtSWEET12</i> (Fig. S9c)	$F_{3,31} = 0.768$	0.622
<i>MtSWEET15c</i> (Fig. S9c)	$F_{3,31} = 3.418$	0.0198
<i>MtSWEET15d</i> (Fig. S9c)	$F_{3,31} = 3.137$	0.0326
<i>MtPT4</i> (Fig. S9a)	$F_{3,31} = 4.395$	0.00775
<i>MtAMT2;3</i> (Fig. S9b)	$F_{3,31} = 7.385$	≤ 0.001

CHAPTER 3: NEUTRAL OR REDUCED GROWTH OF *MEDICAGO TRUNCATULA*
IN TRIPARTITE INTERACTIONS: WAS IT ONLY CARBON COST?

This chapter is formatted and in preparation for submission to "Mycorrhiza"

3.1 Abstract

Legume plants form tripartite interactions with arbuscular mycorrhizal (AM) fungi and rhizobial bacteria. The nutritional benefit of the AM fungi is context dependent. However, the underlying mechanism of the context dependency is largely unknown. *Medicago truncatula* plants were inoculated with either *Rhizophagus irregularis*, or *Ensifer meliloti*, or both symbionts and grown for nine weeks at low (L) or high (H) phosphate (P) and nitrogen (N) nutrient supply conditions. Despite higher P response in shoot and root tissues of the AM inoculated plant across the nutrient regimes, there was high a variability in the plant growth response. Mycorrhization and nodulation was slightly higher in the dual inoculated roots. Interestingly, there was higher carbon and nitrogen concentration in AM roots of those plants which had negative growth response. This higher demand of carbon by the AM roots could be for the assimilation of nitrogen before delivering to the host or to retain within the fungal bodies. To retain some of available nutrients within fungal bodies may be for their survivability/ adaptability, or to offer some nutrients during initial stage of symbiosis with upcoming host plant, or a biological factor to balance the biogeochemical cycle and ecosystem stability.

Keywords *Medicago truncatula*, *Rhizophagus irregularis*, *Ensifer meliloti*, tripartite interactions, growth response, phosphate, nitrogen, carbon

3.2 Introduction

The majority of land plants form a symbiotic relation with arbuscular mycorrhizal (AM) fungi. AM fungi provide nutritional benefits such as phosphate (P), and nitrogen (N) (Bücking and Kafle, 2015; Fellbaum et al., 2012; Hodge and Storer, 2015; Mäder et al., 2000; Ngwene et al., 2013; Püschel et al., 2016; Tanaka and Yano, 2005; Toussaint et al., 2004). In return, AM fungi get photosynthetic carbon (C) from the host to maintain their metabolic and reproductive processes. Plant needs substantial amounts of P, but P is not readily available in soils due to its soil immobility and complex formation with soil substrates. Thus, plants form symbiotic interactions with AM fungi to overcome this P limitation. AM hyphae, because of their small diameter, can penetrate smaller soil compartments, and have access to soil beyond the root depletion zones to scavenge P resources. Besides P and N nutrients, the AM fungi also offer other nutrients to host plants (Nouri et al., 2014) and non-nutritional benefits (Augé, 2001; Gallou et al., 2011; Jung et al., 2012).

The degree of mutualistic association between these two partners depends on the host nutrient demand and soil nutrient condition. For instance, if the host demands more P or N, the AM fungus confers accordingly. In return, the fungus reciprocally receives C from the host to make the mutualism more sustainable (Bücking and Shachar-Hill, 2005; Fitter, 2006; Kiers et al., 2011; Lendenmann et al., 2011). However, sometimes the symbiosis becomes neutral or antagonistic (Jin et al., 2017; Li et al., 2008; Li et al., 2006) particularly when the soil contains high concentration of nutrients. In this context, the symbiosis is not beneficial for the host as root has easy access to available nutrients, but

the host plant allocates C to AM fungi to maintain the symbiosis (Jin et al., 2017; Johnson et al., 1997; Li et al., 2008). The host plant cannot completely remove the AM fungus from its roots once the symbiosis is established even in high soil nutrient conditions but allows a base level of colonization by the fungus for other non-nutritional purposes. In this context, the maintenance of the symbiosis becomes expensive because the host invests C continuously to the fungus. So, this loss of C can cause growth depressions of the host if the AM symbiosis is not beneficial. The variation of the benefits of AM symbiosis from mutualism to antagonism are therefore context dependent, for instance: light and shade (Zheng et al., 2015), nutrient conditions in host and soil (Johnson et al., 2010; Ngwene et al., 2013; Nouri et al., 2014; Püschel et al., 2016). In addition, other factors for example: length of the time for plant growth, pot size, soil pH, and soil water also affect the plant growth response (Koide R.T. 1991; Poorter et al., 2012). Moreover, the degree and extend of mutualism also depends on the functional niche of AM fungi to the host as some fungi are host specific (Lendenmann et al., 2011; Mensah et al., 2015; Wang et al., 2016b).

A few plants especially legumes (Fabaceae family) form symbiotic associations with diazotrophs, generally called rhizobia (R). Rhizobia, localized within a host specialized structure called nodule, convert atmospheric free nitrogen (N_2) into plant useable inorganic N (NH_4^+) using the nitrogenase enzyme (Udvardi and Poole, 2013). Nodules have almost three times higher P concentration than surrounding root tissues, indicating that the bacteria inside the nodules have a high P demand for enzymatic activity (Kafle et al., 2018). The host plant invests between 6% to 30% of recently fixed C to the bacteria for the enzymatic reactions and to maintain bacterial cell mass within the nodule (Provorov and Tikhonovich, 2003). However, benefits from this interaction decreases

when the soil has higher N and consequently lower nodulation (Wang et al., 2016a). The host plant gets benefit from this mutualistic association to meet its N demand under N limiting conditions which has substantial implications in agriculture, economics, and environment (Peoples et al., 2009)

Under natural conditions, legume plant roots are colonized by both AM fungi and rhizobia forming tripartite interactions and interact with each other. Functionally active bacteria/bacteroids, demand a considerable amount of P to maintain their enzymatic activity to fix free nitrogen. Thus, rhizobia and their enzymatic activity in legume nodules are facilitated by the presence of AM fungi. Consequently, these symbionts have synergistic effects to each other and on host plant growth compared to inoculations with only one symbiont (Afkhani and Stinchcombe, 2016; Bournaud et al., 2017; Jia et al., 2004; Kaschuk et al., 2010; Larimer et al., 2014; Mortimer et al., 2008; Wang et al., 2011). However, antagonistic effects of one symbiont to other have also been reported (Catford et al., 2006; Mortimer et al., 2013). Growth responses in tripartite interactions depend on the stage of the symbioses (Mortimer et al., 2008), specificity between host and symbionts (Ide Franzini et al., 2010), soil nutrient status and host nutrient demand conditions. Thus, compatibility and context determine the degree of interaction which can vary from mutualism via neutralism to parasitism (Bournaud et al., 2017; Larimer et al., 2014; Mensah et al., 2015; Mortimer et al., 2008; Ossler et al., 2015; Walder and van der Heijden, 2015; Wang et al., 2016b).

Sucrose from the host photosynthetic leaves is uploaded actively via sucrose transporters (SUTs) and sugars will eventually be exported transporters (SWEETs) into the

phloem for long distance transport. In the root tissues, sugars are unloaded into the cortical cells as energy source and substrates either for root elongation or for transport to different symbiotic partners. During this unloading, many SUT and SWEET transporter are involved in the translocation of sucrose from cell to cell as previously reported (Doidy et al., 2012; Kryvorunchko et al., 2016; Roth et al., 2018). These transporters play an important role to maintain the symbiotic association with AM fungi and rhizobial bacteria as they depend on host carbon for their metabolic activities. How these sucrose transporters are regulated in AM and rhizobial symbioses with legumes in different nutrient supply conditions is currently not well understood.

Considerable amount of information exists on interactions between host and one type of symbiont, either AM fungi (Bonneau et al., 2013; Breuillin-Sessoms et al., 2015; Fellbaum et al., 2014; Javot et al., 2011; Li et al., 2008; Mensah et al., 2015; Wang et al., 2016b) or rhizobia (Jeudy et al., 2010; Sulieman et al., 2013; Udvardi and Poole, 2013). However, there is less information on tripartite associations of the host that are associated with both symbionts under different nutrient supply conditions. Moreover, previous reports have demonstrated the context dependency of host plant growth responses but did not provide information about the underlying mechanism of growth variability. Here, we assessed the growth response, nutrient acquisition, carbon allocation to symbiotic roots in tripartite associations among *Rhizophagus irregularis*, *Ensifer meliloti* (formerly known as *Sinorhizobium meliloti*), and the host *Medicago truncatula* under four different soil nutrient supply conditions as low and high phosphate and nitrogen concentrations.

3.3 Material and methods

3.3.1 Plant, fungal and rhizobial materials

Medicago truncatula A17 seeds were scarified with conc. H₂SO₄, rinsed, and kept at 4°C overnight. On the next day, the seeds were transferred onto sterilized moist filter paper in a Petri plate, sealed with parafilm and kept in the dark until germination/sprouting. These plates, then were kept on the table top to expose the seedlings to light for 5 days until the first single leaflets appeared. Three seedlings were planted (days after planting DAP) into each 700 mL transparent plastic cups containing 500 mL of autoclaved soil substrate containing sand, perlite, vermiculite mix, and organic soil in the ratio of 6:2:1:1 by v: v:v: v. The soil substrate contained 0.134 mM Olsen phosphate, 0.006 mM nitrate, and 0.073 mM ammonium (Ag Lab Express, Sioux Falls, South Dakota, U.S.) at pH 8.7. At the base of the seedlings, 500 spores along with ~0.5g of mycorrhizal root segments or double autoclaved spores and root segments were added to mycorrhizal and non-mycorrhizal pots, respectively. One week after planting, one seedling was removed, so each pot had two seedlings. At DAP 8, the plants were fertilized with modified nutrient solution (Ingestad, 1960) containing 0.005 mM NH₄NO₃ as nitrogen (N) and 0.002 mM KH₂PO₄ as phosphate (P) in the soil substrate. We provided relatively low nutrient concentrations in the soil to facilitate host nutrient demand and therefore stimulate AM fungal and rhizobia bacterial benefits for the host plants. Rhizobia (R) bacteria, *Ensifer meliloti* (formerly *Sinorhizobium meliloti*) were cultured in sterilized tryptone yeast broth media for 16 hours at 28°C in a shaker at 250 rpm, centrifuged at 5000 rpm at 20°C for 5 minutes, and the bacterial pellet was re-suspended by a vortex with autoclaved tap water, and a measured OD of 1.08 at 600 nm. Each plant (at the base of stem) received one mL

of this bacterial solution along with 10 mL of milliQ water at DAP 10 or double autoclaved solution, respectively.

3.3.2 Experimental design and growth conditions

The plants were either inoculated with AM fungus alone, rhizobia alone, or dual inoculated (AM fungus and rhizobia), or non-inoculated controls to compare the effects of individual root symbionts for plant growth response and nutrient acquisition. There were four nutrient regimes: low phosphate and low nitrogen (LPLN); low phosphate and high nitrogen (LPHN); high phosphate and low nitrogen (HPLN); and high phosphate and high nitrogen (HPHN). Each treatment had six replicates except the treatment with rhizobia alone at LPHN that had five replicates, thus totaling 95 pot systems. Nutrients solution was supplied at DAP 17, 24, 38, and 45. The final soil P concentration in the soil at LP or HP conditions was 0.18 mM and 0.70 mM respectively as KH_2PO_4 including original P concentration present in the soil substrate. Similarly, final nitrogen concentration in the soil as LN and HN was 0.5mM and 4 mM respectively as NH_4NO_3 . To reduce nutrient leaching, we supplied water to the tray in which the pots were kept so that water could percolate to the pot substrate through the hole at the base of the pots. Plants were allowed to grow in the growth chamber (model TC30; Conviron, Winnipeg, MB, Canada) at a 14 h photoperiod of photosynthetically active radiation of $225 \mu\text{molm}^{-2}\text{S}^{-1}$, 25°C: 20°C, day: night cycle, and 30% humidity as Fellbaum et al. (2014) until harvest at DAP 65. One day before harvest, the center of the most recent fully exposed leaf was chosen from each plant to measure the chlorophyll content using a SPAD probe.

3.3.3 Biomass, nodule, and AM colonization quantification

After harvest, shoots and roots were kept separately in paper bags. The roots were washed carefully with tap water to remove soil particles. Root aliquots were taken in separate tubes for AM colonization and nodulation assays. The rest of the roots were kept in the paper bags and dried at 70°C for 48 hours. Root aliquots for AM colonization assay were preserved in 50% alcohol during harvesting and kept at 4°C until measurement. For AM fungal colonization assays, the alcohol was discarded, roots were rinsed several times in tap water, and then cut ~2cm length, kept in a water bath at 80°C in 10% KOH for 40 minutes, and stained with 5% ink-vinegar solution at 80°C for 15 minutes as described previously (Vierheilig et al., 1998). The AM fungal colonization rate was determined following the grid line intersection method as percentage of root length colonized (McGonigle et al., 1990). Mycorrhizal growth responses (MGR) were calculated using the formula: $MGR (\%) = (\text{dry biomass of AM plant} - \text{mean dry biomass of non-AM plant} / \text{mean dry biomass of non-AM plants}) \times 100$. A similar approach was used for the dual inoculated plants against rhizobia bacteria alone plants.

3.3.4 Phosphate, carbon, and nitrogen analysis

Dry shoot and root samples were separately grounded and homogenized using a tissue homogenizer (Precellys 24, Cayman Chemical Company, Ann Arbor, USA). For the phosphate (P) measurement, aliquots of the dry powder were digested in 1 mL of 2N HCl at 95°C for 2 hours and later treated with ammonium molybdate as previously described (Kafle et al., 2018; Wang et al., 2016b). Shoot and root total nitrogen (N) and carbon (C) were measured from five gram of dry tissue powder using a ThermoFlash EA1112 flash

analyzer (Department of Soil Science, University of Wisconsin, Madison, Wisconsin, USA). Mycorrhizal phosphate response (MPR) and mycorrhizal nitrogen response (MNR) were calculated as described above for the MGR. Moreover, we measured the C concentration in the root to understand how the host plant allocate carbon to different symbiotic root tissues under different nutrient supply conditions.

3.3.5 Gene expression analysis

We determined the transcript levels in the roots of two AM-inducible plant genes, the P transporter *MtPT4* (Chiou, Liu & Harrison, 2001; Harrison, Dewbre & Liu, 2002; Javot, Penmetsa, Terzaghi, Cook & Harrison, 2007) and the ammonium transporter *MtAMT2;3* (Straub, Ludewig & Neuhäuser, 2014; Breuillin-Sessoms *et al.*, 2015). In addition, we analyzed the expression levels of three plant sucrose transporters from the SUT family, *MtSUT1-1*, *MtSUT2* and *MtSUT4-1* (Doidy *et al.*, 2012), and seven transporters of the SWEET family, *MtSWEET1b*, *MtSWEET6*, *MtSWEET9*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d*. Since *MtSWEET9* showed only low and very inconsistent levels of expression in our experiments, the results of this transporter are not shown. All steps were performed according to the manufacturer's instructions unless stated otherwise. We homogenized the root samples with a mortar and pestle cooled with liquid nitrogen, and extracted total RNA using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNAs were treated with TURBO™ DNase (Thermo Fisher Scientific) and quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNAs were synthesized from 400 or 600 ng of DNase-treated RNAs using the RNA Maxima First Strand cDNA Synthesis Kit with

dsDNase (Thermo Fisher Scientific), and diluted with RNase-free water to a final concentration of 20 ng μl^{-1} if needed. qPCRs were performed using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 1 μl of 20 ng μl^{-1} cDNAs, and 5 μM of forward and reverse primers (Table S1) for each gene in a 20 μl reaction mix using a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). The PCR conditions were as follows: 50°C for 2 min; 95°C for 15 min; 40 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s; dissociation at 95°C for 15 s; 60°C for 15 s; and 95°C for 15 s. We used *MtTefl α* as a reference gene (Gomez et al., 2009) and the expression coefficients were calculated using the $2^{-\Delta\text{Ct}}$ method. The results are based on three to four biological replicates and three technical replicates.

3.3.6 Statistical analyses

Data set of mean \pm sem from six independent biological replicates, if not stated otherwise, were analyzed using Statistix analytical software (Statistix9, Tallahassee, Florida, U.S.A.). Prior to statistical analyses, the data set were tested for the assumption of normality and homogeneity using Shapiro-Wilk and Leven's testn respectively. The variability among the data sets were measured by ANOVA and at $p \leq 0.05$, further multiple pairwise comparisons were conducted using the LSD test. The same analytical package was used for the regression analyses between shoot P acquisition and root C concentration; and root N and C concentration.

3.4 Results

3.4.1 *Effect of symbionts and nutrient regimes on shoot, root biomass, AM colonization, and nodulation*

Plants inoculated with rhizobia alone or with AM fungi and rhizobia had a higher shoot and root biomass compared to plants that were non-inoculated or plants that were only inoculated with AM fungi at LPLN, LPHN, and HPLN (Figure 3.1). When plants that were only inoculated with AM fungi were compared to non-inoculated plants, AM fungi had a neutral effect on shoot and root biomass or reduced plant biomass under HPHN conditions (Figure 3.1, Table 3.1, Table S3.1). Interestingly, dual inoculated plants had a lower biomass than R plants at LPHN, whereas dual inoculated plants at HPHN conditions had a higher biomass ($p \leq 0.05$) than R plants (Figure 3.1, Table S3.1). The highly significant interaction between symbionts (none, AM alone, R alone, and dual) and nutrient regimes (LPLN, LPHN, HPLN, and HPHN) indicates that the symbionts respond differently to the nutrient supply conditions (Table S3.3).

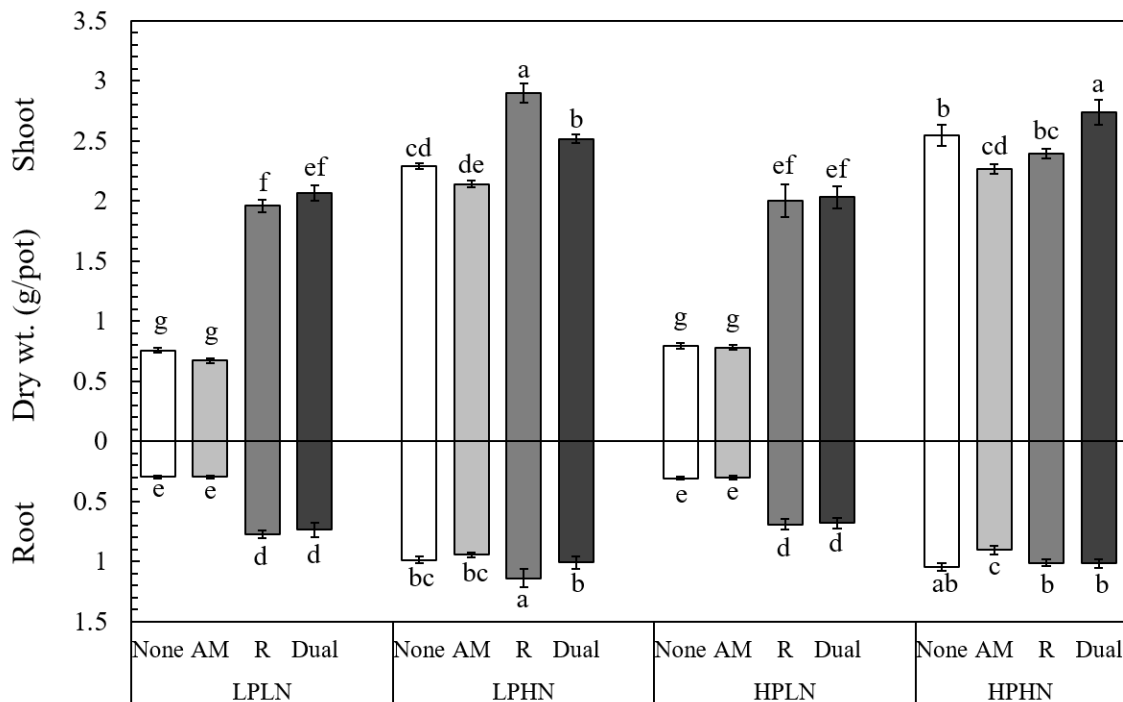


Figure 3.1. Shoot (above) and root (below) dry biomass of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None), or were inoculated with *Rhizopagus irregularis* (AM) alone, or *Ensifer meliloti* (R) alone or both symbionts (Dual). The vertical bars represent the means (n=6) with standard error of means. Different letters refer to statistically significant differences at p values of ≤ 0.05 after ANOVA and LSD test. Analyses of variance (one way and two way) results are shown in Table S3.1 and S3.3.

Root AM colonization was not significantly different between dual inoculated plants and plants that were only inoculated with AM fungi except at HPLN (Figure 3.2a). The colonization was relatively lower under HPHN conditions. Similarly, nodule dry weight was not statistically different between R alone and dual inoculated plants (Figure 3.2b). Notably, nodule weight was reduced ($p \leq 0.05$) under LPHN supply conditions.

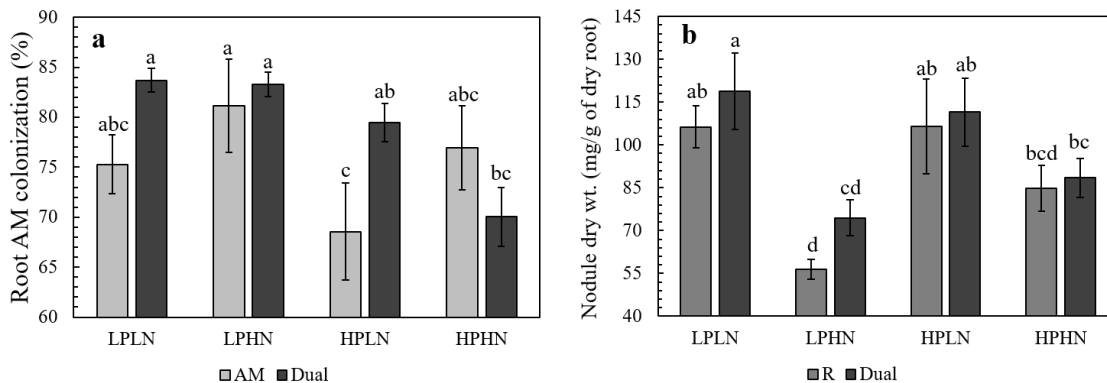


Figure 3.2. Root AM colonization (a) and nodulation (b) *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None), or were inoculated with *Rhizopagus irregularis* (AM) alone, or *Ensifer meliloti* (R) alone or both symbionts (Dual). The vertical bars represent the means (n=6) with standard error of means. Different letters refer to statistically significant differences at p values of ≤ 0.05 after ANOVA and LSD test. Analyses of variance (one way and two way) results are shown in Table S3.1 and Table S3.3

Shoot and root phosphate (P) concentrations were higher ($p < 0.05$) in AM plants than non-inoculated plants (Figure 3.3a, b, Table 3.1, Table S3.2). Considering the similar plant biomass, AM colonization increased the P concentrations of the shoots compared to non-inoculated plants by 9.5% to 23% at HPHN and LPLN, respectively (Figure 3.3a, Table 1). In the same way, considering the similar plant biomass, dual inoculated shoots had a by 30% to 71% higher ($p \leq 0.05$) P concentration than plants that were only inoculated by rhizobia at HPHN and LPLN (Figure 3.3a, Table 3.1). Shoot P concentration in the dual inoculated plants was consistently higher than either only rhizobial, or only AM or non-inoculated plants except at HPLN. The root P concentrations had a similar pattern than the shoot. Notably, roots that were only inoculated with AM had higher ($p \leq 0.05$) P concentrations than non-inoculated roots under LN supply conditions (Figure 3.3b). Interestingly, the root P concentration was higher ($p \leq 0.05$) in AM plants (Figure 3.3b) that had lower chlorophyll contents in leaves (Figure S3.1). The shoot P content was

significantly higher in the dual inoculated plants than either only rhizobial, or only AM, or non-inoculated plants. Dual inoculated shoots had up to 80% higher ($p \leq 0.05$) P contents than plants that were only inoculated with rhizobia across all nutrient treatments (Figure 3.3c, Table 3.1). However, compared to the non-inoculated controls, the AM symbiosis did not increase the shoot P contents (Table S3.1), despite higher P concentrations (Figure 3.3c). In contrast, the root P contents were consistently higher ($p \leq 0.05$) in AM inoculated plants than in non-inoculated roots (Figure 3.3d, Table S3.1).

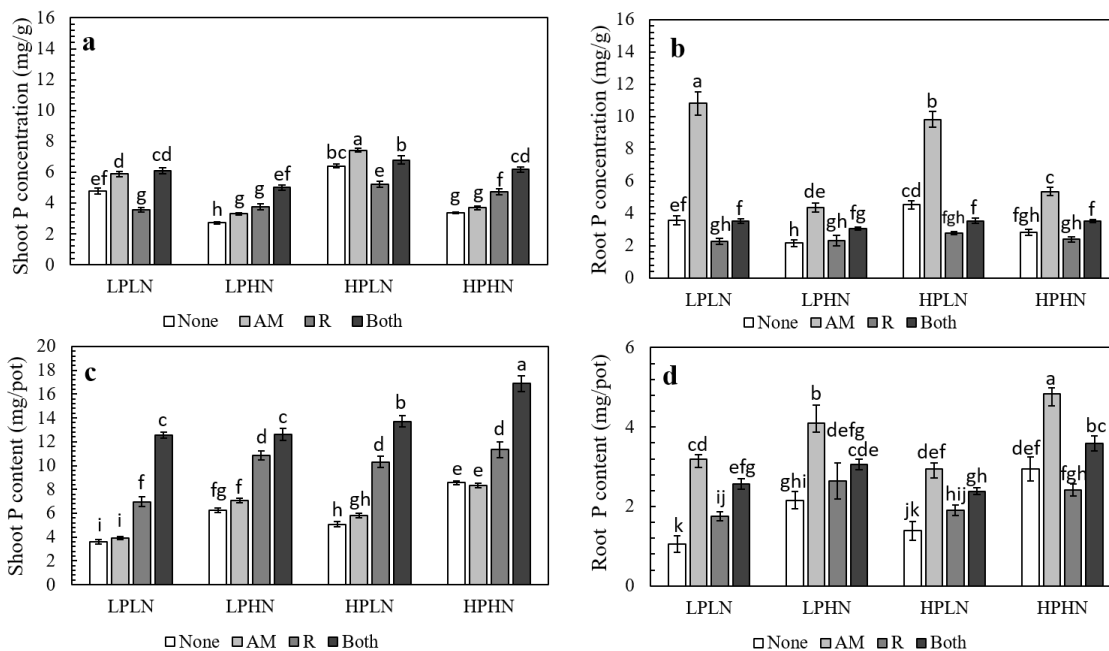


Figure 3.3 Shoot and root phosphate (P) concentration (a and b) and content (c and d) of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None), or were inoculated with *Rhizophagus irregularis* (AM) alone, or *Ensifer meliloti* (R) alone or both symbionts (Dual). The vertical bars represent the means ($n=6$) with standard error of means. Different letters refer to statistically significant differences at p values of ≤ 0.05 after ANOVA and LSD test. Analyses of variance (one way and two way) results are shown in Table S3.1 and Table S3.3.

Shoot nitrogen (N) concentration and content in the dual inoculated plants was higher than only rhizobia, or AM, or non-inoculated plants at LPLN supply conditions (Figure 3.4 a, c). Shoot N concentrations and contents of AM inoculated plants did not differ from non-inoculated plants within nutrient treatment (Figure 3.4a, c). Similarly, the shoot N concentration did not differ between dual inoculated plants and plants that were inoculated with R alone except at LPLN. Root N concentration was higher in the dual inoculated plants than other either inoculated with either symbiont or non-inoculated plants. Compared to non-inoculated roots, the inoculation with only AM fungi led to higher root N concentrations at LPLN and HPHN conditions but did not differ at LPHN and HPLN conditions (Figure 3.4b, Table 3.1, Table S3.2). Similarly, dual inoculated roots had higher ($p \leq 0.05$) N concentrations plants that were only inoculated with R, notably under LPLN and LPHN supply condition (Figure 3.4b). For instance, N concentration in dual inoculated root had pronouncedly higher, as much as 25% more N than R alone inoculated root (Figure 3.4b, Table 3.1) in LPHN supply conditions, whereas other dual inoculated roots had not such noticeable different with R alone inoculated roots. In consistent with shoot N concentration, AM alone inoculated and none inoculated plants had similar N content in shoot (Figure 3.4c, Table S3.1). However, shoot N content in dual inoculated plants had higher ($p \leq 0.05$) than none inoculated shoot in LPLN and HPHN supply conditions. Dual inoculated root had relatively higher N content than R alone inoculated, particularly at LPHN (Figure 3.4c, Table 3.1, Table S3.1).

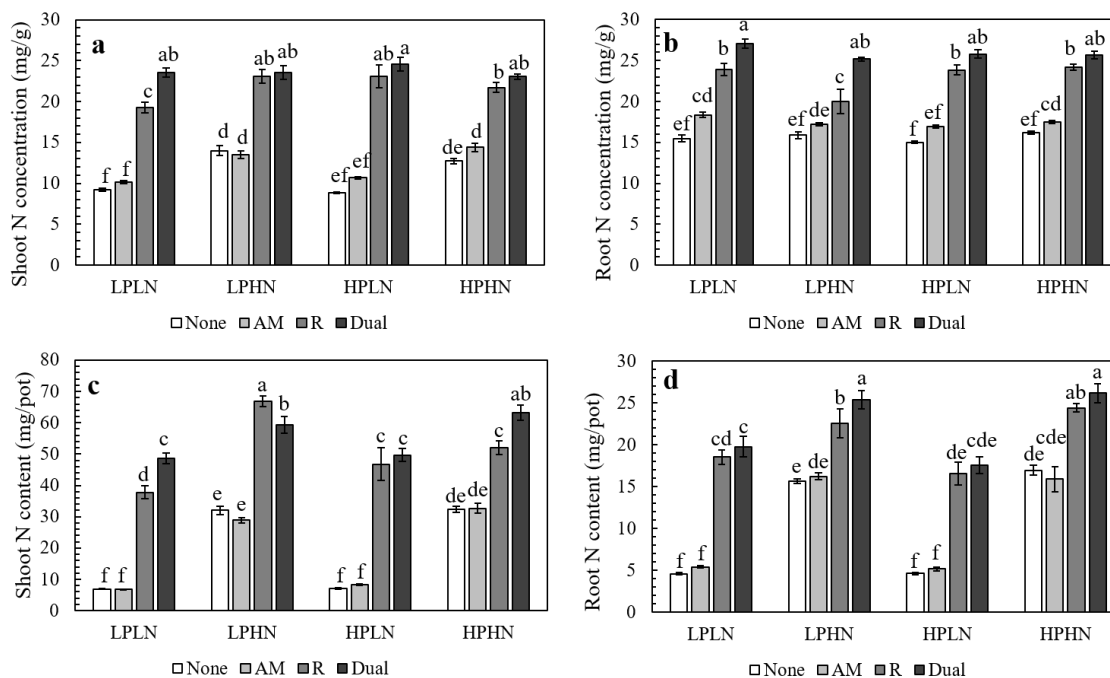


Figure 3.4. Shoot and root nitrogen (N) concentration (a and b) and content (c and d) of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None), or were inoculated with *Rhizophagus irregularis* (AM) alone, or *Ensifer meliloti* (R) alone or both symbionts (Dual). The vertical bars represent the means (n=6) with standard error of means. Different letters refer to statistically significant differences at p values of ≤ 0.05 after ANOVA and LSD test. Analyses of variance (one way and two way) results are shown in Table S3.1 and Table S3.3.

Shoot carbon (C) concentration was in increasing trend from non-inoculated to dual inoculated plants (Figure 3.5a) in LPLN and HPLN supply conditions. Whereas root C concentration in only AM inoculated plants was higher than non-inoculated plants when one or both nutrients were in limiting supply but no different at HPHN supply condition (Figure 3.5b, Table 3.1). Root C concentration in dual inoculated was higher than R alone inoculated plants and this different was notably higher ($p \leq 0.05$) in LPHN supply conditions (Figure 3.5b, Table 3.1, Table S3.2). Similarly, root C content of AM plants was relatively higher than non-inoculated plants except in HPHN supply conditions (Table S3.1).

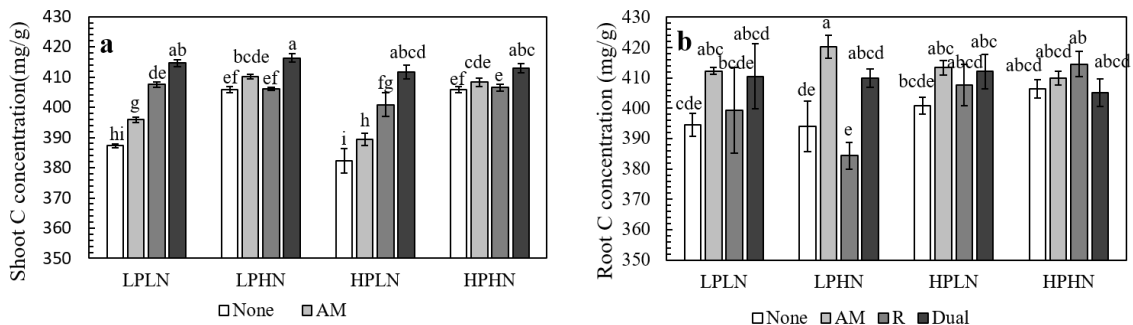


Figure 3.5. shoot (a) and root (b) carbon concentration of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None), or were inoculated with *Rhizopagus irregularis* (AM) alone, or *Ensifer meliloti* (R) alone or both symbionts (Dual). The vertical bars represent the means (n=6) with standard error of means. Different letters refer to statistically significant differences at p values of ≤ 0.05 after ANOVA and LSD test. Analyses of variance (one way and two way) results are shown in Table S3.1 and Table S3.3.

Table 3.1. Shoot and root dry wt., phosphate (P), nitrogen (N), and carbon (C) responses *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate.

	Comparisons	LPLN	LPHN	HPLN	HPHN
Shoot biomass response	AM/None	-11.23% (d)	-6.4% (e)	-1.6% (b)	-10.9% (c)
	R/None	159.2% (c)	26.5% (b)	152.8% (a)	-6% (c)
	Dual/None	173.1% (bc)	9.9% (d)	156.8% (a)	7.5% (b)
	R/AM	192% (ab)	35.3% (a)	157.1% (a)	5.6% (b)
	Dual/AM	207.6% (a)	17.5% (c)	161.2% (a)	20.8% (a)
	Dual/R	5.3% (d)	-13.1% (f)	1.6% (b)	14.3% (ab)
Shoot phosphate response	AM/None	23.10 (b)	21.28% (cd)	15.90% (b)	9.55 % (c)
	R/None	-25.78% (d)	37.77% (b)	-18.65% (c)	40.25% (b)
	Dual/None	27.52% (b)	83.71% (a)	5.97% (b)	83.45% (a)
	R/AM	-39.71% (e)	13.59% (d)	-29.81% (d)	28.02 % (b)
	Dual/AM	3.59% (c)	-26.32% (e)	12.21% (b)	8.06 % (c)
	Dual/R	71.84% (a)	33.36% (bc)	30.28% (a)	30.80 % (b)
Root phosphate response	AM/None	202.97% (a)	101.49 % (a)	116.79 % (a)	88.86% (a)
	R/None	-36.23% (d)	6.98 % (cd)	-38.83 % (e)	-15.22% (e)
	Dual/None	-1.1% (c)	40.59 % (b)	-21.95 % (d)	24.43% (c)
	R/AM	-0.78% (c)	-0.46 % (d)	-0.71 % (c)	-0.55% (d)
	Dual/AM	-0.67% (c)	-0.30 % (d)	-0.64 % (c)	-0.34% (d)
	Dual/R	55.06% (b)	31.42 % (bc)	27.58 % (b)	46.77 % (b)
Shoot nitrogen response	AM/None	10.28% (e)	-3.7% (c)	20.60% (d)	13.39% (d)
	R/None	109.19% (c)	99.34% (a)	157.20% (ab)	98.44% (a)
	Dual/None	156.06% (a)	68.14% (b)	177.73% (a)	81.40% (b)
	R/AM	89.67% (d)	71.37% (b)	116.58% (c)	50.60% (c)
	Dual/AM	132.18% (b)	74.61% (b)	130.27% (bc)	59.97% (c)
	Dual/R	22.40% (e)	1.89% (c)	6.32% (d)	6.21% (d)
Root nitrogen response	AM/None	18.86% (c)	8.13% (d)	12.59% (c)	7.72% (c)
	R/None	0.54% (d)	0.25% (e)	0.58% (d)	0.49% (d)
	Dual/None	75.20% (a)	58.35% (a)	71.91% (a)	58.30% (a)
	R/AM	0.30% (d)	0.16% (e)	0.41% (d)	0.38% (d)
	Dual/AM	47.39% (b)	46.43% (b)	52.68% (b)	46.95% (b)
	Dual/R	13.36% (c)	25.74% (c)	8.18% (c)	6.22% (c)
Shoot carbon response	AM/None	2.22% (cd)	1.03% (b)	1.86% (ab)	0.61% (bc)
	R/None	5.22% (b)	0.03% (c)	9.85% (a)	0.16% (cd)
	Dual/None	7.05% (a)	2.57% (a)	7.69% (a)	1.73% (a)
	R/AM	2.93% (c)	-0.99% (d)	7.83% (a)	-0.44% (d)
	Dual/AM	4.71% (b)	1.52% (b)	5.71% (ab)	1.11% (abc)
	Dual/R	1.73% (d)	2.54% (a)	-1.96% (b)	1.55% (ab)
Root carbon response	AM/None	4.51% (a)	6.66% (a)	3.09% (a)	0.87% (ab)
	R/None	1.23% (a)	-2.46% (c)	1.68% (ab)	2.01% (a)
	Dual/None	4.06% (a)	4.02% (b)	2.79% (a)	-0.30% (abc)
	R/AM	-3.13% (a)	-8.55% (d)	-1.36% (b)	1.13% (ab)
	Dual/AM	-0.43% (a)	-2.47% (c)	-0.29% (ab)	-1.16% (bc)
	Dual/R	2.79% (a)	6.65% (a)	1.08% (ab)	-2.27% (c)

3.4.3 Effects of symbionts on the expression of genes at different nutrient supply conditions

We examined the expression of nine different plant sugar transporter genes to understand how their expression is affected by different symbionts under different nutrient supply conditions (Figure 3.6a-i). In general, we observed high transcripts levels of the three different sugar uptake transporters genes (*MtSUT1-1*, *MtSUT2* and *MtSUT4-1*) in non-inoculated and in AM roots, while roots that were colonized only rhizobia or with rhizobia and AM showed comparatively lower transcript levels. The highest expression levels of *MtSUT1-1* were observed in non-mycorrhizal roots under HPLN and HPHN conditions (Figure 3.6a).

The transcript levels of *MtSWEET1b* were highly expressed in AM roots under all nutrient supply conditions but especially at LPLN and HPLN (Figure 3.6d), while non-inoculated roots, or roots that were colonized with rhizobia showed significantly lower expression levels. In contrast, *MtSEET11* was only expressed in rhizobia inoculated plants (Figure 3.6f) particularly under low nitrogen supply conditions (LPLN and HPLN). Expression levels of *MtSWEET12* were higher in AM roots particularly at LPHN (Figure 3.6g), while *MtSWEET15c* and *MtSWEET15d* were highly expressed in non-inoculated roots (Figure 3.6 h, i).

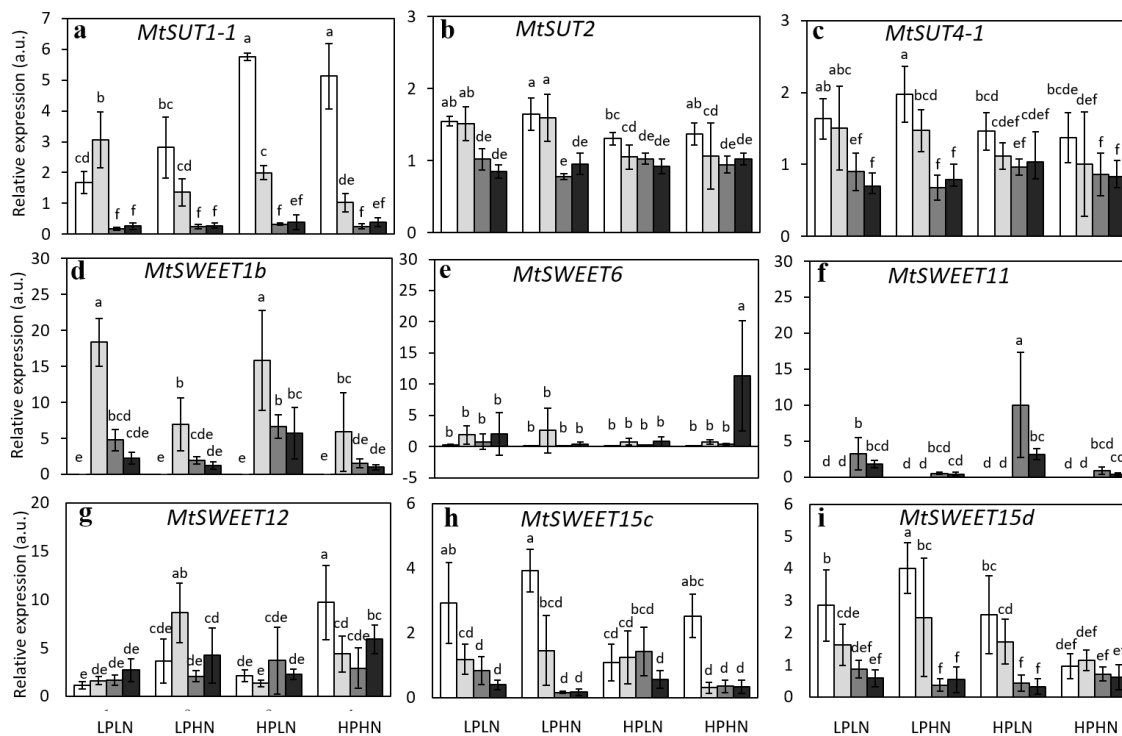


Figure 3.6. Relative expression of three sucrose transporters (*MtSUT1-1*, *MtSUT2*, and *MtSUT4-1*) and six SWEETS (Sugars Will Eventually be Exported Transporter; *MtSWEET1b*, *MtSWEET6*, *MtSWEET11*, *MtSWEET12*, *MtSWEET15c*, *MtSWEET15d*) genes of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None, white bar), or were inoculated with *Rhizophagus irregularis* (AM, light grey bars) alone, or *Ensifer meliloti* (R, medium grey bar) alone or both symbionts (Dual, dark grey bars). The vertical bars represent the means (n=3) with standard error of means. Different letters refer to statistically significant differences at p values of ≤ 0.05 after ANOVA and LSD test.

We detected significantly higher transcript levels of AM fungal specific phosphate transporter (*MtPT4*) and ammonium transporter (*MtAMT2;3*) exclusively when plants were inoculated with AM fungus whereas these transcripts were highly suppressed or not detected in dual inoculated roots (Figure S3.2 a, b). Among all nutrient supply conditions, expression of *MTPT4* and *AMT2;3* was higher under LPLN than under the other nutrient supply conditions.

3.5 Discussion

Phosphate (P) and nitrogen (N) in soil are critical nutrients for crop productivity. Here, *Medicago truncatula* plants were kept non-inoculated, were inoculated with only the AM fungus *Rhizophagus irregularis*, or were only inoculated with *Ensifer meliloti*, or with both symbionts (dual) and supplied with different nutrient supply conditions in the soil to evaluate the host plant growth response, nutrient acquisition, and genes involved in sugar transport to symbiotic roots. We found that plants that were colonized with both symbionts had relatively higher shoot biomass than plants that were only colonized with rhizobia when both nutrients (P and N) were under limiting conditions (LPLN supply conditions) (Figure 3.1, Table S3.1) which supports previous findings (Mortimer et al., 2012; Wang et al., 2011). However, plant growth responses were neutral or negative when one of the nutrients was limiting in the soil (Mortimer et al., 2012; Vázquez et al., 2001). For instances, Vázquez et al. (2001) and Correa et al. (2014) reported that AM fungal benefits decreased under high N supply conditions. Increased shoot and root biomasses in dual inoculated systems and plants that were only colonized with rhizobia at LPLN and HPLN indicate that plants were under N limiting conditions, but that P limitation was not as severe since shoot and root biomasses had similar response at LPLN and HPLN. This variability in host growth benefits in tripartite interactions is the function of context dependency as suggested before (Catford et al., 2006; Larimer et al., 2014). For instances, variability of growth response occurred between different experimental set up (Facelli et al., 2014) light intensity, soil nutrient conditions, fungal species (Lendenmann et al., 2011), growth period, and size of pot could probably also affect the fungal benefits to host plant. The variability in our study could be due to exchange of resources: P, N, and C between the host plant and

symbiotic partners (Jin et al., 2017). In addition, plants inoculated with only the AM fungus had a neutral growth response in comparison to non-inoculated plants under all nutrient supply conditions (Figure 3.1). This neutral response of AM fungi for the plant growth in this study agrees with previous findings (Bonneau et al., 2013; Leigh et al., 2009; Li et al., 2008; Mäder et al., 2000; Rezacova et al., 2018). Generally, positive effects of AM symbiosis are observed in a large volume of soil where AM fungi have access to soil nutrients beyond the direct root uptake pathway (Facelli et al., 2014; Kafle et al. 2018). However, in this study, plants were grown in a relatively small volume of soil (500 mL of soil substrate) for nine weeks where roots had easy access to the supplied nutrients therefore reducing the functional niche of AM fungi. In addition, nutrient gain by the host plant that did not outweigh the C cost for the symbiosis could be another possible explanation for neutral growth response (Jin et al., 2017; Leigh et al., 2009; Lerat et al., 2003).

Plants that were inoculated with both symbionts had higher shoot N concentrations and contents than plants that were only inoculated with rhizobia under LPLN conditions (Figure 3.4a, Table 3.1, Table S3.2), indicating a beneficial (direct or indirect) role of AM fungus for N allocation to shoots in the tripartite interactions consistent with Mortimer et al. (2012). Root N concentration in the dual inoculated plants was consistently higher than only rhizobia inoculated roots across the nutrient treatments (Figure 3.4b, 3.4d, Table 3.1, Table S3.2). Notably, N concentration and content in the dual inoculated root was significantly higher than only rhizobial roots at LPHN suggesting that mycorrhizal root retained a significant amount of N which may be a probable reason why we observed reduced shoot growth of dual inoculated plants at LPHN (Figure 3.1, Table 3.1). This

higher N accumulation in the dual inoculated root suggested that some the supplied N could have been assimilated in the mycorrhizal structures (hyphal, vesicles) within the roots as reported previously (López-Pedrosa et al., 2006; Ngwene et al., 2013; Tomè et al., 2015; Vázquez et al., 2001). It has been reported that in AM symbiosis, substantial amount of extra radicle mycelium (ERM) mass of AM fungi exist in the soil outside the host root (Johnson et al., 2015; Leigh et al., 2009; Lendenmann et al., 2011). This ERM demands more N to maintain extending hyphal structures and fruiting bodies as AM fungi are biological entity which therefore need some N to run their life process. Moreover, Ngwene et al. (2013) and Tomè et al. (2015) claimed that the part of the supplied N gets assimilated within the hyphae rather than exporting into the host to satisfy the fungal internal demand of N. For instance, Jin et al. (2005) detected considerable amount of ^{15}N labelled arginine in the ERM including spores even after 6 weeks of N application indicating that AM fungus partly retained some of N despite its contributing of N to the host. Thus, in our study, IRM and ERM might have used/retained part of the supplied N for their growth and development which could be the consequences of neutral or negative growth of the mycorrhizal plants. This functionality of the AM fungus to retain some of the N within its hyphal structures may have bigger implications in the natural system, for instance, to provide N at the early stage of the plant development for the next season plants during symbiosis (Hodge and Fitter, 2010).

Plants inoculated with dual symbionts consistently had higher P concentration and content than only rhizobia inoculated plants in shoot and root tissues depicting the contribution of AM fungus for plant P acquisition (Figure 3.3a-d). In return of P supply, mycorrhizal roots received proportionally higher of C from the host plant than rhizobial

roots (Figure 3.5b) supporting previous reciprocal rewards findings (Bücking and Shachar-Hill, 2005; Fitter, 2006; Kiers et al., 2011; Lendenmann et al., 2011; Zheng et al., 2015). These previous studies demonstrated strong positive correlation between mycorrhizal P supply and C allocation to the mycorrhizal root (Kiers et al., 2011; Zheng et al., 2015) which is in conjunction with our findings. Remarkably, plants allocated a higher proportion of C to dual inoculated roots at LPHN than only rhizobial inoculated roots. Investment of C to the dual roots in this LPHN condition could be for the P demand of the host plant to assimilate N as there was higher supplied N in the soil (Figure 3.5b, Table S3.2). However, there was no plant growth despite higher AM contribution for plant P acquisition (Li et al., 2008), as it probably became an expensive for the plant to invest C in return to P (Graham and Abbott, 2000; Johnson et al., 1997). Plants invest a substantial amount of resources and energy to produce carbohydrates during the photosynthesis, so any loss of this carbohydrates affects the plant growth. Therefore, higher C investment to the dual inoculated roots might have caused the growth depression of plant at LPHN. We are not vehemently claiming that the AM fungus in this context veer from the mutualism to parasitism as it was still providing P. The continued supply of C to the mycorrhizal roots could be the investment for the future “paying for the insurance when you are healthy”. This investment of C to belowground soil through network of mycorrhizal hyphae might have larger implications to balance the carbon cycle and ecosystem stability (Smith et al., 2009; Wurzburger et al., 2017).

Plants inoculated with only AM fungus had similar expression of AM fungal specific P transporter *MtPT4* between lower and higher P soil concentration (LPHN and HPLN) (Figure S3.2 a) which suggest that this transporter may not be a good predictor

with P uptake in the plants (Facelli et al., 2014). Surprisingly, undetected expression of *MtPT4* was observed in dual inoculated roots in all nutrient supply conditions which could be the effect of rhizobia in the same roots. It has been demonstrated that dual inoculated *Medicago truncatula* roots had higher expression of transcripts for phosphatase (Afkhami et al., 2016), which could be an antagonistic effect of rhizobia against AM fungus for the expression of *MtPT4*. Rhizobia may trigger plants to uptake P by direct uptake mechanism to minimize another competitor (AM fungus) for host C. Besides *MtPT4*, recent evidence has suggested that AM fungus also induces host root for the expression of another AM fungal specific P transporter *MtPT8* (Breuillin-Sessoms et al., 2015) which might have associated for the P transport into the host cytoplasm in our study.

Similarly, we detected higher expression of AM fungal specific ammonium transporter (*MtAMT2;3*) specifically when the plants were inoculated with only AM fungus but did not detect in dual inoculated roots (Figure 3.2b). Moreover, this expression was more pronounced in LPLN and HPLN conditions, clearly demonstrating contribution of AM fungus for ammonium uptake in the host plant when host plants are under N demand conditions (Breuillin-Sessoms et al., 2015; Kafle et al., 2018). However, in the dual inoculated plants, rhizobia might have supplied N demand of the host, consequently there was significantly reduced expression of *MtAMT2;3*. These AM fungal specific phosphate and ammonium transporters have extensively studied in only AM inoculated legume plants (*Medicago* and soybean) (Breuillin-Sessoms et al., 2015; Fellbaum et al., 2014; Koba et al., 2010) but rarely in the tripartite symbiotic association (Kafle et al., 2018). However, studying legume plant like *Medicago* and soybean without considering both symbionts

may not provide enough information as legumes are simultaneously colonized with AM fungi and rhizobia in the same root forming tripartite interaction.

Plants invest considerable amount of photosynthetic C to roots so that roots can extend further to forage soil nutrients. It has been reported that there are several clades of carbon (in the form of sucrose) transporters involved in loading from the source of C synthesis and uploading into the sink tissues such as either symbiotic or non-symbiotic root tissues (Doidy et al., 2012; Kafle et al., 2018; Kryvoruchko et al., 2016; Sugiyama et al., 2017). To have an insight knowledge, how the host plants regulate their carbon transporters in their root tissues in different nutrient conditions with different symbiotic partners. We observed that the expression of *MtSUT1* was highly upregulated in only AM inoculated roots at LPLN conditions (Figure 3.6 a), supporting previous findings (Doidy et al., 2012) indicating that host plants invested higher C to AM symbiotic roots under nutrient limiting conditions. Higher expression of *MtSUT1* in non-inoculated plants than dual or only rhizobia inoculated plants probably could be the effect of nutrient starvation especially N in non-inoculated plants as these plants had lower chlorophyll content in their leaves (Figure S3.1). Similarly, expression *MtSUT2* and *MtSUT4-1* were reduced in only rhizobia and dual inoculated roots than non-inoculated and only AM inoculated roots in LPLN and LPHN conditions, indicating that these transporters are not only symbiotic specific. Rather, higher expression of these transporters in LPLN conditions (Figure 3.6 b, c) suggests that they are involved in C unloading to sink roots in nutrient limited soil so that roots can use this C for further extend into the soil for nutrients.

Moreover, we measured the expression of *SWEETs* in host roots which are involved in transporting C from phloem tissues into cortical cells of roots. We detected higher transcripts level of *SWEET11* in only rhizobia inoculated roots in LPLN and HPLN conditions suggesting that this transporter plays a significant role in delivering C to N₂ fixing bacteroids in the nodulated roots. However, as we supplied more N in the soil, the expression of this transcript reduced significantly which thus verified its role in C flux towards the nodulated roots (Kafle et al., 2018; Kryvoruchko et al., 2016). Similarly, higher expression of *MtSWEET1b* in only AM fungus inoculated roots suggest that this transporter is AM fungal specific for C transport to AM symbiosis. A comprehensive functional understanding of SUT, SWEET, and other sugar transporters for the timing and their localization of expression during AM and rhizobial symbioses in different nutrient regimes in the soil may have a broader impact and implications in improving legumes like *Medicago* and Soybean.

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3.7 Supplemental information

Figure S3.1. Chlorophyll content (SPAD unit) of *Medicago truncatula*

Figure S3.2. Relative expression AM fungal specific AMPT4 and AMT2;3 in roots of *Medicago truncatula*

Table S1. Shoot and root dry wt., phosphate (P), nitrogen (N), and carbon (C) content of *Medicago truncatula*

Table S2. Shoot and root phosphate (P), nitrogen (N), and carbon (C) concentration of *Medicago truncatula*

Table S3. One Way and Two Ways ANOVA of plant attributes of *Medicago truncatula*

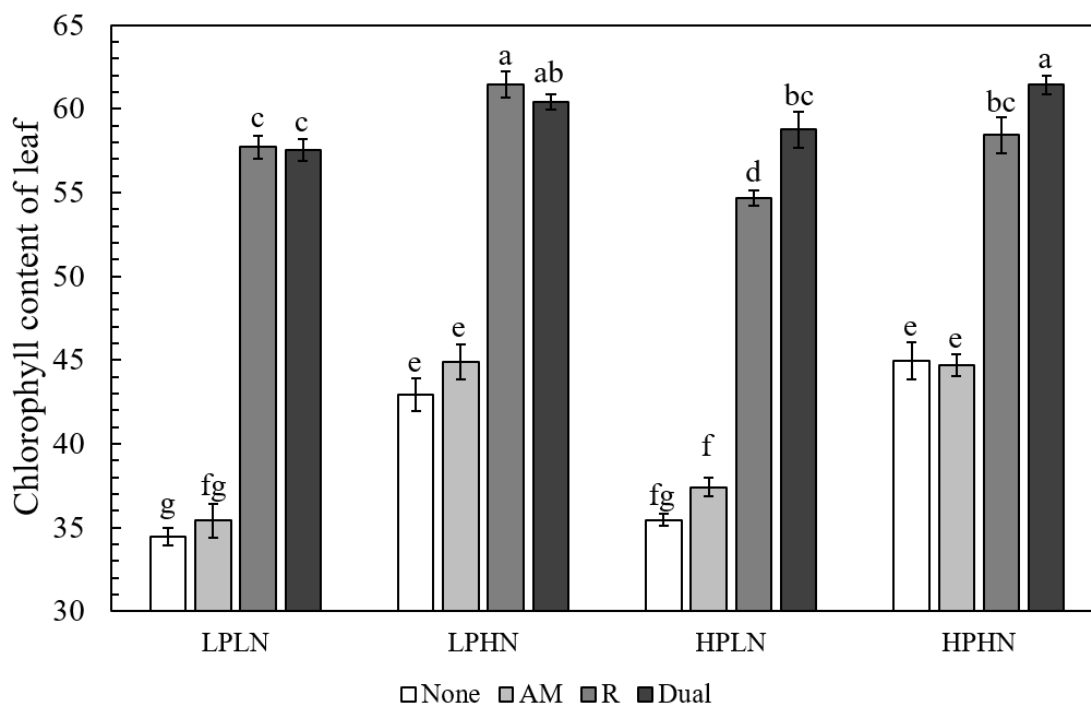


Figure S3.1. Chlorophyll content (SPAD unit) of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None), or were inoculated with *Rhizophagus irregularis* (AM) alone, or *Ensifer meliloti* (R) alone or both symbionts (Dual). The vertical bars represent the means (n=6) with standard error of means. Different letters refer to statistically significant differences at p values of ≤ 0.05 after ANOVA and LSD test.

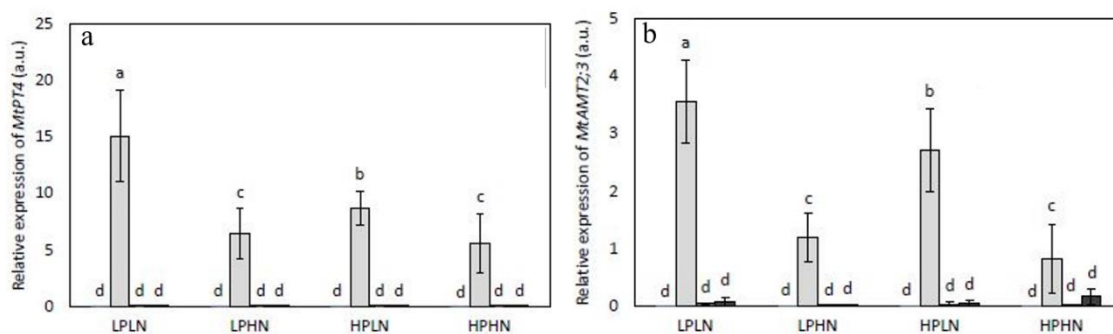


Figure S3.2. Relative expression AM fungal specific phosphate transporter (*MtPT4*) and ammonium transporter (*AMT2;3*) of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None, white bar), or were inoculated with *Rhizophagus irregularis* (AM, light grey bars) alone, or *Ensifer meliloti* (R, medium grey bar) alone or both symbionts (Dual, dark grey bars). The vertical bars represent the means (n=3) with standard error of means. Different letters refer to statistically significant differences at p values of ≤ 0.05 after ANOVA and LSD test.

Table S3.1. Shoot and root dry wt., phosphate (P), nitrogen (N), and carbon (C) content of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate in the growth chamber. Plants were kept either non-inoculated (None), or inoculated with only *Rhizophagus irregularis* (AM), or with only *Ensifer meliloti* (Rhizobial) or both symbionts (Dual). The values are given as mean \pm se of six biological replicates otherwise mentioned. The lower case behind the values are from One Way ANOVA comparing dataset within the nutrient treatment (row).

	Nutrient regime	None	AM	Rhizobial	Dual
Shoot dry wt. (g)	LPLN	0.756 \pm 0.018 b	0.671 \pm 0.019 b	1.961 \pm 0.051 a	2.066 \pm 0.062 a
	LPHN	2.290 \pm 0.024 c	2.141 \pm 0.0273 d	2.898 \pm 0.077 a	2.516 \pm 0.036 b
	HPLN	0.791 \pm 0.024 b	0.778 \pm 0.020 b	2.001 \pm 0.135 a	2.033 \pm 0.091 a
	HPHN	2.546 \pm 0.086 ab	2.266 \pm 0.038 c	2.395 \pm 0.041 bc	2.738 \pm 0.101 a
Root dry wt. (g)	LPLN	0.296 \pm 0.010 b	0.296 \pm 0.011 b	0.776 \pm 0.032 a	0.736 \pm 0.061 a
	LPHN	0.988 \pm 0.026 b	0.9446 \pm 0.021 b	1.138 \pm 0.076 a	1.010 \pm 0.050 ab
	HPLN	0.307 \pm 0.011 b	0.2999 \pm 0.015 b	0.690 \pm 0.047 a	0.682 \pm 0.041 a
	HPHN	1.045 \pm 0.031 a	0.904 \pm 0.036 b	1.010 \pm 0.029 a	1.017 \pm 0.037 a
Shoot P content (mg/pot)	LPLN	3.618 \pm 0.181 c	3.942 \pm 0.126 c	6.967 \pm 0.423 b	12.536 \pm 0.241 a
	LPHN	6.243 \pm 0.183 c	7.0796 \pm 0.179 c	10.845 \pm 0.355 b	12.615 \pm 0.502 a
	HPLN	5.074 \pm 0.203 c	5.780 \pm 0.190 c	10.311 \pm 0.476 b	13.712 \pm 0.487 a
	HPHN	8.552 \pm 0.184 c	8.348 \pm 0.203 c	11.345 \pm 0.661 b	16.885 \pm 0.649 a
Root P content (mg/pot)	LPLN	1.055 \pm 0.088 d	3.185 \pm 0.202 a	1.749 \pm 0.114 c	2.564 \pm 0.137 b
	LPHN	2.158 \pm 0.242 c	4.098 \pm 0.219 a	2.639 \pm 0.457 bc	3.060 \pm 0.137 b
	HPLN	1.391 \pm 0.092 d	2.952 \pm 0.235 a	1.903 \pm 0.137 c	2.380 \pm 0.083 b
	HPHN	2.938 \pm 0.147 c	4.833 \pm 0.303 a	2.412 \pm 0.154 c	3.588 \pm 0.193 b
Shoot N content (mg/pot)	LPLN	6.945 \pm 0.118 c	6.796 \pm 1.009 c	37.800 \pm 1.979 b	48.629 \pm 1.740 a
	LPHN	32.027 \pm 1.261 c	28.851 \pm 0.826 c	66.765 \pm 1.685 a	59.324 \pm 2.589 b
	HPLN	7.042 \pm 0.3031 b	8.298 \pm 0.237 b	46.766 \pm 5.230 a	49.728 \pm 1.985 a
	HPHN	32.320 \pm 1.003 c	32.717 \pm 1.512 c	52.085 \pm 2.244 b	63.130 \pm 2.448 a
Root N content (mg/pot)	LPLN	4.576 \pm 0.134 b	5.427 \pm 0.161 b	18.546 \pm 0.863 a	19.782 \pm 1.236 a
	LPHN	15.667 \pm 0.264 b	16.237 \pm 0.386 b	22.558 \pm 1.752 a	25.389 \pm 1.121 a
	HPLN	4.617 \pm 0.147 b	5.178 \pm 0.235 b	16.557 \pm 1.369 a	17.568 \pm 1.026 a
	HPHN	16.968 \pm 0.553 b	15.899 \pm 0.803 b	24.405 \pm .521 a	26.159 \pm 1.148 a
Root C content (mg/pot)	LPLN	117.30 \pm 5.796 b	122.04 \pm 4.754 b	311.09 \pm 19.255 a	299.23 \pm 17.309 a
	LPHN	388.41 \pm 5.129 a	397.08 \pm 10.220 a	436.77 \pm 26.501 a	413.64 \pm 19.029 a
	HPLN	123.38 \pm 4.270 b	126.90 \pm 6.987 b	282.35 \pm 22.074 a	280.98 \pm 17.107 a
	HPHN	424.74 \pm 12.740 a	372.95 \pm 19.698 b	419.05 \pm 14.396 a	411.88 \pm 14.390 ab

Table S3.2. Shoot and root phosphate (P), nitrogen (N), and carbon (C) concentration of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate. Plants were kept either non-inoculated (None), or inoculated with only *Rhizophagus irregularis* (AM), or with only *Ensifer meliloti* (Rhizobial) or both symbionts (Dual). The values are given as mean±se of six biological replicates otherwise mentioned. The lower case behind the values are from One Way ANOVA comparing dataset within the nutrient treatment (row).

	Nutrient regime	None	AM	Rhizobial	Dual
Shoot P (mg/g dry sample)	LPLN	4.776±0.176 b	5.880±0.167 a	3.544±0.153 c	6.091±0.205 a
	LPHN	2.726±0.076 d	3.306±0.081 c	3.756±0.180 b	5.009±0.173 a
	HPLN	6.404±0.120 b	7.422±0.105 a	5.209±0.191 c	6.786±0.278 b
	HPHN	3.367±0.069 c	3.689±0.115 c	4.723±0.203 b	6.177±0.176 a
Root P (mg/g dry sample)	LPLN	3.566±0.276 b	10.804±0.728 a	2.273±0.184 c	3.525±0.119 b
	LPHN	2.163±0.202 c	4.359±0.290 a	2.314±0.332 bc	3.042±0.097 b
	HPLN	4.525±0.269 b	9.811±0.491 a	2.768±0.102 c	3.531±0.166 c
	HPHN	2.829±0.188 c	5.344±0.255 a	2.398±0.172 c	3.520±0.102 b
Shoot N (mg/g dry sample)	LPLN	9.196±0.174 c	10.142±0.186 c	19.237±0.645 b	23.548±0.582 a
	LPHN	14.00±0.605 b	13.484±0.440 b	23.107±0.847 a	23.545±0.862 a
	HPLN	8.841±0.0901 b	10.664±0.166 b	23.095±1.378 a	24.556±0.853 a
	HPHN	12.712±0.284 c	14.415±0.519 b	21.711±0.627 a	23.060±0.307 a
Root N (mg/g dry sample)	LPLN	15.461±0.415 d	18.378±0.330 c	23.985±0.762 b	27.088±0.574 a
	LPHN	15.900±0.410 a	17.193±0.193 c	20.022±1.516 b	25.177±0.251 a
	HPLN	15.009±0.122 d	16.899±0.190 c	23.851±0.589 b	25.803±0.496 a
	HPHN	16.23±0.191 c	17.487±0.206 b	24.192±0.372 b	25.697±0.0461 a
Root C (mg/g dry sample)	LPLN	394.49±3.734 a	412.29±1.166 a	399.35±14.092 a	410.51±10.724 a
	LPHN	394.06±8.243 b	420.32±3.794 a	384.35±4.388 b	409.92±2.937 a
	HPLN	400.91±2.819 a	413.33±2.364 a	407.67±6.756 a	412.11±5.610 a
	HPHN	406.35±2.946 a	409.90±2.223 a	414.54±4.177 a	405.13±4.572 a

Table S3.3. One Way and Two Ways ANOVA of plant attributes of *Medicago truncatula* grown for nine weeks in 500 mL of soil substrate in the growth chamber. Plants were kept either non-inoculated (None), or inoculated with only *Rhizophagus irregularis* (AM), or with only *Ensifer meliloti* (R) alone or both symbionts (Dual). The values are given as mean \pm se of six biological replicates otherwise mentioned.

	Symbionts		Nutrient		Symbionts*Nutrient	
	F value	p value	F value	p value	F value	p value
Shoot biomass (g)	F(3,94)= 12.25	p < 0.0001	F(3,94)=35.66	p < 0.0001	F(9,94)= 30.58	p < 0.0001
Root biomass (g)	F(3,94)= 5.90	p = 0.0010	F(3,94)= 59.13	p < 0.0001	F(9,94)= 11.25	p < 0.0001
Shoot p (mg/g)	F(3,94)= 9.93	p < 0.0001	F(3,94)= 29.52	p < 0.0001	F(9,94)= 26.82	p < 0.0001
Root p (mg/g)	F(3,94)= 47.04	p < 0.0001	F(3,94)= 4.65	p = 0.0045	F(9,94)= 25.30	p < 0.0001
Tot. shoot p content (mg)	F(3,94)= 87.40	p < 0.0001	F(3,94)= 6.71	p = 0.0004	F(9,94)= 5.26	p < 0.0001
Tot. root p content (mg)	F(3,94)= 29.30	p < 0.0001	F(3,94)= 12.35	p < 0.0001	F(9,94)= 2.27	p = 0.0254
Shoot N (mg/g)	F(3,93)= 197.06	p < 0.0001	F(3,93)= 1.03	p = 0.3812	F(9,93)= 6.25	p < 0.0001
Root N (mg/g)	F(3,91)= 228.85	p < 0.0001	F(3,91)= 0.78	p = 0.5107	F(9,91)= 3.74	p = 0.0006
Tot. shoot N content (mg)	F(3,93)= 65.97	p < 0.0001	F(3,93)= 8.28	p = 0.0001	F(9,93)= 6.55	p = 0.000
Tot. root N content (mg)	F(3,91)= 33.47	p < 0.0001	F(3,91)= 16.03	p < 0.0001	F(9,91)= 3.84	p = 0.0005
AM root colonization (%)	F(1,47)= 1.92	p = 0.1729	F(3,47)= 2.86	p = 0.0477	F(3,47)= 2.88	p = 0.0478
Nod dry wt. (mg/g of aliq. dry root)	F(1,46)= 0.92	p = 0.3425	F(3,46)= 8.86	p = 0.0001	F(3,46)= 0.21	p = 0.8894

CHAPTER 4: APPLICATIONS OF ARBUSCULAR MYCORRHIZAL FUNGAL
ADDITIVES INCREASE PLANT BIOMASS AND SEED YIELD OF SOYBEANS
UNDER GREENHOUSE AND FIELD CONDITIONS

This chapter is formatted and in preparation for submission to journal “*Agronomy*”.

4.1 Abstract

Soybeans form tripartite interactions and simultaneously interact with both arbuscular mycorrhizal (AM) fungi and rhizobia bacteria to maximize their nutritional benefits. Several studies have been conducted to observe effects AM fungi and rhizobia bacteria mostly in greenhouse but sporadically in field conditions. However, AM fungi produced in the lab are practically not sufficient in amount to apply and test in the field trials. Here, we examined effects of commercially produced AM additives on different soybean cultivars for plant growth response, nutrient uptake, and seed yield both in greenhouse and field conditions. In both greenhouse and field experiments, commercial AM inoculum, “MycoApply”, performed better in plant growth response, phosphate (P) acquisition, and seed yield. Commercial AM inoculum had high variability in root colonization among experiments in the greenhouse. For field experiment, application of MycoApply had positive plant growth response and seed yield than other commercial inocula, “Nature Solution Mycorrhizae” and “Bio-Organics.” Plants treated with MycoApply under low nutrient conditions, produced similar seed yield to that of plants not treated with MycoApply at high nutrient supply conditions. Soybean cultivar Channel1405 demonstrated better responsiveness for the plant growth and seed yield than cultivars

AG1636 and AG1733. Taken together, AM fungal additives are beneficial for the plant growth and seed yield of soybean in low nutrient conditions which have higher implications for farmers prospective.

4.2 Introduction

Soybean (*Glycine max*) is an important legume crop as it is a major source of protein and oil for daily human consumption. Consequently, the global demand of soybeans is increasing rapidly (Krishnan and Nelson, 2011; Vance, 2001). For example, the U.S. alone increased the planted acres on which soybeans are grown from 72 million acres to 90 million acres which represents a 25% increase between 1998 and 2017 (USDA NASS, 2018). Out of the 348 million tons of soybeans that are produced worldwide, the U.S. produced 116 million tons of soybean seeds in 2017 or 33.3% of the world production (USDA NASS, 2018). Of the total seed production, nearly 47% is exported to other countries, and here mainly to China which has contributed more than \$16 billion to the national economy in 2016 (USDA NASS, 2016). Therefore, U.S.A is the largest producer and exporter of soybeans in the world, demonstrated the significance of soybeans for the U.S. economy. To use high yielding soybean cultivars, and to apply more chemical fertilizers, and pesticides are the general management practices to increase soybean seed production (Tilman et al., 2002; Tylka and Mullaney, 2016; Wang et al., 2010). However, conventional tillage, fertilizer and pesticide usage lead to unintended ecological and human health consequences (Damalas and Eleftherohorinos, 2011; Jiao et al., 2012; Kafle, 2013). Therefore, researchers are working on the development of appropriate biofertilizers that

can enhance crop production under low input conditions and improve the environmental sustainability of crop production.

The majority of land plants including soybeans forms symbiotic interactions with arbuscular mycorrhizal (AM) fungi (Wang and Qiu, 2006). The host plant gets nutritional benefits mainly phosphate (P), nitrogen (N), and potassium from the AM symbiosis (Bücking and Kafle, 2015; Bücking and Shachar-Hill, 2005; Garcia and Ané, 2017; Kafle et al., 2018; Smith and Smith, 2011). Besides the nutritional benefits, many studies have demonstrated that AM plants have a higher tolerance against drought, and pathogens (Porcel et al., 2006; Song et al., 2010). In return of these benefits, the host plant allocates photosynthetic carbon to AM fungi in a reciprocal system (Fellbaum et al., 2014; Kiers et al., 2011). Once the fungus has established the successful symbiosis with host plant, the fungus extends its hyphae beyond the root depletion zone to search for soil nutrients and transfers these nutrients to the host. Inside the host root, AM fungi form special structures called arbuscules which are known to serve as the exchange sites for resources between both symbiotic partners. The plasma membrane of host cortical cells surrounds the arbuscules to increase the efficiency with which nutrients can be taken up that are released by the AM fungus. Functionally active arbuscules induce the host root plasma membrane to specifically upregulate P and N transporters that can take up the nutrients released into the mycorrhizal interface (Breuillin-Sessoms et al., 2015; Gomez et al., 2009). The nutritional benefit of AM fungi is more pronounced under nutrient limitation, which favors the development of larger arbuscules (Breuillin-Sessoms et al., 2015). Thus, the benefit of AM fungi is highly important in our agriculture to increase the production in a sustainable way.

The majority of legume plants including soybeans form also a symbiotic interaction with atmospheric nitrogen (N_2) fixing bacteria generally called diazotrophs or rhizobia. As in the AM symbiosis, the host plant gets N from rhizobia and the bacteria can contribute between 65% to 95% to the host N demand (Bolger et al., 1995; Herridge et al., 2008). In return, the host provides photosynthetic organic carbon to the bacteria inside the nodule (Kafle et al., 2018; Paul and Kucey, 1981). A recent study has demonstrated that a legume plant with higher photosynthetic rate had a higher nodulation and nitrogenase activity suggesting that the host shared more carbon to functional bacteria which consequently resulted in more plant biomass (Gebriel et al., 2015). Root nodules offer a conducive environment for rhizobia for N_2 fixation. The mechanisms of rhizobial symbiosis with legume plants are well reviewed in previous literature (Lodwig and Poole, 2003; Oldroyd et al., 2011). Bacteroids inside the nodule possess a unique nitrogenase enzyme complex that acts as a template to reduce N_2 into plant assimilable N form mainly NH_3 which later changes into NH_4^+ and get converted into amino acids in host cytosol (Udvardi and Poole, 2013). Bacteroids need large amounts of P to generate energy in the form of ATP to function the enzyme complex for N_2 fixation. Because of this P demand, nodules are sink organs for P in legume plants (Kafle et al., 2018; Sulieman et al., 2013). The dependency of host plants on rhizobia is high under low N availability, indicating that the incorporation of rhizobia bacteria in legume crops can replace N fertilizer applications in crop fields (Heath et al., 2010).

In natural environments, legume plants are simultaneously colonized and interact with AM fungi and rhizobia and form tripartite interactions (Ossler et al., 2015). The host plant maximizes its nutritional benefits during tripartite interactions since AM fungi and

rhizobia are complimentary in providing P and N (Püschel et al., 2017). As mentioned above, bacteria inside the nodules have a high P demand which is effectively provided by AM fungi. Therefore, bacteria are stimulated in their N₂ fixing efficiency when plants are colonized by AM fungi and consequently have higher plant growth (Püschel et al., 2017). In addition to their contribution to the P supply, AM fungi also provide zinc, iron, manganese, and molybdenum to the host plant (Antunes et al., 2006; Chen et al., 2003; Ibiang et al., 2017). Some of these minor but vital elements are essential for the activity of the nitrogenase enzyme in fixing N₂. Therefore, tripartite interaction can synergistically improve crop yield in natural environments (Larimer et al., 2014; Ossler et al., 2015). For example, higher plant productivity and yield were observed along with higher P and N delivery to the host in tripartite interactions under limited soil P and N (Meghvansi et al., 2008; Pellegrino et al., 2012; Wang et al., 2011). Higher AM fungal colonization and nodulation were reported in tripartite interactions than in plants that were only inoculated with one symbiont (Wang et al., 2011). However, antagonistic effects to either one of the partners are also known when plants are colonized by both root symbionts. Synergistic and antagonistic effects depend on the environmental context (Larimer et al., 2014), functional niche of AM fungi and rhizobial bacteria (Bournaud et al., 2017), and the compatibility between symbiotic partners (Meghvansi et al., 2008; Mensah et al., 2015). For instance, the rhizobial strain STM 7183 is more compatible than STM 7282 with the AM fungus *Rhizophagus clarus* for nitrogenase activity, nodulation, and host plant growth response (Bournaud et al., 2017). Similarly, *Bradyrhizobium japonicum* is more compatible with the AM fungus *Rhizophagus irregularis* than with *Acaulospora tuberculata* or *Gigaspora gigantea*, resulting in better soybean productivity and seed yield (Meghvansi et al., 2008).

In most studies, tripartite interactions improve plant productivity, nutrient acquisition, and seed yield (Bournaud et al., 2017; Jia et al., 2004; Kaschuk et al., 2010; Larimer et al., 2014; Mortimer et al., 2008; Wang et al., 2011). Thus, symbiotic benefits of both AM fungi and rhizobia bacteria have a big impact on seed yield of legume crops and enhance productivity with minimum inputs of agrochemicals.

To meet the growing demand of soybeans, researchers have been paying considerable attention to exploring possible ways to increase soybean production in low input agriculture systems. AM fungi offer multiple benefits to the host plant and are therefore also called bio-fertilizers or bio-enhancers. Promoting the effectiveness of AM fungal strains by deliberate applications into the field could be an option to increase the seed production (Pellegrino et al., 2012). Due to technical difficulties, it is difficult to maintain and reproduce AM fungal inoculum continuously by farmers on a large scale for field applications. To fill this gap, many companies have started to produce commercial AM inoculum in recent years (Corkidi et al., 2004; Faye et al., 2013; Niwa et al., 2018). These companies use inert carrier material to protect and maintain the viability of AM fungal propagules in the commercial products before application in the field. Commercial AM inocula have demonstrated a high variation in plant growth responses and grain yields in a variety of crops (Corkidi et al., 2004; Eulenstein et al., 2016; Niwa et al., 2018). However, results with ineffective commercial products have also been published (Berruti et al., 2013; Corkidi et al., 2004; Eulenstein et al., 2016; Faye et al., 2013). Farmers are aware of rhizobia bacteria as seed companies provide frequently information about the importance of bacteria particularly on soybeans. However, many farmers are not familiar with the importance of AM fungi. Commercial AM products have been tested mostly on

corn, and wheat (Corkidi et al., 2004; Eulenstein et al., 2016; Pellegrino et al., 2012), but only very recently on soybean (Niwa et al., 2018). Many countries and the European Union, Australia, Japan, Brazil, and India promote using AM fungal and rhizobia bacteria for agronomic practices by providing tax exemptions. Additionally, some countries have regulatory branches to maintain the quality of AM inoculum (Owen et al., 2015). Frequent tillage, continuous fertilizers use, and practices of mono-cropping systems reduce AM fungal communities and also reduce their functional benefits to host crops (Chagnon et al., 2013; Oehl et al., 2010; Verbruggen et al., 2010). Most of the previous research questions were based on symbiotic effects of only AM fungi (Kobae et al., 2010; Wang et al., 2016) or only rhizobia bacteria (Damodaran et al., 2017; Manavalan et al., 2009), but fewer with both (dual) symbionts on soybean (Bulgarelli et al., 2017; Wang et al., 2016). Moreover, conclusions are drawn from either greenhouse or field experiments but rarely with both greenhouse and field experiments. Soybean is the one of the top cash crops in the state of South Dakota in the U.S.A, contributing 6% yield nationally equivalent to \$2.3 billion (USDA-NASS 2016). Here, we evaluated the effects of commercial AM additives on plant growth response, nutrient acquisition, and seed yield of soybean in greenhouse and field experiments.

4.3 Material and methods

4.3.1. Greenhouse experiment

4.3.1.1. Plant, fungal, and rhizobial materials

We performed three independent experiments. In all experiments, soybean seed cultivars were surface sterilized with 8% bleach (sodium hypochlorite for two minutes with

constant shaking and rinsed several times with autoclaved distilled water. Seeds were then subsequently treated with 70% ethanol for 2 minutes and rinsed several times. Seeds were soaked in autoclaved distilled water overnight before sowing into pots. Five seeds were sown in each pot containing an autoclaved (121°C for 2 hours) growth substrate mixture of sand: perlite+ vermiculite: organic soil in the ratio of 5: 2:2: 1: (v: v: v: v) respectively.

The soil substrate contained plant available Olsen phosphate (9.2 mg/kg), nitrate (5.2 mg/kg), ammonium (0.04 mg/kg), pH (8.97) in experiment I and phosphate (12.84 mg/kg), nitrate (27.35 mg/kg), ammonium (6.35 mg/kg), and pH (8.42) in experiment II and III (AgLab Express, Sioux Falls, South Dakota, USA). Before sowing the seeds, ~2 g of a commercial AM fungal soil additive (MycoApply- Mycorrhizal Applications, Grants Pass, Oregon, USA) was added and mixed well five centimeters below the top surface and covered with growth substrate (see below). The products contain the four different AM fungal species *Rhizophagus intraradices*, *Glomus mosseae*, *G. aggregatum*, and *G. etunicatum* each with 70 propagules/g. Seeds were put one centimeter below the soil substrate and covered with steam autoclaved perlite to minimize seed drying. Seedlings were thinned and kept to two per pot after one week after seed germination and treated with rhizobia (*Bradyrhizobium japonicum* USD 110). The bacterial strain was cultured in Vincent reagent in a shaker (RPM) at 28°C for 3 days, centrifuged at 3500 rpm for 10 minutes, and resuspended the pellets with MgSO₄·7H₂O (0.125 mM). Ten mL of the bacterial suspension with an optical density of 0.08 was applied at the base of the seedling stem to the respective pots.

4.3.1.2 Experimental design and growth conditions

We conducted three experiments to assess the effects of the commercial AM fungal additive MycoApply on different soybean cultivars under different nutrient regimes and with (*B. japonicum*, USDA 110) or without rhizobia. In experiment I, we chose the soybean cultivar AG1234, as one of the mostly used cultivars for soybean seed production in this region of South Dakota. We kept the soybean plants either as non-inoculated controls (None), or inoculated the plants with only Mycoapply (AM), or only rhizobia (R), or with both MycoApply and rhizobia. There were in total 80 pots and thus 20 pots were allocated to each symbiotic treatment. We supplied basic nutrient solution (0.06 mM as NH_4NO_3 and 0.01 mM as KH_2PO_4) to all plants in the first and second week after seed germination along with distilled water (Ingstad, 1960). We treated the plants with either low (L) and or high (H) nitrogen (N) and phosphate (P) as NH_4NO_3 and KH_2PO_4 in the third, fourth, and fifth week and had the following treatments: LPLN, LPHN, HPLN, HPHN using a modified Ingstad (1960) nutrient solution. The concentration of nutrients that were added to the soil was: LP (0.025 mM), HP (0.125 mM), LN (0.25 mM), and HN (1 mM) along with KCl (0.077 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.125 mM), Fe-EDTA (0.0017 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.078 mM), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.378 μM), H_3BO_3 (0.202 μM), Zn-EDTA (0.012 μM), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.0036 μM). The final nutrient concentration in the soil were 0.2 mM P (LP) or 0.5mM P (HP) as KH_2PO_4 , and 1mM or N (LN) or 3.2 mM (HN) as NH_4NO_3 . There were five independent biological replicates per nutrient treatment in each symbiotic treatment. Plants were randomized three times during the experimental period and watered with distilled water regularly until harvested in the seventh week. The teemerature in the greenhouse ranged from 16°C to 18°C and 23°C to 26°C during night and day, respectively.

In experiment II, we examined plant growth response and seed yield of soybean cultivar AG1234 under tripartite interaction at different nutrient regimes. Unlike in experiment I, all plants were inoculated with rhizobia bacteria and half the plants were inoculated with both rhizobia and AM fungi (MycoApply). We provided nutrients in the fifth, seventh, and ninth week as low (L), medium (M), and high (H): LPLN, LPMN, MPLN, MPMN, HPLN, HPMN, MPHN, HPHN. We did not provide any nutrients to the LP or LN treatments because the soil substrate had already basic concentration of phosphate and N (nitrate and ammonium). The concentration of nutrients added to the soil each time was: MP (0.06 mM), HP (0.12 mM), MN (0.66 mM), and HN (1.33 mM). Therefore, final nutrient concentration in the soil were as: LP (0.14 mM), MP (0.25 mM), HP (0.5 mM), LN (0.104 mM), MN (2 mM), HN (4 mM). Plants were harvested after 15 weeks and evaluated for their growth response, P content, mycorrhization, nodulation, and seed yield.

In experiment III, we tested three different soybean cultivars (Channel1405, AG1636, and AG1733) for their growth response using the same symbionts as described before. The experimental plants were grown in the same environment as in experiment I. The experimental design was the same as in experiment I except that here only two nutrient regimes were used, LPLN or HPHN. We provided nutrients in the fifth and sixth week and harvested the plants in the eighth week. Since the soil substrate had already 12.8 mg/L of plant available phosphate, 27 mg/kg of nitrate, and 6.35 mg/kg of ammonium, we did not provide any nutrients to half of the pot systems (48 pots) to be treated as LPLN. The concentration of nutrients that was added each time to the soil was: HP (0.20 mM), and HN (2 mM). Therefore, the final nutrient concentrations in the soil were: LP (0.13 mM),

HP (0.5 mM), LN (0.8 mM), and HN (4 mM). We had three cultivars, four symbiotic factors, two nutrient regimes, and four biological replicates thus totalling 96 plant systems. All plants were on a single bench, watered as needed with distilled water, and randomized three times during the experimental period.

4.3.2 Field experiment

4.3.2.1 Field location, nutrient conditions, plant, and fungal materials

All three field experiments were conducted at the Aurora Research Field Station, South Dakota State University. The field soil contained plant available phosphate (P) 12.4 mg/kg, ammonium 1.5 mg/kg, nitrate 10.3 mg/kg, and pH 5.64. We planted soybean seeds at the rate of 1,60,000 seeds per acre. Soybean seeds were provided by the South Dakota State University, Agriculture Experiment Station office. For the first two experiments, we used soybean cultivar AG1234, while for the third experiment we used Channel1405, AG1636, and AG1733. The commercially available mycorrhizal inoculum, MycoApply was used for experiment I, and experiment III. To examine the effect of different commercial AM products on plant performance, we used MycoApply, Nature Solution Mycorrhizae (NSM) (Nature's Solutions, 2330 Bird St, Oroville, CA 95965), and Bio-Organics in experiment II. According to the recipe MycoApply had four different AM fungal species: *Rhizophagus intraradices*, *Glomus mosseae*, *G. aggregatum*, and *G. etunicatum* each with 70 propagules/g. Similarly, there were five different AM fungal strains in NSM *Glomus intraradices*, *G. mosseae*, *G. claroideum*, *G. coronatum*, and *G. microaggregatum* each with 180 propagules/g and nine different AM fungi in Bio-Organics (Bio-Organics, 2799 Creamery Rd, New Hope, PA 18938) as *Glomus intraradices*, *G.*

mosseae, *G. aggregatum*, *G. etunicatum* each with 10 spores/cc and *G. clarus*, *G. deserticola*, *G. monosporus*, *Gigaspora margarita*, and *Paraglomus brasilianum* each with 2 spores/cc. We used standard agronomic practices of 3 meters (10 feet) width with four rows for seeding.

4.3.2.2 Experimental Design

For field experiment I, we had three treatments: control, fungicide, and MycoApply each with four replicates of an area of 18m² (6 m x 3 m by length and width). For the AM inoculation, we mixed MycoApply with water and applied by spraying manually close to the base of the stem at a rate of 1.9 g/m² (525 propagules/m²) four weeks after seed plantation. To suppress existing natural AM fungal community, we sprayed the fungicide (TopsinM) at a rate of 1.25 g/m² every two weeks until seed maturity. We harvested four plants from the inner two rows for biomass, mycorrhizal colonization, and nodulation, and phosphate analyses after 14 weeks. We harvested seeds from the inner two rows using a harvester machine after complete seed maturity in the 17th week after planting.

In field experiment II, we examined the effects of three commercial mycorrhizal inocula on the soybean plant growth response and seed yield of cultivar AG1234. Field soil condition, seeding rate, width of rows, fungicide and mycorrhizal inoculum application method and rate were as in experiment I. We had five replicates for control, fungicide, and for each fungal inoculum, and each plot was 9 m² (3 m x 3 m by length and width). We applied the commercial inocula: MycoApply, Nature Solution Mycorrhizae (NSM), and Bio-Organics in the respective plot two and four weeks after plantation at 525 propagules/m² for each inocula. As in experiment I, we applied the fungicide Topsin M

every two weeks to suppress naturally present AM fungal communities in the soil day after seed plantation until plant maturity at a rate of 1.25 g/m². We harvested four plants from the inner two rows to assess nodulation, mycorrhization, and phosphate content in the shoot and root tissues after 16 weeks. We harvested seeds after complete maturity from the inner two rows after 19 weeks using plant harvester machine.

Similarly, we had nutrient treatment plots each with a 2 m length and 3 m width. We had control, fungicide, and MycoApply treated plots with four different nutrient supply conditions: 100%, 50%, 25%, and 0% of phosphate in the form of KH₂PO₄ (P) and nitrogen (N) as urea. The recommended fertilizer dose rate for soybeans in South Dakota is 18.14 kg of P/Acre and 7.71 kg of N/Acre according to the USDA. We used this rate as 100% and reduced proportionally to get 50% and 25% 5 weeks after plantation. We applied only water for 0% treatment. We had four replicates per nutrient regime totalling 48 plots. We harvested seeds after 19 weeks to examine the effects of MycoApply on seed yield at different nutrient regimes.

In field experiment III, we examined the effects of MycoApply on three different soybean cultivars: Channel1405, AG1636, AG1733 at three different nutrient regimes: 100%, 50%, and 0% of P and N as described before. We had three soybean cultivars, three nutrient regimes, two symbionts (MycoApply and control), and four replicates thus making 72 plots, each having 3 m length and 3 m width. As before, we mixed mycorrhizal inoculum with water and sprayed at the base two weeks after plantation and applied nutrients at 100%, 50%, and 0% four weeks after plantation. We harvested four plants from each inner two rows for their growth response, mycorrhization, nodulation, and P content in the shoot

in 9 week old plants. We harvested seeds after 19 weeks to examine the effect of MycoApply on seed yield of the different soybean cultivars under different nutrient regimes.

At harvest, shoots were kept in paper bags for drying at 70°C for 48 hours. Similarly, roots were washed to remove soil substrate particles with running tap water, blotted with paper towel, and the fresh weight was recorded. Aliquots of the root were removed and stored in 15 mL tubes with 50% ethanol for the mycorrhizal colonization assay from the respective roots. Similarly, nodulated roots were kept in cold room (4°C) before separating and counting nodules as described above.

4.3.3 Rhizobial nodulation and AM colonization assay

One day after harvesting, all visible nodules from the roots were removed, counted, and measured for their dry weight after drying in an oven at 70°C at 48 hours. To determine the AM colonization, the preserved roots were rinsed with tap water to remove alcohol, water bathed with 10% KOH solution at 80°C for 30 minutes, rinsed several times with tap water, and stained with 5% ink at 80°C for 15 minutes (Vierheilig et al., 1998). We analysed a minimum of 150 root segments to determine the percentage of AM root colonization by using the gridline intersection method (McGonigle et al., 1990).

4.3.4 Quantification of P

Dry shoot and root tissues were pulverized with a tissue homogenizer (Precellys 24, Cayman Chemical Company, Ann Arbor, MI, USA). We digested an aliquot tissue with 2N HCl for 2 h at 95°C and determined the P content spectrophotometrically at 436

nm after adding ammonium molybdate vanadate solution (Fisher Scientific, Pittsburgh, USA) as described (Wang et al., 2016). Plant P content was calculated by considering the plant tissue biomass.

4.3.5 Data analysis

Plant growth response, mycorrhizal colonization, rhizobial nodulation, seed yield, phosphate acquisition in shoot, root and nodule on different soybean cultivars were analyzed using Statistix 9 analytical software (Tallahassee, Florida, USA). Mean values were calculated using Microsoft Excel 2016 (Microsoft Company, USA). The mean differences of plant traits were compared using ANOVA and if found significance at $P \leq 0.05$, a LSD multiple pairwise comparison was performed.

4.4 Results and discussion

4.4.1 Greenhouse experiment

4.4.1.1 Effect of mycorrhizal inoculum on plant biomass, phosphate content, and seed yield

In greenhouse experiments, plants treated with the commercial mycorrhizal inoculum MycoApply (here after- AM fungi) had higher shoot biomass than non-inoculated plants. In experiment I, soybean plants inoculated with only AM fungi demonstrated a significantly higher ($P \leq 0.05$) shoot growth in comparison to non-inoculated plants particularly when supplied with high nitrogen (N) supplied (Figure 4.1A). For the root growth; only AM inoculated plants had higher root biomass while plants inoculated with others had similar growth response (Figure 4.1B). In experiment II, plants

inoculated with dual symbionts had significantly ($P \leq 0.05$) higher shoot and root biomass under limiting nutrient (LPLN) supply conditions (Figure 4.2A and B). However, under high nutrient supply conditions (HPHN), the shoot and root biomass were similar between the plants that were only inoculated with rhizobia and dual inoculated plants.

Mycorrhizal symbiosis increased plant P uptake in both experiments I and II compared to non-AM plants. In experiment I, plants inoculated with only AM fungi had significantly ($P \leq 0.05$) higher shoot P contents than non-inoculated plants except at LPLN (Figure 4.1C). A similar pattern was observed in the dual inoculated plants compared to the plants that were only inoculated with rhizobia except at LPLN conditions. The difference in the root P contents between AM and non-inoculated plants was not as pronounced as in the shoot tissues (Figure 4.1D). We observed a similar pattern in the P concentration in shoot and root tissues (Figure S4.1). Plants that were inoculated with both (dual) symbionts had significantly ($P \leq 0.05$) and consistently higher P contents in the shoot and roots than plants that were inoculated with only rhizobial bacteria (Figure 4.2A and B). This higher P content in the plant tissue in dual inoculated plants clearly demonstrated the significance of AM fungi for rhizobia during tripartite interactions especially in limited N supply conditions.

We observed a high variation in the AM colonization between two experiments. The mean AM fungal root colonization was below 13% in experiment I, as high as 60% in experiment II (Figure 4.1E and Figure 4.2E). The AM fungal root colonization did not differ among the treatments in experiment I but differed among the nutrient treatments in experiment II where we observed low colonization in high nutrient supply conditions

(Figure 4.2 E). Nodule dry weight was significantly higher under low N supply conditions than under high N supply conditions in both experiments (Figure 4.1F and 4.2F). Nodule dry weight was higher in the dual inoculated plants in experiment II, while there was no difference in experiment I.

In experiment II, we examined effect of AM fungi on seed yield of soybean cultivar AG1234 in different nutrient supply conditions. Seed yield was significantly ($P \leq 0.05$) and consistently higher at dual inoculated plants than only rhizobial inoculation except at high N supply conditions (Figure 4.2G).

In our study, soybean plants inoculated with AM fungi had positive effects on shoot growth (Figure 4.1A and 4.2A). This beneficial effect of AM fungi for shoot growth could be explained by possible mechanism of AM fungi for P and N contribution to the host. It is long known that the AM symbiosis increases the plant P acquisition (Bücking and Shachar-Hill, 2005; Mensah et al., 2015; Wang et al., 2016b). Higher shoot P contents in AM inoculated plants under high N supply conditions (Figure 4.1C) could be the effects of cross talk between P and N as suggested by (Bonneau et al., 2013; Correa et al., 2015; Nouri et al., 2014). As more P is needed by plants to assimilate supplied N. Thus, this higher P content in the shoot could have been mediated by AM fungi. Since we removed all visible nodules for the nodulation assay from only rhizobial and dual inoculated roots, these roots (R and dual roots) had lower P contents at low N conditions than the non-nodulated roots i.e. only AM and non-inoculated roots (Figure 4.1D). Since the weight of nodules was higher under low N supply conditions (Figure 4.1F) and therefore likely to have more P, as nodules are P sink (Kafle et al., 2018; Sulieman et al., 2013). However, only rhizobia

and dual inoculated roots at high N supply conditions had similar P content (Figure 4.1D) as the weight of nodules was lower which is further explained below.

Besides P, contribution of AM fungi to N supply of the host has been reported (Bücking and Kafle, 2015; Courty et al., 2014; Tanaka and Yano, 2005; Vázquez et al., 2001). For example, Vázquez et al. (2001) discussed that the AM symbiosis is more effective than nodulated plants for delivering N to the host. Similarly, soybean plants with dual inoculation had significantly higher ($P \leq 0.05$) shoot biomass than plants that were only inoculated rhizobia except at low P and low N supply conditions (Figure 4.1B). Soybean plants form tripartite interactions with AM fungi and rhizobia (dual inoculation) in which host plants can maximize their nutritional benefits. During tripartite interactions, higher plant growth is observed due to a synergistic response of AM fungi and rhizobia bacteria for P and N delivery which has been demonstrated in previous reports (Afkhami and Stinchcombe, 2016; Kafle et al., 2018; Wang et al., 2011). In return of these nutritional benefits, the host plants provide photosynthetic carbon to both root symbionts to balance the mutualistic benefits. Interestingly, plants inoculated with only AM fungi had a higher shoot biomass than dual inoculated plants under high N supply conditions. Since, the host plants need to share photosynthetic carbon to both symbionts that could cause smaller shoot growth responses in dual inoculated plants than in AM only inoculated plants under high nitrogen supply conditions (LPHN and HPHN). In this case, N demand of the host might have been supplied via only AM fungi (Vázquez et al., 2001) and therefore host plants had to share their carbon substrates only with AM fungi.

In our experiments, we quantified total AM fungal structures inside and on the surface of roots after cleaning and staining (McGonigle et al., 1990; Vierheilig et al., 1998) but did not separately quantify arbuscules, vesicles, intraradical or extraradical hyphae. Arbuscules are the exchange sites of fungal mediated nutrients for plant carbon substrates and are thus linked to plant growth. However, AM fungal root colonization, in general, does not always correspond with plant growth (Corkidi et al., 2004). This is consistent with our findings since we observed higher plant growth responses regardless of the AM colonization (Figure 4.1A and B and Figure 4.8A). Earlier studies reported that the application of commercial AM fungal inocula had high variation in AM colonization and presence of other microbial contaminants (Berruti et al., 2013; Corkidi et al., 2004; Faye et al., 2013; Garmendia and Mangas, 2014; Tilak et al., 1995). For instances, Corkidi et al. (2004) and Faye et al. (2013) experienced the presence of other microbes that were not listed in the inoculum composition and other growth promoting contaminants. Nevertheless, benefits of commercial AM inocula have been reported so further work should focus on improving the quality and host specificity (Berruti et al., 2016).

High nodulation during the tripartite interactions under low N supplied in our study (Figure 4.2F) support previous findings (Kafle et al., 2018; Püschel et al., 2017; Wang et al., 2011). During the tripartite interactions under limited N supply conditions, AM fungi facilitate for P delivery to the host plant. To operate nitrogen fixing operation using nitrogenase enzyme, bacteria inside the nodule demand more P, therefore AM fungi facilitate for P delivery and consequently higher nodulation. Plants under N demand conditions allocate more carbon photosynthates to nodulated roots and thereby more nodulation and nitrogen fixation. However, plants not under N demand, reduce allocation

of C to nodulated roots thus lower nodulation and nitrogen fixation. Recent evidence demonstrated that phloem contains higher concentration of asparagine when supplied more N to the legume plants (Mortimer et al., 2012). This higher asparagine concentration is believed to participate in negative feedback signalling for nodulation at the root. Outcome of this mechanism is consistent with our results having lower nodulation in high soil N (Mortimer et al., 2012).

High seed yield during tripartite interactions (dual inoculated plants) in our study (Figure 4.2G) corroborated the studies of Cely et al. (2016); and Meghvansi et al. (2008) in which a higher seed production was found when soybean plants were inoculated with both AM fungi and R bacteria. Since, AM fungi and rhizobia bacteria are complementary for P and N delivery, consequently, the host plants are well fitted for the seed production (Kaschuk et al., 2010). Interestingly, plants inoculated with dual symbionts reached their maximum yield potential under low nutrient supply conditions, while plants that were inoculated by only rhizobia bacteria reached a similar yield only under high nutrient supply conditions. Instead of applying P fertilizer to the soybean field, alternative biological approach (AM fungal partner) is necessary to reach higher seed yields (Cely et al., 2016).

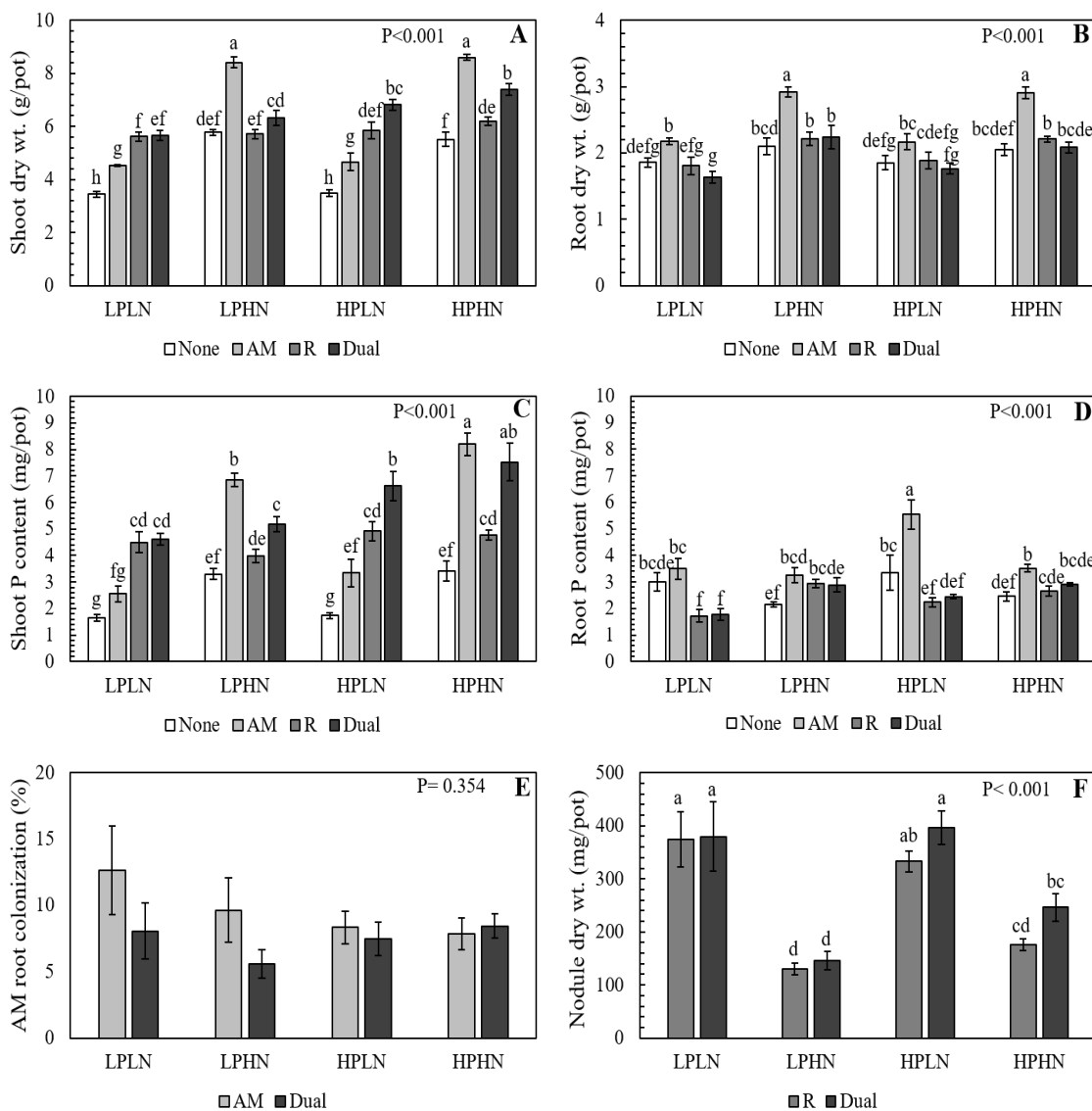


Figure 4.1. Shoot (A) and root (B) dry biomass, shoot (C) and root (D) phosphate content, AM root colonization (E) and nodule dry weight (F) of soybean cultivar AG1234 grown for eight weeks in two liters of soil substrate in the greenhouse. Plants were remained non-inoculated (None), or inoculated either with only MycoApply (AM), or only *Bradyrhizobium japonicum*, USDA 110 (R) or were dual inoculated (AM+R). The vertical bars represent mean of five biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

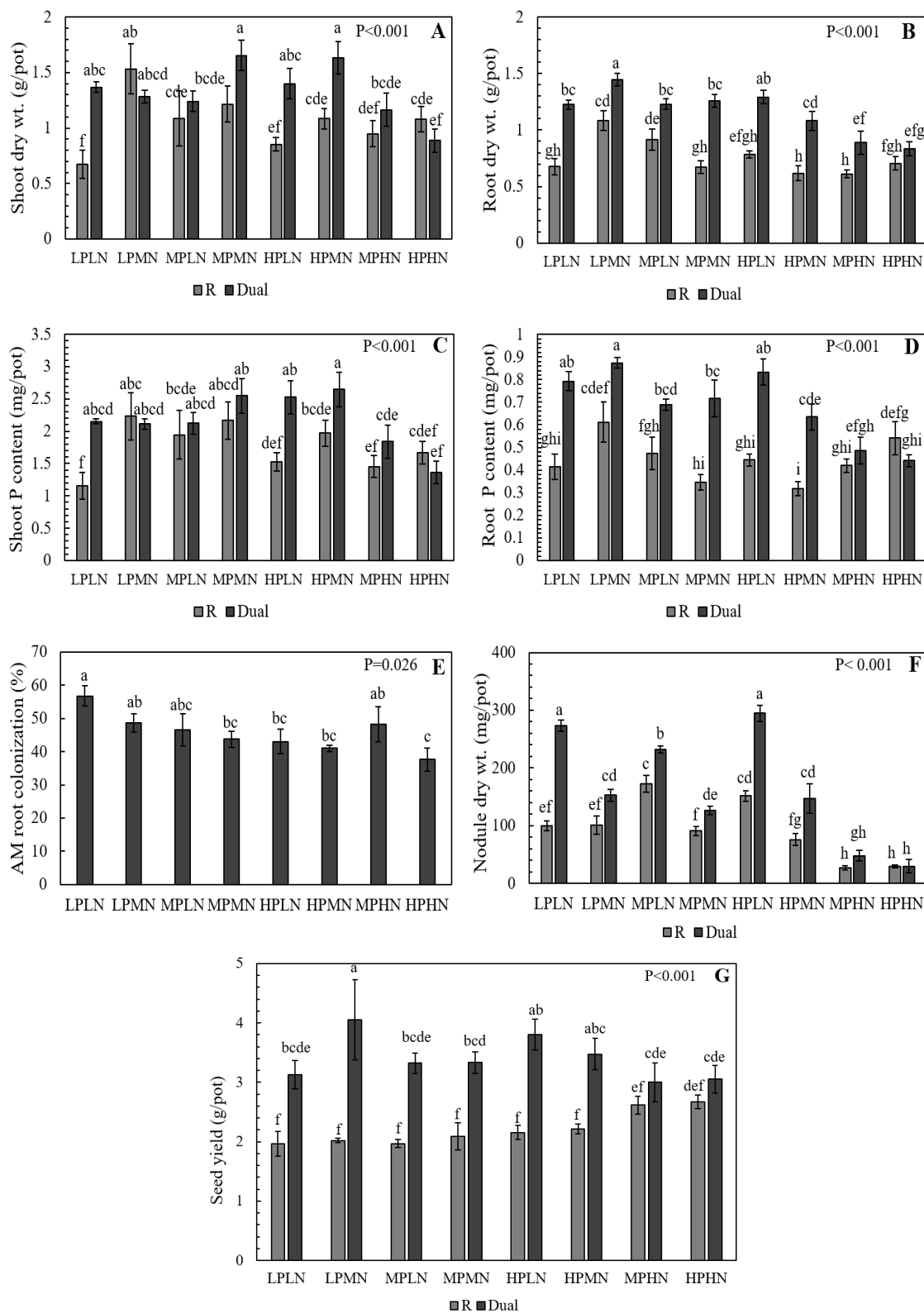


Figure 4.2. Shoot (A) and root (B) dry biomass, shoot (C) and root (D) phosphate content, AM root colonization (E) and nodule dry weight (F), and seed yield (G) of soybean cultivar

AG1234 grown for 15 weeks in two liters of soil substrate in the greenhouse. Plants were inoculated either with only *Bradyrhizobium japonicum*, USDA 110 (R) or were inoculated with both symbionts (Dual) (AM+R). The vertical bars represent the mean of five biological replicates with standard error of mean. Different letters on the bar indicate statistically significant differences at P values at ≤ 0.05 after ANOVA and LSD.

In experiment III, we examined three soybean cultivars for their growth response with AM fungi. All three soybean cultivars had higher shoot dry weight when plants were inoculated with AM fungi in compared to non-inoculated control under low P and low N supply conditions (Figure 4.3A). Plant growth was not statistically different when plants were inoculated with only rhizobial bacteria and both (dual) symbionts. However, plants that were inoculated with dual symbionts had higher shoot biomass than plants inoculated with only rhizobial bacteria. Soybean cultivars under HPHN supply conditions had statistically similar ($P= 0.92$) growth response among symbionts (None; AM; R; Dual) (Figure 4.3A). Root growth response among microsymbionts treatment were statistically similar at low P and low N ($P= 0.95$), under high P and high N ($P= 0.38$) supply (Figure 4.3B).

We tested difference in plant P acquisition efficiency among three soybean cultivars under two different nutrient supply conditions. We found that the cultivar Channel1405 was more efficient for P uptake than the other two cultivars: AG1636 and AG1733 at LPLN conditions (Figure 4.3A). Moreover, all three cultivars inoculated with only AM fungi had significantly higher P contents than their corresponding non-inoculated controls. However, dual inoculated plants had a similar P content among all cultivars. In comparison plants that were only inoculated with rhizobia, the dual inoculated cultivars Channel1405 and AG1733 had a higher shoot P content at LPLN. Under LPLN supply conditions, we

observed a similar P uptake response by these soybean cultivars under HPHN conditions between AM fungal and non-inoculated plants (Figure 4.3C). However, shoot P content between dual and only rhizobial plants was not different among the cultivars. Shoot and root P concentration did not differ within nutrient regimes among the different symbiotic treatments (Figure S4.3 A and B). Only AM fungi inoculated Channel 1405 had higher root P contents than its corresponding non-inoculated plants at LPLN ($P \leq 0.05$) (Figure 4.3C). Channel 1405 showed a higher P uptake than the other two cultivars under LPLN conditions (Figure 4.3C). Root P content was not statistically different ($P=0.136$) among cultivars and symbiotic partners under HPHN condition. However, there was a trend of higher P contents in the roots of dual inoculated plants. Contrary to the nodule P concentration (Figure S4.4), dual inoculated plants had higher P contents in their nodules than rhizobial inoculated plants at LPLN (Figure 4.3E).

The AM root colonization was statistically similar between only AM and dual inoculated plants under LPLN conditions, but the colonization was proportionally higher in the dual inoculated plants. However, the AM colonization reduced under HPHN conditions and was similar between only AM and dual inoculated (Figure 4.3F). The AM colonization had similar response among soybean cultivars.

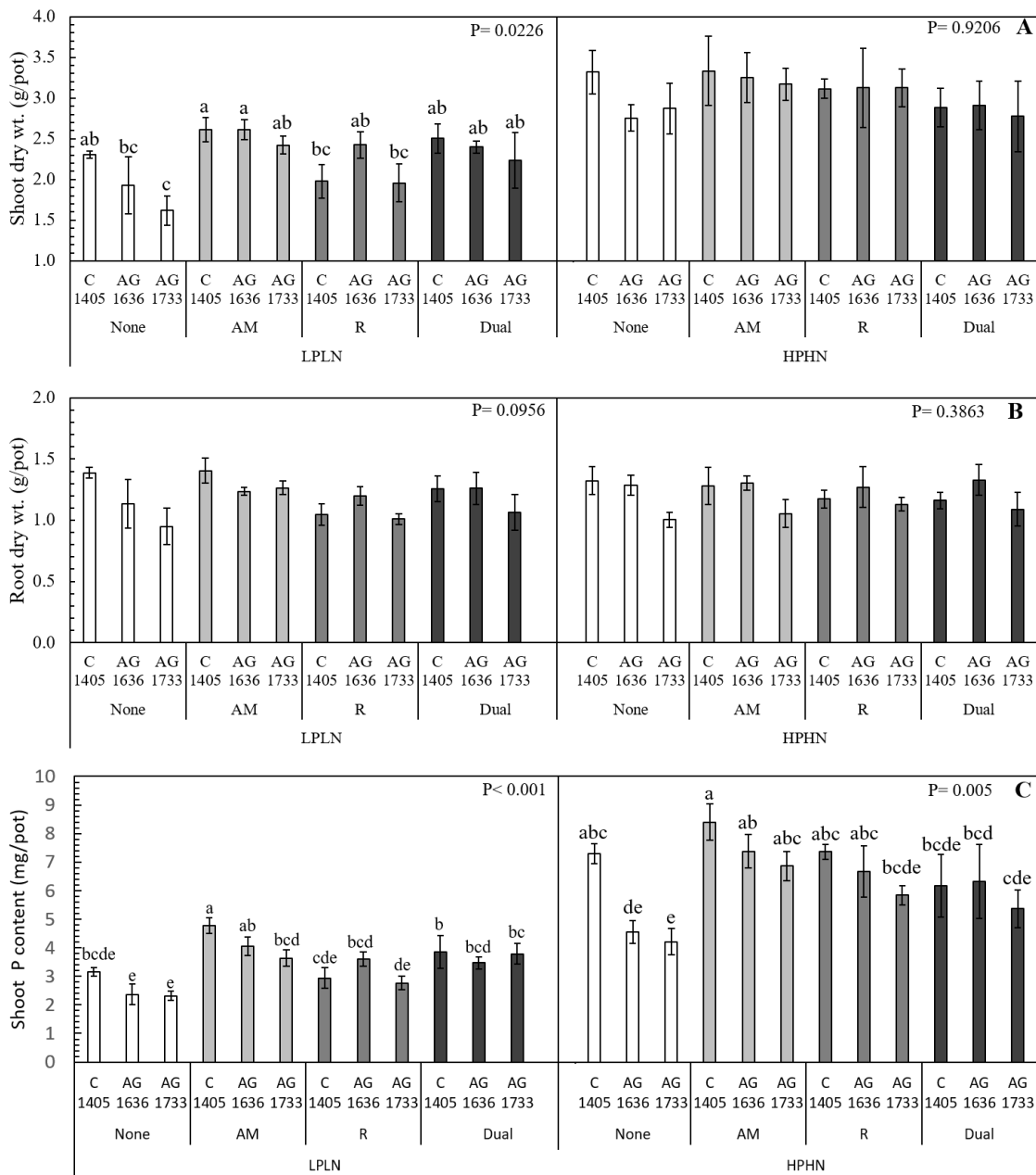
Nodule dry weight was significantly higher at low N supplied conditions but decreased significantly at high N supplied conditions (Figure 4.3G). Nodule dry weight was higher in the dual inoculated plants than those plants which were inoculated with only rhizobial bacteria under LPLN conditions.

Higher growth response of soybean inoculated with AM fungi in our experiment (Figure 4.3A) is congruent with Wang et al. (2011) where they presented higher soybean shoot biomass in mycorrhizal plants than in non-inoculated plants. Moreover, Wang et al. (2011) demonstrated that soybean plants inoculated with only AM fungi had higher shoot biomass than plants inoculated with only rhizobia under low nutrient supply conditions which is in support of our findings. When the different cultivars that were inoculated with AM fungi were compared, plants had similar shoot growth responses indicating the cultivars do not differ in plant growth when treated with AM fungi as observed in Wang et al. (2016b).

We examined and demonstrated that Channel1405 performed better than the other two cultivars especially under low P and low N supply conditions (Figure 4.3C and D). The degree of AM benefits depends on host plant type, cultivar, and environmental condition (Nemec and Datnoff, 1993). For example, Smith and Goodman (1999) reported that different alfalfa cultivars differed in their response to AM fungi, which supports our findings. Plant P acquisition of different soybean cultivars has been attributed to different root architectures (Wang et al., 2011). In this study, we observed higher P benefits in the shoot and root of dual inoculated plants compared to plants that were only inoculated with rhizobia at LPLN conditions (Figure 4.3C and D) confirming that tripartite interactions facilitate nutritional benefits to the host (Meghvansi et al., 2008). However, there was no difference in P uptake among cultivars in tripartite interactions (Figure 4.3C and D) which could be sink effects of nodulated rhizobial bacteria in the roots (Figure 4.3E).

The AM root colonization was similar when the plants were inoculated with only AM fungi and when the plants were inoculated with dual symbionts (Figure 4.3G). This similar rate of AM colonization fits with growth response where all cultivars had similar shoot biomass (Figure 4.3A). However, plants inoculated with AM fungi had higher shoot P content and consequently higher shoot growth response as observed by Wang et al. (2016b).

High nodulation during the tripartite interactions under low N supplied in our study (Figure 4.3G) support previous findings (Kafle et al., 2018; Püschel et al., 2017; Wang et al., 2011). During the tripartite interactions under limited N supply conditions, AM fungi facilitate for P delivery to the host plant. To operate nitrogen fixing operation using nitrogenase enzyme, bacteria inside the nodule demand more P, therefore AM fungi facilitate for P delivery and consequently higher nodulation. Plants under N demand conditions allocate more carbon photosynthates to nodulated roots and thereby more nodulation and nitrogen fixation. However, plants not under N demand reduce allocation of C to nodulated roots thus lower nodulation and nitrogen fixation. Recent evidence demonstrated that phloem contains higher concentration of asparagine when supplied more N to the legume plants (Mortimer et al., 2012). This higher asparagine concentration is believed to participate in negative feedback signaling for nodulation at the root. Outcome of this mechanism is consistent with our results having lower nodulation in high soil N (Mortimer et al., 2012).



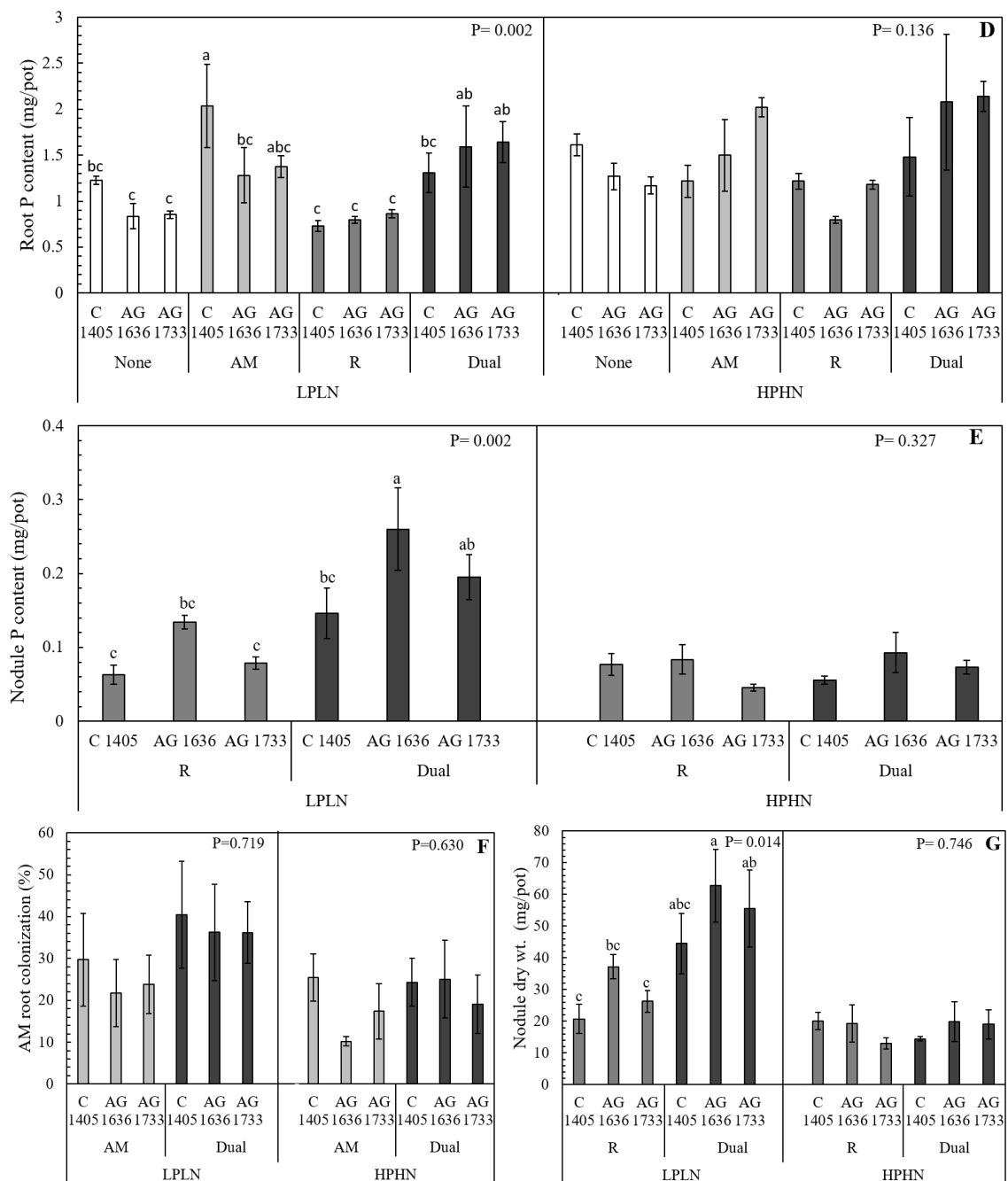
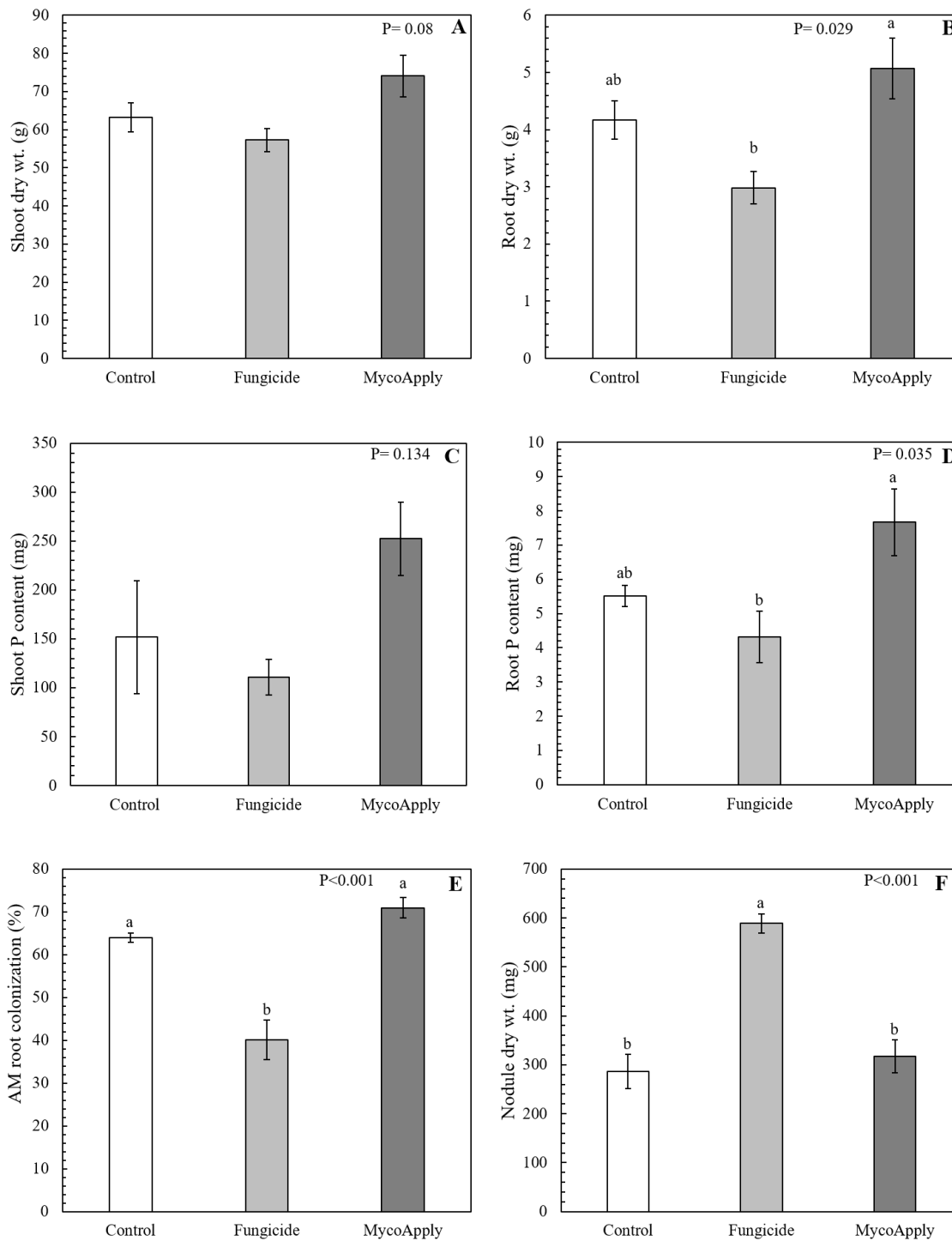


Figure 4.3. Shoot (A) and root (B) dry biomass, shoot (C) root (D), and nodule P content (E), AM root colonization (F) and nodule dry weight (G) of soybean cultivar AG1234 grown for 15 weeks in two liters of soil substrate in the greenhouse. Plants were inoculated either with only *Bradyrhizobium japonicum*, USDA 110 (R) or were inoculated with both symbionts (Dual) (AM+R). The vertical bars represent the mean of five biological replicates with standard error of mean. Different letters on the bar indicate statistically significant differences at P values at ≤ 0.05 after ANOVA and LSD.

4.4.2 *Field experiment*

4.4.2.1 Effect mycorrhizal inoculum on soybean plant biomass, phosphate content, and seed yield

We examined effects of commercial mycorrhizal additives for soybean plant growth and seed yield under field conditions. In field experiment I, we observed similar shoot ($P = 0.08$) dry weights but there were statistical significant differences in root dry weight among the treatments: control, fungicide, and MycoApply (Figure 4.4A and B). In field experiment II, we observed significantly higher ($P \leq 0.05$) shoot and root biomass when the plots were treated with MycoApply (Figure 4.5A and B). In field experiment III, we analyzed shoot and root growth of three soybean cultivars in each nutrient treatment and found no statistical difference among cultivars except at 50% of the recommended rate of N and P (Figure 4.6A) in which plants treated with MycoApply had significantly higher ($P \leq 0.05$) shoot dry weight. Soybean cultivar Channel1405 demonstrated higher shoot growth response when the plants were treated with MycoApply than untreated control under 0% nutrient application (Figure 4.6A) while the same cultivar was unresponsive under 100% nutrient application.



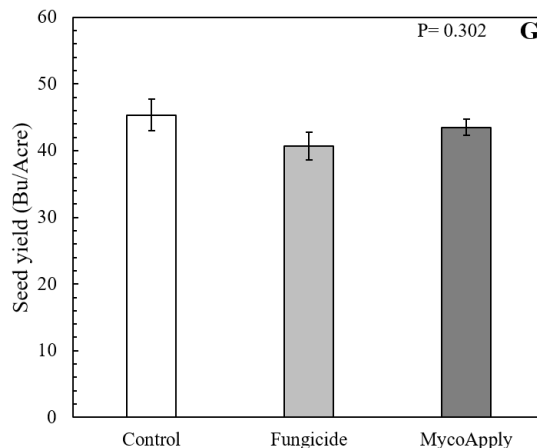


Figure 4.4. Shoot (A) and root dry weight (B), shoot (C) root (D) phosphate content, AM root (E) and nodule dry weight (F), and seed yield (G) of soybean cultivar AG1234. Four plants were harvested after 14 weeks of planting for biomass, P content and inner two rows for seed yield. Plants were either control plots or were treated with the fungicide Topsin M or with MycoApply. The vertical bars represent means of four biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

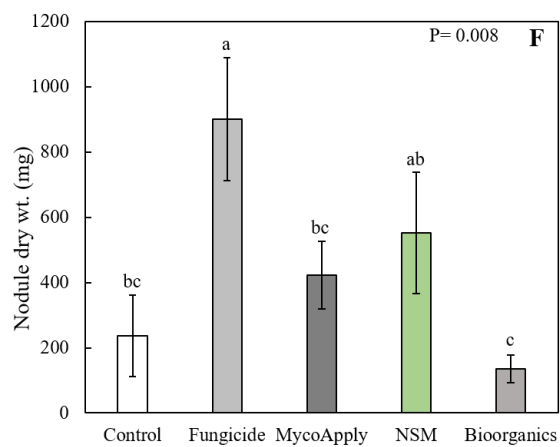
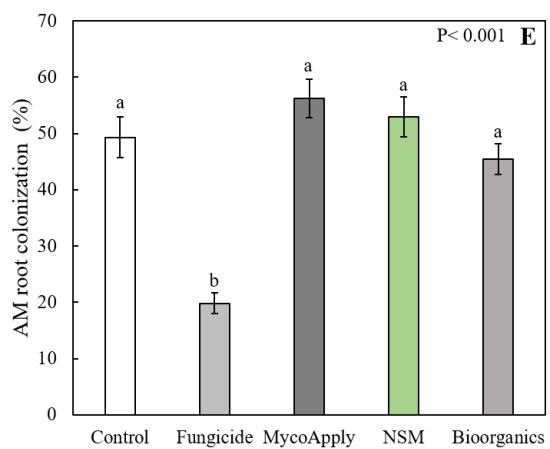
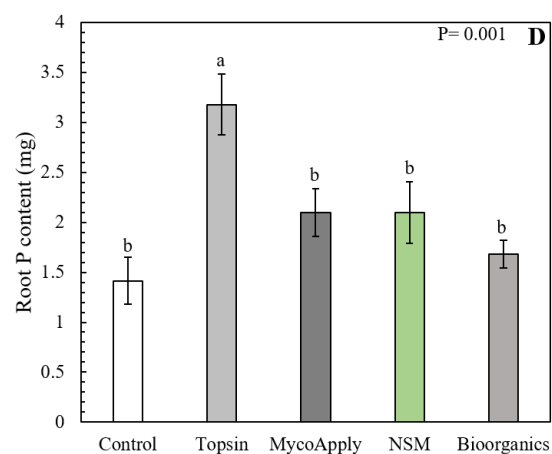
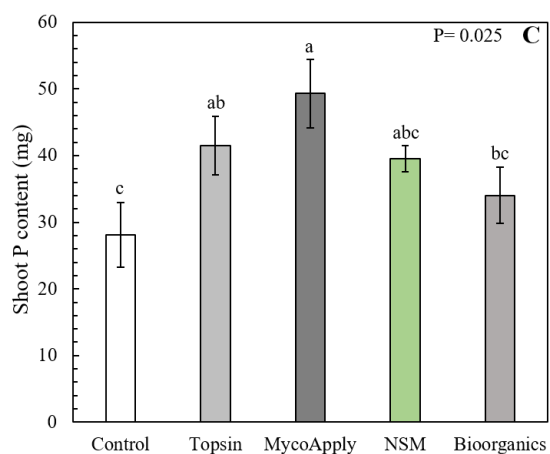
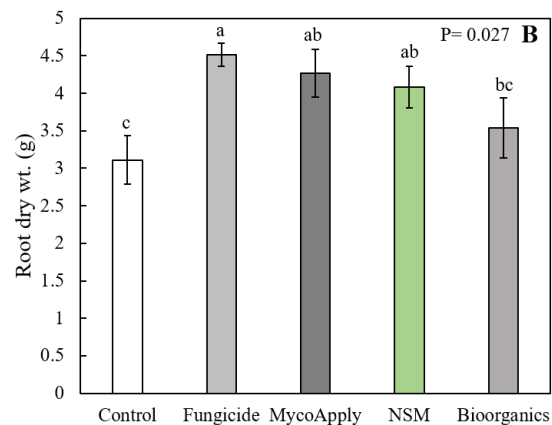
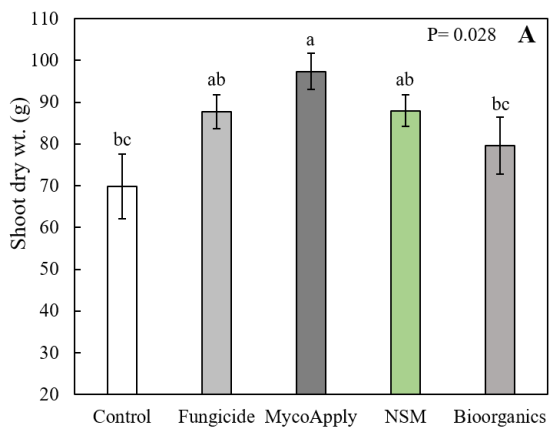
Application of commercial mycorrhizal inoculum did not demonstrate higher plant P uptake in field experiment I among control, fungicide, and MycoApply treated plots (Figure 4.4C and D). Whereas, in experiment II, MycoApply treated plots had significantly higher ($P \leq 0.05$) shoot P content (mean= 49.3 mg) in compared to control treatment (mean= 28.0 mg) but no difference of P content in root tissues (Figure 4.5C and D). Among commercial mycorrhizal inocula, MycoApply outperformed other two inocula for P content in shoot tissues where P content in shoot was 49.3 mg, 39.5 mg, and 34.0 mg respectively at MycoApply, NSM, and Bio-organics inocula applied plants. However, shoot and root P concentration between control and inocula applied plots was statically no different (Figure S4.5). MycoApply treated plants, in experiment III, had lower shoot P concentration than control plots at 0% and 50% of NP application (Figure S4.6).

MycoApply contributed higher plant P acquisition in the shoot (mean weight= 208 mg) especially in cultivar Channel 1405 under 0% nutrient application in compared to control (mean weight= 146 mg) (Figure 4.6C). However, this positive effect of MycoApply for P acquisition in plant tissues, was not observed under 100% nutrient application.

Mycorrhizal colonization between control and AM fungal inocula treated soybean roots was not statistically different in all experiments (Figure 4.4E, Figure 4.5E, Figure 4.6 D). However, the colonization rate significantly reduced at the fungicide treated plants (Figure 4.4E and 4.5E). Nodule dry weight between control and MycoApply treated plants was similar in all three experiments (Figure 4.4F, 4.5F, 4.6E). Surprisingly, we observed significantly higher nodule dry weight in fungicide treated plants in experiment I and II (Figure 4.4F and 4.5F).

Seed yield in experiment I and III was not statistically different among treatments (Figure 4.4G and Figure 4.6F). However, we observed significantly higher ($P \leq 0.05$) seed yield at MycoApply treated plants in comparison to control plants in experiment II (Figure 4.5G). Similarly, among inocula, MycoApply performed the best for the seed yield (Figure 4.5G). Notably, fungicide treated plants in experiment II had significantly higher ($P \leq 0.05$) seed yield than the control plants. Despite examining efficiency of different commercial fungal inocula, we further tested effects of nutrients with or without MycoApply additives on seed yield (Figure 4.5H). We found positive effects of MycoApply on the seed yield under 0% and 25% NP nutrient supply condition, where seed yield increased by 14% and 8% in MycoApply treated over control at 0% and 25% NP respectively (Figure 4.5H). This trend progressively decreased as we increased nutrients supply. Seed yield in the control

without MycoApply proportionally increased as we supplied more nutrients: from 45.6 bushel/Acre at 0% nutrient to 53.0 bushel/Acre at 100% nutrient. Interestingly, in the case of MycoApply, we did not observe such linear trend of seed yield increase despite adding more nutrients to the field. Seed yield at 0% nutrient with MycoApply was similar with 100% nutrient application without MycoApply.



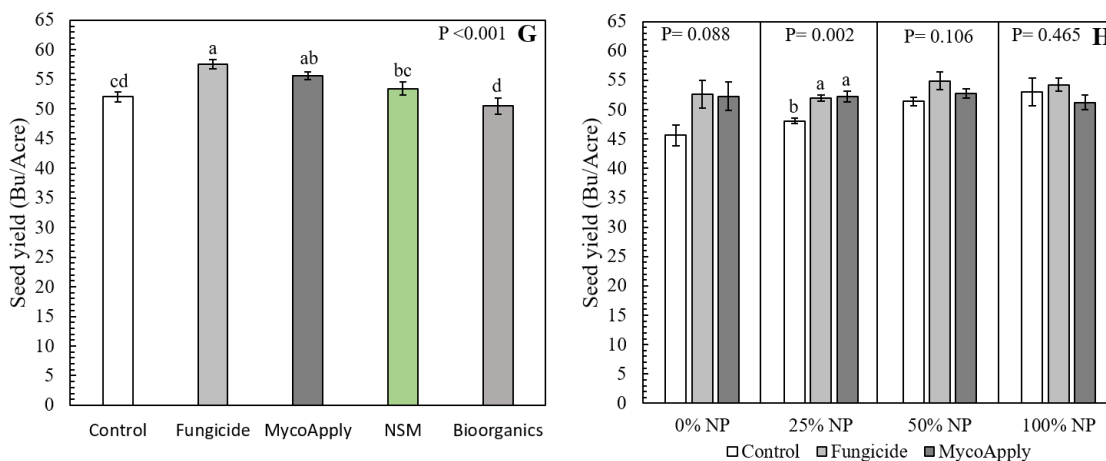
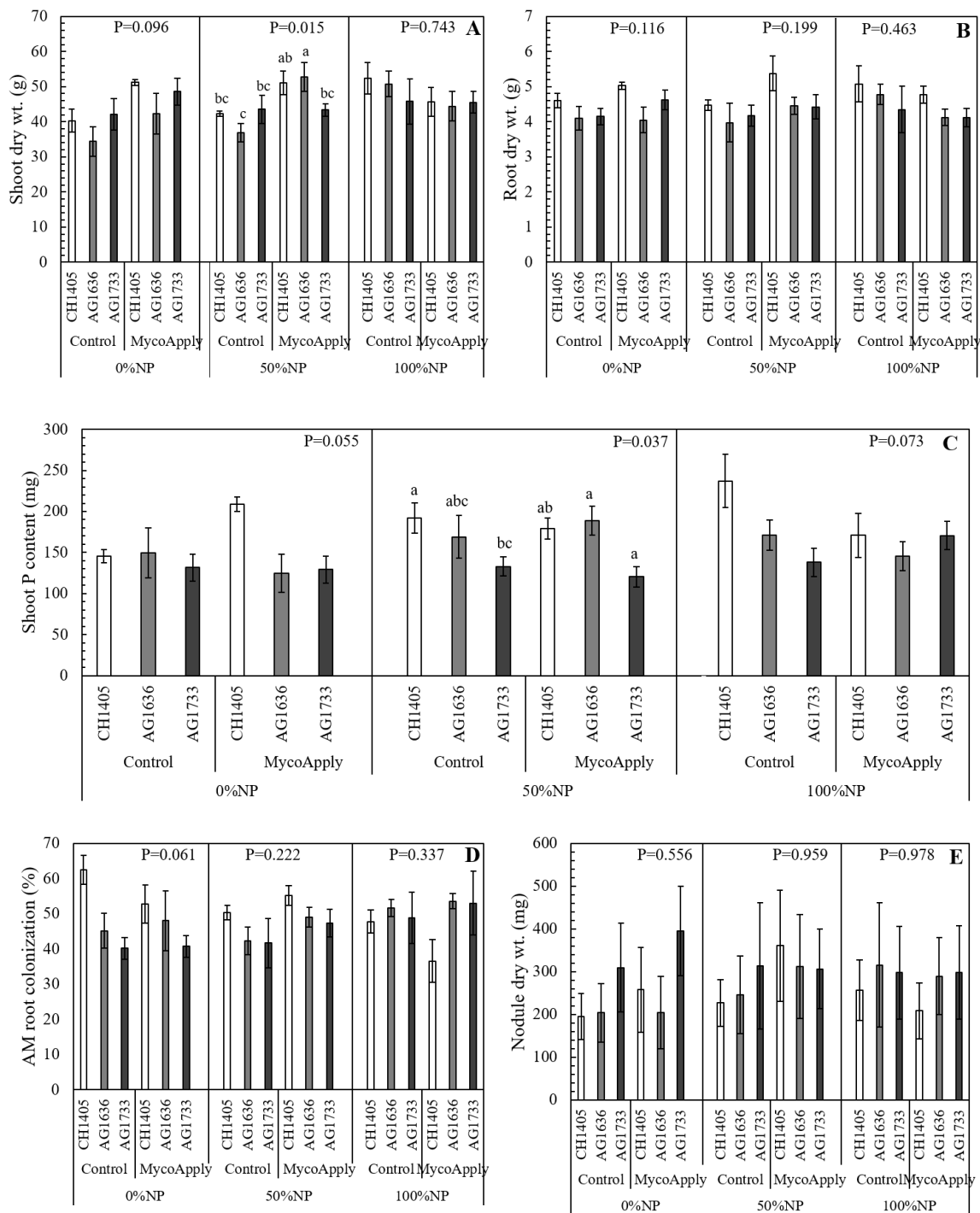


Figure 4.5. Shoot (A) and root dry weight (B), shoot (C), root (D) phosphate content, AM root colonization (E) and nodule dry weight (F), and seed yield (G and H) of soybean cultivar AG1234. Four plants were harvested after 16 weeks of plantation from field experiment for biomass, phosphate content and inner two rows for seed yield. Plants were either non-additive (Control), or treated with Topsin fungicide, or treated with commercial inocula: MycoApply, Nature Solution Mycorrhizae (NSM), and Bioorganics. The vertical bars represent mean of five biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

This increasing trend of soybean shoot weight in our field studies (Figure 4.5A and Figure 4.6A) demonstrated the positive effect of commercial mycorrhizal inoculum that was also observed in earlier studies (Mahanta et al., 2014; Ortas, 2012). Particularly, positive effects of mycorrhizal inoculum especially MycoApply on shoot and root weight was significant (Figure 4.5A and B). However, previous reports have suggested higher variation in the performance of the inocula for the plant growth could exist because of cumulative impacts of multiple factors in the field conditions (Verbruggen et al., 2013). For instance, existing bacterial, fungal, and other soil organisms have niche competition with the introduced commercial inoculum which can lead to a higher variability in plant growth responses (Niwa et al., 2018). In experiment II, despite having higher diversity of AM species in NSM and Bio-organics inocula, their response for shoot and root growth was lower than MycoApply which has only four different AM fungal species. It has been

suggested that many species having functional redundancies to offer benefits to the host plant and thus these species compete for the host photosynthetic carbon and other sources (Jansa et al., 2008). Thus, continuous allocation of host carbon to these diverse AM species in NSM and Bio-organics inocula might be a probable reason of lower shoot and root weight of plants treated with these inocula as suggested in previous studies (Garmendia and Mangas, 2014; Pearson et al., 1993). Moreover, different AM species have different response to the host (Klironomos, 2003). However, experiment in the controlled environment generally demonstrated that different AM fungal species are able to provide macro and micro nutrients benefits to the host (Hart and Forsythe, 2012). Experimental outcomes between controlled and field conditions are not in similar directions because of complexity of natural factors. Channel1405 cultivar performed better than other two cultivars at 0% nutrient application (Figure 4.6A and B). This variability of plant growth response in our soybean cultivars treated with AM inoculum is concomitant with findings in different cultivars of soybean (Hayashi et al., 2018), alfalfa (Lambert et al., 1980), corn (Sawers et al., 2017). But, symbiotic benefits to the host plant reversed in 100% of recommended dose of nutrient application as compared to 0% and 50% of recommendation, which is consistent with Chu et al. (2013) and Hetrick et al. (1996) where they observed reduced growth of AM plants than control in high nutrient supply conditions. If soil has already higher nutrients (100% nutrients application), host plants have easy access to these nutrients so reduced growth of host plants are observed as investment of host photosynthetic carbon to root symbionts (Williams et al., 2017).



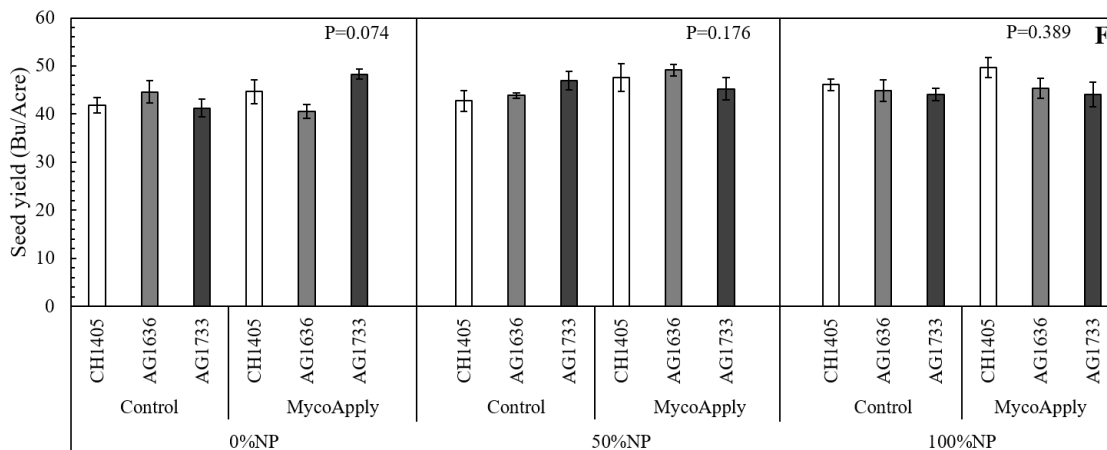


Figure 4.6. Shoot (A) and root dry weight (B) shoot phosphate content (C), AM root colonization (D), nodule dry weight (E), and seed yield (F) of three soybean cultivars Channel1405, AG1636, and AG1733 four plants were harvested after nine weeks of plantation from field experiment inner two rows for seed yield. Plants were either non-additive (Control) or treated with MycoApply. Plots were treated with the recommended rate of nitrogen (N) urea (7.71 kg of N/Acre) and phosphate (P) KH_2PO_4 (18.14 kg of P/Acre) for soybean in South Dakota as 100% and reduced proportionally to 50% and 0%. The vertical bar represents mean of four biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

Application of mycorrhizal inoculum increased shoot and root P content in our field studies (Figure 4.4C and D, Figure 4.5C, Figure 4.6C) which is consistent with other field studies (Labidi et al., 2015; Ortas, 2012; Tawaraya et al., 2012). This increased P content in mycorrhizal applied plants could be a possible mechanism of increased plant biomass in this study. It has been well recognized the contributions of AM fungi for P delivery to the host plant. However, environmental conditions, formulation of commercial AM inocula, and other factors regulate the functionality of AM fungi in field as suggested by Owen et al. (2015) in their review paper. We observed different commercial AM fungal additives had different P benefits to soybean, as MycoApply performed the best among three (Figure 4.5C). In consistent with our findings, Garmendia and Mangas (2014) observed one type of commercial product had higher performance for plant P acquisition than others. Higher shoot P content in one cultivar (Channel1405) treated with AM inoculum without any fertilizer application (0% NP application) clearly suggest that soybean cultivars have different response to AM fungi as in other crops (Sawers et al., 2017). This higher contribution of AM fungal soil additives for P delivery to soybean signifies the importance in our agriculture for soybean production. As soybean is nodulated legume plants that need higher amount of P for the atmospheric N₂ fixation by bacteria residing inside the root nodule (Kafle et al., 2018; Sulieman et al., 2013).

The similarity of AM colonization between AM soil additives and control in our findings (Figure 4.4E, Figure 4.5E, and 4.6D) is in accordance with other studies (Cely et al., 2016; Eulenstein et al., 2016; Janoušková et al., 2013). We measured total AM fungal colonization, but we do not know either this colonization is from introduced or indigenous AM fungal species. This similarity of AM root colonization between AM fungal soil

additives and control plant suggest that our field research area had already higher abundance of AM fungal community. Indeed, soil of prairie land (UpperMidwest) in the U.S.A. has higher AM fungal community abundance (Wilson and Hartnett, 1998). For instance, Monier (PhD dissertation, 2018) found 18 different AM species within one family Glomeraceae. Moreover, in our study plots, maize was a preceding crop before our soybean experiment. Maize is one of the highly responsive crops to AM fungi in the field conditions in terms of colonization and nutritional benefits (Benitez et al., 2017). Similarly, there was similar nodule weight between control and AM fungal inocula treated plants (Figure 4.4F, Figure 4.5F, and 4.6F) Soil in the eastern part of South Dakota is generally enriched with phosphate, which most probably why we observed similar nodulation between control and AM inocula additives. The fungicide might have detrimental effects on the pathogenic fungi at the rhizosphere. Reduction of such fungal communities (both AM fungi and pathogenic fungi) could have produced favorable environment (more photosynthetic carbon allocated) for rhizobial bacteria which therefore we observed higher nodulation at fungicide treated plots (Figure 4.4F and Figure 4.5F).

Application of commercial AM inoculum in our studies under field conditions had positive effects (either statistically significant or not) on soybean yield which is consistent with other studies for soybean yield (Cely et al., 2016; Klironomos, 2003; Meghvansi et al., 2008), maize yield (Sawers et al., 2017), and alfalfa (Pellegrino et al., 2012). For instance, meta-analysis of AM fungi on legumes (Kaschuk et al., 2010) referred that yield ranges from -4% to +24% and average about 9% which is in support of our studies where we found 14% higher yield in AM treated plants (Figure 4.5G and H). Niwa et al. (2018) and Owen et al. (2015) suggested that the variability of seed yield using agronomic

practices in the field conditions is cumulative effects of multiple environmental factors, therefore seed yield ranges from negative to positive effects. In our study, higher production of soybean seed might be the effects of nutritional benefits by AM fungi as there was higher P content in MycoApply treated plants than control (Figure 4.5C) consistent with Tawaraya et al. (2012). Here, we used fungicide to suppress natural existing AM fungal communities in the soil. Despite suppressing AM fungal communities, the applied fungicide might have reduced saprophytic and pathogenic fungi in the soil as suggested by Wilson and Williamson (2008) which therefore consequently improved soybean biomass and yield compared to control plants (Figure 4.5C and Figure 4.5G and H). Indeed, application of fungicide in the field and greenhouse conditions increased vegetation growth of grasses and forbs (Hartnett and Wilson, 1999). However, wide variations in soybean seed production with fungicide treatments have been reported (Cordeiro et al., 2015; Schreiner and Bethlenfalvay, 1997; Zilli et al., 2009). Moreover, other field and greenhouse studies found no effects of fungicide for the AM root colonization, nutrient acquisition, and plant growth when they treated seeds with fungicides (Cameron et al., 2017; Jin et al., 2013). Dose, frequency, and environmental factors affect the response of fungicide on plant growth (Buysens et al., 2015). For instance, Cameron et al. (2017) did not observe difference between AM root colonization, P acquisition, and plant growth when they treated seeds with fungicide before applying into the soil. This one-time application of fungicide before seed germination might not be sufficient enough to inhibit both AM and soil pathogenic fungi. Application of fungicide in different time point is crucial for the initial establishment of seedlings and later for the successful

flowering and pod formation against pathogenic fungi (Koenning and Wrather, 2010; Munkvold, 2009).

Furthermore, effect of mycorrhizal fungal additives (MycoApply) for soybean seed yield was more pronounced under no (0%NP) or moderate (25%NP) nutrient supply conditions than applying higher (100% NP) concentration (Figure 4.5G and H). Indeed, it is apparent that AM fungi and rhizobial bacteria perform better for plant productivity and seed yield in limited soil P and N (Hayashi et al., 2018). For example, recent evidence (Hayashi et al., 2018) showed no difference in seed yield between limited nutrient and extra nutrient added plots, indicating the importance of AM fungi under limited soil nutrient conditions which supports our findings where MycoApply treated plots had same yield between 0% and 100% NP fertilizer (Figure 4.5G and H).

4.5 Conclusions

Application of commercial AM fungal inoculum especially MycoApply was beneficial for better plant growth and seed yield of soybean both in greenhouse and field experiments. The benefits of application of AM fungi was higher when soil had limited phosphate and nitrogen. Therefore, application of AM fungi to field crops may be beneficial in terms of plant growth, nutrient acquisition, and seed production. However, further greenhouse and field trials need to be tested before concluding as we observed higher variation in the AM root colonization in the greenhouse experiments with commercial AM product, MycoApply. Availability of commercial AM fungal products with higher abundance of viable spores along with high performing strains would be beneficial for farmers in upper Midwest of the U.S.A. as climate of this region is suitable

for soybean. Further, effects of exogenous application of commercial AM additives and fungicide on indigenous AM fungal community, and their interactions would help us to understand how AM fungal community changes in the field conditions. Moreover, isolation, propagation, and application of the top few dominant AM fungal species in such interaction may be beneficial for plant fitness under natural conditions of major agriculture crops soybean, corn, and wheat.

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4.6 Supplemental information

Figure S4.1. Shoot (A) and root (B) phosphate concentration of soybean cultivar AG1234 of greenhouse experiment

Figure S4.2. Shoot (A) and root (B) total phosphate content of three different soybean cultivars Channel1405, AG1636, and AG1733 of greenhouse experiment

Figure S4.3. Root nodule phosphate concentration of three different soybean cultivars Channel1405, AG1636, and AG1733 of greenhouse experiment

Figure S4.4. Shoot (A) and root (B) phosphate concentration of soybean cultivar AG1234 of field experiment of field experiment

Figure S4.5. Shoot and root phosphate (P) content of soybean cultivar AG1234 of field experiment

Figure S4.6. Shoot phosphate concentration of three soybean cultivars Channel1405, AG1636, and AG1733 of field experiment

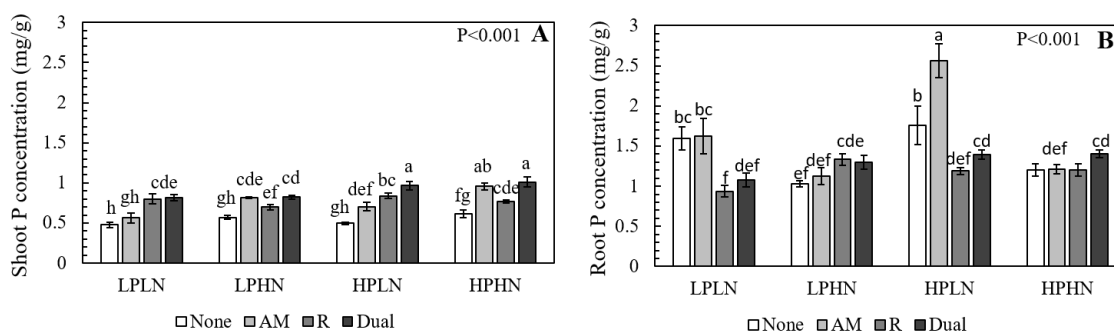


Figure S4.1. Shoot (A) and root (B) phosphate concentration of soybean cultivar AG1234 grown for eight weeks in two liters of soil substrate in greenhouse. Plants were remained non-inoculated (None), or inoculated either with only MycoApply (AM), or only *Bradyrhizobium japonicum*, USDA 110 (R) or dual (AM+R). The vertical bars represent mean of five biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

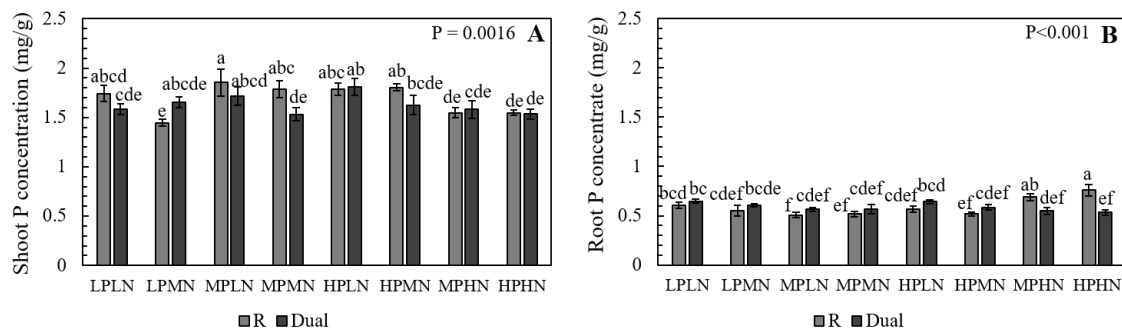


Figure S4.2. Shoot (A) and root (B) phosphate concentration of soybean cultivar AG1234 grown for 15 weeks in three liters of soil substrate in the greenhouse. Plants were inoculated with only *Bradyrhizobium japonicum*, USDA 110 (R) or dual inoculated with both root symbionts (AM+R). The vertical bars represent mean of five biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

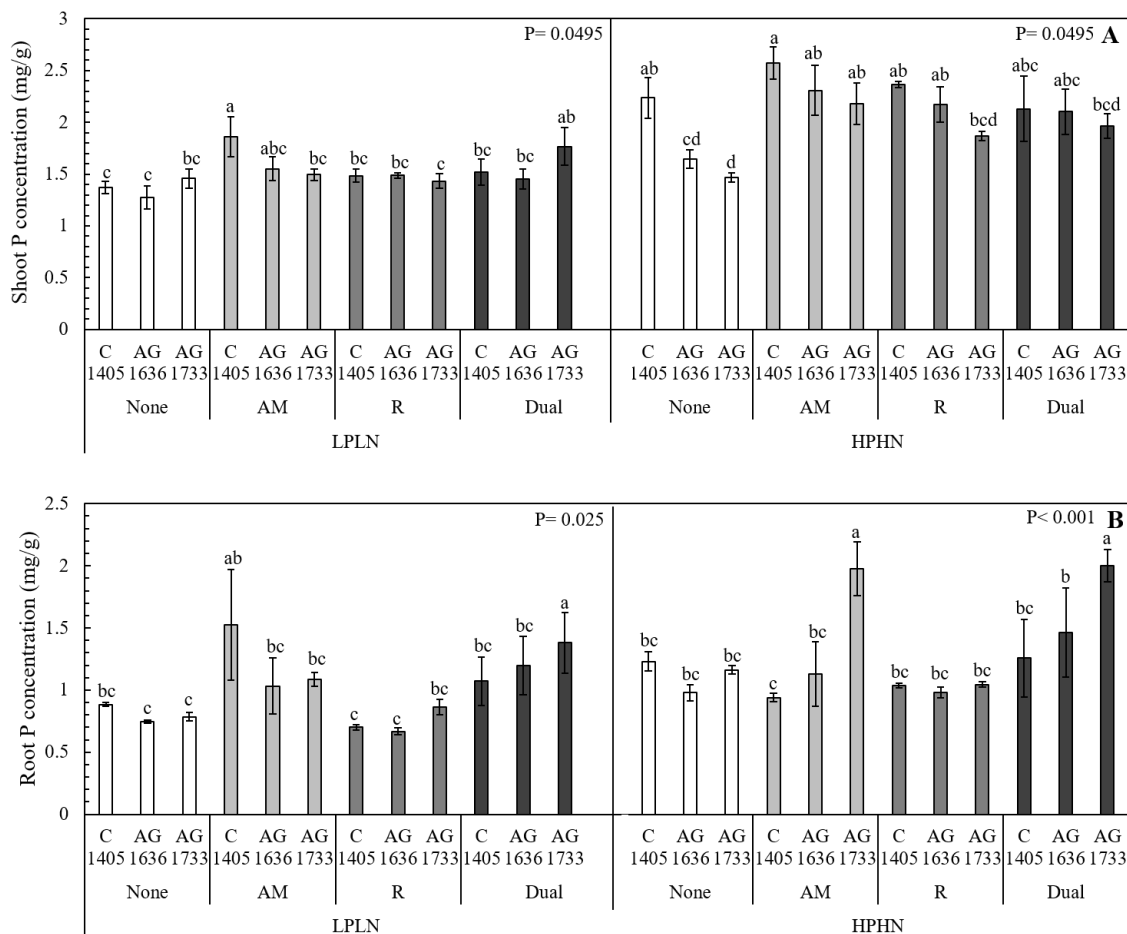


Figure S4.3. Shoot (A) and root (B) total phosphate content of three different soybean cultivars grown for eight weeks in two liters of soil substrate in greenhouse. Plants were remained non-inoculated (None), or inoculated either with only MycoApply (AM), or only *Bradyrhizobium japonicum*, USDA 110 (R) or dual (AM+R). The vertical bars represent mean of four biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test. Three cultivars of soybean were as: Channel1405, AG1636, AG1733.

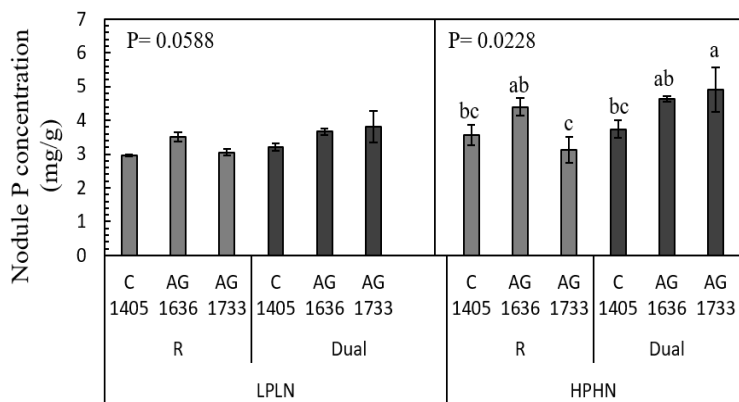


Figure S4.4. Root nodule phosphate concentration of three different soybean cultivars grown for eight weeks in two liters of soil substrate in greenhouse. Plants were inoculated with only *Bradyrhizobium japonicum*, USDA 110 (R) or dual (AM+R). The vertical bars represent mean of four biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test. Three cultivars of soybean were as: Channel1405, AG1636, AG1733.

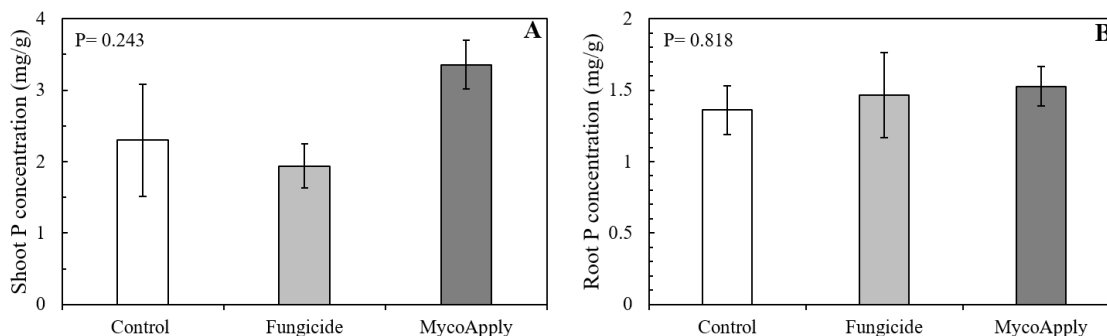


Figure S4.5. Shoot (A) and root (B) phosphate concentration of soybean cultivar AG1234 four plants harvested after 14 weeks of plantation from field experiment. Plants were either non-additive (Control) or treated with Topsin fungicide or MycoApply. The vertical bars represent mean of four biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

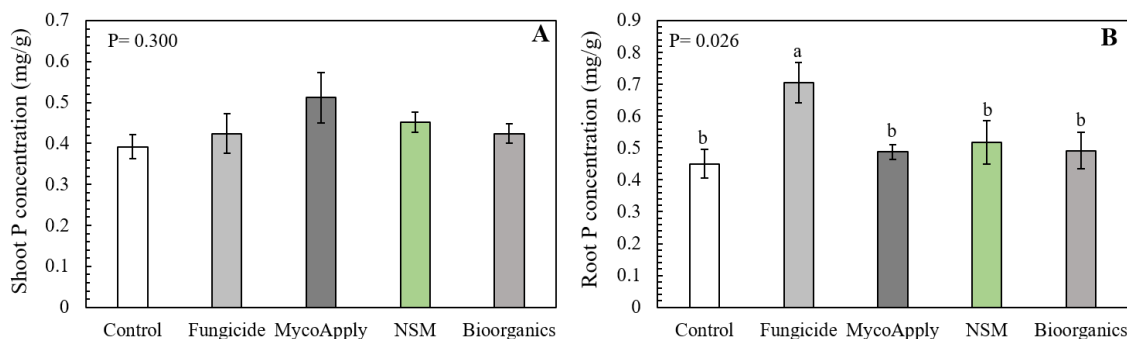


Figure S4.6. Shoot and root phosphate (P) content of soybean cultivar AG1234 four plants harvested after 16 weeks of plantation from field experiment. Plants were either non-additive (Control), or treated with Topsin fungicide, or treated with commercial inocula: MycoApply, Nature Solution Mycorrhizae (NSM), and Bioorganics. The vertical bars represent mean of five biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

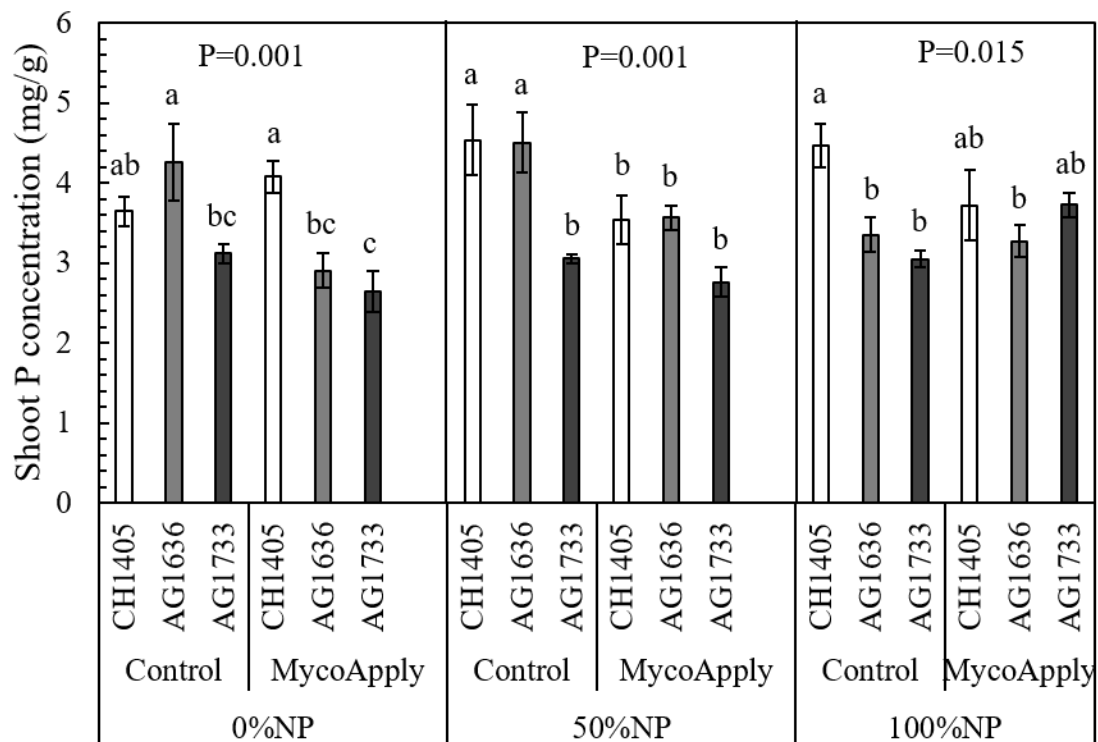


Figure S4.7. Shoot phosphate concentration of three soybean cultivars Channel1405, AG1636, and AG1733, four plants harvested after nine weeks of plantation from field experiment. Plants were either non-additive (Control) or treated with MycoApply. Plots were treated with the recommended rate of nitrogen (N) urea (7.71 kg of N/Acre) and phosphate (P) KH_2PO_4 (18.14 kg of P/Acre) for soybean in South Dakota as 100% and reduced proportionally to 50% and 0%. The vertical bars represent mean of four biological replicates with standard error of mean, different letters on the bar refer difference at P values at ≤ 0.05 after ANOVA and LSD test.

CHAPTER 5: CONCLUSIONS AND FUTURE QUESTIONS

Tripartite interactions of legumes with arbuscular mycorrhizal (AM) fungi and rhizobial bacteria have a bigger implication to maintain sustainable agriculture. Most of the previous research literatures focused on studying interactions between legume and one of the symbionts, either AM fungus, or rhizobial bacteria. However, understanding legume with only one of the symbionts at a time does not provide enough information about dynamic of nutrient exchanges process between symbiotic partners, as legume in natural conditions forms symbiotic relations simultaneously with AM fungi and rhizobial bacteria forming tripartite interactions. The main goal of this study was to understand physiological and molecular mechanisms of the tripartite interactions of legumes in association with AM fungi and rhizobial bacteria. Moreover, what is the importance and potential of tripartite interactions for agronomic purpose.

Our studies have clearly demonstrated that the tripartite interactions significantly facilitate for the plant growth response along with phosphate and nitrogen uptake of the plant. We found that the nutrient demand of the host, and the fungal access to nutrients are important factors that control the carbon allocation to individual root symbionts in tripartite interactions. The host plant allocated more photosynthetic carbon to nodulated root half under nitrogen demand conditions. However, host plant strategically allocated more carbon to AM root half when exogenous nitrogen was supplied to the plant. This discriminatory capability of the host plant to allocate its carbon to the most beneficial partner supporting previous findings of biological market dynamics in plant-beneficial microbes interactions. Additionally, this is the first study to demonstrate gene expression of several plant

transporters of the Sucrose Uptake Transporter (SUT) and Sugars Will Eventually be Exported Transporter (SWEET) family that controls the carbon flux to different symbiotic partners during the tripartite interactions. Therefore, we provided insight into physiological and molecular mechanisms of resources exchange during tripartite interactions.

Tripartite interactions have a synergistic effect on the host plant growth response as AM fungi deliver phosphate from soil beyond root access and rhizobial bacteria provide nitrogen through biological nitrogen fixation process to the host plant. However, neutral or antagonistic responses have also been reported. It has been suggested that synergistic or neutral or suppressive host growth responses are the effects of environmental context in which experiments were conducted. We investigated that in our pot experiment, tripartite interactions did not facilitate for the plant growth response. However, despite neutral or negative plant growth, phosphate uptake of the host plant was significantly higher in AM plants. The probable reason for the neutral to negative growth response could be: nutrients gained by the host plant did not outweigh the carbon cost for the symbioses. Indeed, host plant allocated relatively higher carbon to tripartite root system than only rhizobial root system. This investment of host carbon to root and probably to soil through network of AM hyphae might have bigger implications to balance carbon cycle and ecosystem stability.

We tested effects of commercial AM fungal inocula plant growth and seed yield of soybean cultivars in greenhouse and field conditions. We found that the application of AM inoculum increased plant biomass and seed yield in greenhouse and field conditions. Response of AM inocula on plant growth and seed yield was a notably higher in limited soil nutrient conditions. Additionally, different commercial AM fungal inocula have

different efficiency on soybean yield. For instances, of three commercial AM fungal inocula tested, MycoApply outperformed other two inocula for plant biomass and seed production. Also, one soybean cultivar, Channel 1405 demonstrated better responsiveness to fungal inoculum. Taken together, application of commercial AM fungal inocula have positive effects on plant productivity and seed yield especially in limited supply of nutrients which could be an alternative option against chemical fertilizers for soybean seed production. Fungicide not only acted on AM fungi but probably also on other pathogenic fungi in the soil which consequently fungicide treated plots had higher plant growth and seed yield.

In a split root system study, we examined the amount of ^{13}C carbon (^{13}C) labelling in the symbiotic root of host plant during tripartite interactions with relation to benefits conferred by symbionts. It would be better to understand how much carbon has been allocated to fungal hyphae inside and outside the host root. Therefore, further exploration should be conducted to adopt/develop technique that enable to quantify carbon exclusively in the fungal tissues. Additionally, we measured gene expression for SUT and SWEET that are putatively responsible for carbon flux to symbionts. However, we did not know where these transporters were localized in the colonized roots, therefore understanding the localization of these transporters in the colonized root further clarify functional role of these transporters in symbioses.

In simple a pot experiment, we observed neutral to negative growth response of AM plant in compared non-AM plant despite higher phosphate nutrition to host. Further investigation should be addressed to figure out what are the probable factors for growth

variability. These factors could explain the variability: volume of soil/pot size, duration of experiment, nutritional profile of the soil substrate. Additionally, measurement of carbon and nitrogen in root free soil before and after experimental period may provide some information of growth variability. In larger context, role of AM fungi for carbon sequestration in the soil also can be quantitatively addressed in the future study.

Application of AM fungi to increase crop productivity in the field conditions is probably an alternate option to reduce dependency on chemical fertilizer. Furthermore, crop plants including soybean, corn, alfalfa have many biotic stressors in the field during growing period. What is the underlying molecular mechanism of certain soybean cultivars that demonstrate favorable response to AM fungi? How AM fungi can be used to minimize biotic stressors and increase plant productivity? How application of AM fungal inocula interact with existing soil AM fungal community? For farmer perspective, how can we reduce cost of commercial AM inocula? How the efficiency of commercial AM inocula can be increased? Future work on these questions may be useful to ameliorate application AM fungal community in agronomic field to increase productivity in a sustainable way.