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Beneficial Plant-Microbe Interactions to Improve Nutrient Uptake and Biotic Stress Response in Crops

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BENEFICIAL PLANT-MICROBE INTERACTIONS TO IMPROVE NUTRIENT UPTAKE AND BIOTIC STRESS RESPONSE IN CROPS

BY

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

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I would like to dedicate my thesis to my parents, Ram Bahadur Yakha Rai and Lila Maya Yakha Rai, and my dear husband Dr. Rajesh Pathak.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMF: Arbuscular mycorrhizal fungi

ANOVA: Analysis of variance

C: Carbon

CMN: Common mycelial networks

DP: Direct plant pathway

ERM: Extraradical mycelium

Fix+: *E.meliloti* rhizobia that fixes atmospheric nitrogen

Fix-: *E.meliloti* rhizobia that do not fix atmospheric nitrogen

IRM: Intraradicle mycelium

LSD: Least significance difference

Mt: *Medicago truncatula*

MP: Mycorrhizal Pathway

MycoApply: MycoApply commercial AM inoculum

N: Nitrogen

N_2: Nitrogen gas

NH_3: Ammonia

NH_4^+: Ammonium

P: Phosphate

qPCR: Real time quantitative PCR

ROC: Axenic Ri T-DNA transformed root organ culture

SCN: soybean cyst nematode

SUT: Sucrose uptake transporter

SWEET: Sugars will eventually be exported Transporter

T^{Fix+}: Tripartite system inoculated with Fix+ rhizobia and AMF

T^{Fix-}: Tripartite system inoculated with Fix- rhizobia and AMF

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

BENEFICIAL PLANT-MICROBE INTERACTIONS TO IMPROVE NUTRIENT UPTAKE AND BIOTIC STRESS RESPONSE IN CROPS

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Mutualism is a very common phenomenon among living organisms on earth. Legumes because of their high protein content, serve as a great nutrient resource for animals. This group of plants can form a mutualistic symbiosis with beneficial microbes. For example, Alfalfa (Medicago) and soybean (Glycine max) can get colonized with arbuscular mycorrhizal fungi (AMF) and rhizobia bacteria simultaneously forming a complex tripartite interaction for nutrient benefits. Most of the previous research evaluated individual symbionts, either rhizobia bacteria or AMF, but not both. There are only a few reports which discuss the nutrient exchange mechanisms in a tripartite interaction. Thus, there is a lack of fundamental understanding of how the resources are exchanged in tripartite interactions.

Nitrogen (N) and phosphorus (P) are essential nutrients for plant growth; AMF can supply both P and N, while rhizobia bacteria can only supply N to their host plant. Both root symbionts can provide other benefits like abiotic and biotic stress tolerance. In return, the host plant distributes a substantial amount of its photosynthetic carbon (C) produced in the leaves to its root symbionts. However, the regulation mechanisms on C resources allocation by the host plant to its root symbionts is not well understood.

In my first experiment, I hypothesized that the N-fixing capability of the rhizobia bacteria affects the C allocation pattern in a tripartite system with AMF. I evaluated C allocation to the symbionts under in a tripartite interaction with various nutrient access scenarios including the use of a rhizobial strain that lacks biological nitrogen fixation (BNF) capability and AMF having access to a labeled N source. The dual inoculation of N fixing rhizobia (Fix+) and AMF results in a synergistic increase in shoot biomass, enhanced N and P uptake in the sink (roots) but low delivery toward the source (leaves). On the other hand, tripartite interactions of Fix- rhizobia that lack biological N fixation activity and AMF lead to a significant increase in N uptake and delivery towards the source but a significant drop in carbon allocation towards Fix- rhizobia root. Consistent with these findings, we found changes in SUCROSE UPTAKE TRANSPORTER (SUT) and SUGAR WILL EVENTUALLY BE EXPORTED TRANSPORTER (SWEET) genes. These results provide substantial new information about how host plants control their carbon allocations under the different status of N demand in presence of rhizobia and AMF inoculation.

During tripartite interactions, rhizobia bacteria are restricted to the host roots but extraradical mycelia (ERM) of AMF can go beyond, colonizing another host root. This leads to the development of common networks among two or more plants which are known as the common mycelial Network (CMN), creating a biological market for nutrient transport. The nitrogen-fixing capability of rhizobia bacteria can affect the transport of nitrogen (N) by AMF to host plants connected by CMNs. In the second experiment, I hypothesized that access of exogenous 15N to AMF would allocate more N to host plants colonized by Fix- rhizobia that lack BNF capability than those colonized by Fix+ rhizobia. We found that co-inoculation with Fix- rhizobia with AMF or non-mycorrhizal control

plants resulted in elevated 15N enrichment in the shoot of the host plant. This suggests that AMF allocates most of the N they uptake from the soil to the host plant with a greater N demand due to the lack of access to fixed nitrogen. As expected, we found that AMF does not transfer as much N with host plants colonized by Fix+ rhizobia because their N demand can be fulfilled by the rhizobia bacteria.

Plant diseases can be managed in various ways, including the use of disease-resistant and/or tolerant crop varieties, chemical controls, and biological controls. A disease-resistant variety can lose its resistance due to the development of a new variant of the pathogen. Chemicals used in agriculture and other systems can have a very adverse effect on the environment. The use of Microbes for controlling plant diseases is safer and offers environmental sustainability compared to chemical pesticides. In my third experiment, I evaluated if AMF could mitigate the destructive effect of Soybean cyst nematode (SCN: *Heterodera glycines*), one of the most dreadful pests in soybean. Soybean plants infested with SCN do not show any aboveground symptoms in most of the cases, so the field gets unrecognized for a long time. Through the AMF symbiosis, plant hosts receive protection from pathogens as well among other benefits. In this experiment, we evaluated the effects of a commercially available AMF soil additive called MycoApply® (consists of an equal ratio of *Glomus mossaea, Rhizophagus irregulare, G. etunicatum, G. aggregatum*) under greenhouse and field conditions on the reproduction of SCN and the soybean growth and yield increase. We observed increased shoot weight for AMF-treated SCN susceptible variety (Williams-82) infested with SCN but no effect on the resistant variety, Jack (PI88788) in a greenhouse but no differences were found in SCN egg number. However,

soybean seed yield was increased up to 40 % in mycorrhizal treated plots than non-mycorrhizal plots (they do have a natural community of AMF). Our results show that commercially available AMF inoculum can be used to increase soybean production even in the field infested with SCN. However, further investigation should be conducted to know the actual mechanism of how these fungi are able to increase soybean production without any change in AM colonization rate and reduction in SCN egg population in the soil.

In summary, tripartite interactions of legumes with AM fungi and rhizobia bacteria led synergistically increase in plant growth independent of N fixing capability of rhizobia. However, delivery of N by AMF towards shoot increased when plants only have AMF for N source. Consistent with the biological market model, the host plant allocates a significant amount of C to benefit root symbionts. Similar trends were found when plants were interconnected via CMNs. On the other hand, AMF does not provide nutritional benefits but also can provide biotic stress tolerance such as enhanced SCN tolerance. All these indicated a bigger potential role for beneficial microbes in sustainable agriculture.

CHAPTER 1: LITERATURE REVIEW

1 Background

Microbial communities residing on or around host plants play a crucial role in plant functions. The Rhizospheric microbial communities, associated with root systems, and phyllospheric microbes residing aboveground have been shown to directly influence crop productivity through their roles in bio accessibility of mineral nutrients, protection against pathogens and release of phytohormones to stimulate plant growth. However, the relationship cannot always be beneficial; it can also be a neutral, or detrimental one. Beneficial microorganisms that are associated with plants hold enormous potential to be developed into biofertilizers or bio pesticides and new biotechnological tools to increase the nutrient efficiency and stress (biotic and abiotic) tolerance of crops, and environmental sustainability of agroecosystems. Legumes are ranked as the second largest family among food crops, vegetables, forages, and cover crops; They are grown worldwide and contribute to 27% of world food production for 33% of the dietary nitrogen (N) needs of humans [1, 2]. Due to their wide distribution in the diverse ecosystems, the influences of legume on soil ecological processes have been intensively investigated, particularly the influences on the biogeochemical cycling of nutrients. Dinesh et al. (2006) reported that long-term (12 years) cover cropping with four leguminous species significantly improved the N and C cycling driven by soil microbes, resulting in higher levels of total organic C, dissolved organic C and N, labile organic N, and etc. [3].

Legumes form unique symbioses that are not formed by most other plants, including Arabidopsis. Rhizobium-legume symbioses with a high level of host-symbiont specificity is one of the most well-studies plant-microbe interactions [4]. Legumes also

form symbiosis with arbuscular mycorrhizal fungi (AMF) that are ubiquitous in soils around the globe. They belong to the phylum Mucoromycota, sub-phylum Glomeromycotina, and form a symbiotic association with more than 70% of land plant species, including many agronomically important crops [5, 6]. In addition, legumes are associated with fungal and bacterial endophytes that live inside their plant host for at least part of their lives by colonizing inter and /or intra-cellularly inside the healthy tissue without causing apparent disease symptoms. Plant endophytes exhibit phytostimulation, improved biological nitrogen fixation through diazotrophic endophytes, the biosynthesis of ACC (1- aminocyclopropane-1-carboxylate) deaminase, solubilizing phosphate by P solubilizing bacteria and helps to synthesize an d release of antimicrobial metabolites or siderophores that may help to reduce pathogenic microbes [7]. Similarly, endophytic fungi associations is important for the plant immune system [8], disease suppression [9] nutrient acquisition [10]), and tolerance to abiotic stresses [11]

Medicago truncatula has been used extensively for symbiosis research with arbuscular mycorrhizal fungi and nitrogen-fixing rhizobia as well as to study plant–pathogen interactions. The dual functionality of this system opens exciting possibilities for symbiosis research. We summarize here the effects of different beneficial microbes' nutrient uptake and allocation, yield, and stress resistance of *M. truncatula* and soybeans, and identify knowledge gaps that hinder the application of these interactions to their full potential in production systems of leguminous plants.

2 Soybean-microbe interactions and benefits

Soybean is used as oilseed, feed for livestock, diet for human and also as the biofuel feedstock worldwide [2, 12]. Soybean is one of the oldest and widely grown crops first

started its cultivation from northern and central region of China[13]. In United states, it was first cultivated in 1765 in Georgia from China through London by Samuel Bowen for different purposes [14]. In recent years, soybeans have been used as the biofuel in United states about 1750 million gallons of biodiesel were produced in 2014 and expected to be 2.1 billion gallons for 2018 [15].

Soybean are grown with corn and wheat as rotational crop and are second largest cultivated crop after corn in context of yield and the area occupied. Among the world production, US only produces 34% followed by Argentina, Brazil and then China [16]. Illinois is the leading state for production of soybean followed by Iowa and Minnesota [17, 18]. The total bushels (3,969 million) production in the, south Dakota alone is 230 million bushels (8%) that signifies the importance of the soybean [15].

2.1 Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) can be an important source for nitrogen (N) for supporting productivity of plants. N is an essential component of various forms biomolecules, for example, nucleic acids and other organic nitrogenous compounds and is a basic material for synthesizing proteins. BNF is an alternative to fertilizers to meet our agricultural nitrogen needs. Unfortunately, no plant species can convert atmospheric dinitrogen to ammonia and use plant growth and development. A group of prokaryotes, termed diazotrophs are able to reduce the atmospheric nitrogen to plant useable form [19]. The most important examples include aerobic azotobacter, anaerobic Clostridia or in symbiosis with certain higher plants e.g., Rhizobia with legumes (soybean, Medicago) or *Azolla Anabaena Azollae* with *Azolla* and widely studied cyanobacteria. However, the biochemical machinery required for nitrogen fixation is common in all diazotrophs, which

is provided by the nitrogenase enzyme system. The overall reaction for dinitrogen reduction by nitrogenase is:

$$N_2 + 16ATP + 8e^- + 8H^+ \longrightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Promoting BNF in agricultural systems could not only reduce the dependency on chemical fertilizers but also results in economic benefits and environmental sustainability. BNF is naturally a practical alternative. It has been estimated that 17 million tons of nitrogen fertilizer to the soil which translated to a direct economic benefit of US $28 to $148 billion by using biologically fixed nitrogen including recycling of nitrogen-rich plant residues even before the food supply value addition [20] To adapt the N scarcity in soil, plants can obtain the biologically fixed nitrogen through associations with various types of nitrogen fixing organisms. These associations can be broadly classified into three major types: free-living, associative, and symbiotic.

2.1.1 Free living nitrogen fixation

Free-living diazotrophs are bacteria that live in soil and can survive without the direct influence of plant roots [21]. *Azotobacter*, *Bacillus*, *Clostridium*, and *Nostoc* are some of the examples of free-living bacteria. As nitrogenase can be inhibited by oxygen, these organisms act as anaerobes or microaerophiles while fixing nitrogen. These bacteria respond to root exudates via chemotaxis and colonize the rhizosphere but do not penetrate the plant tissues. The energy required for nitrogen fixation is mainly obtained by oxidation of organic molecules released by other organisms or from decomposition. As there are not enough carbon and energy sources for free-living organisms, they have less contribution to global nitrogen fixation. In wheat rotating farming system, It was revealed that free-living microbes provided 20 kg hectare^{-1}year^{-1} [22].

2.1.2 Associative nitrogen fixation

Associative bacteria, such as *Azospirillium*, also regarded as rhizosphere bacteria and form a close association with the surface of the roots. Some strain of *Azospirillum* has specific mechanisms to interact with roots and some are activated by plant root exudates and are attracted by root mucilage [23]. Flavonoids are important plant signals for interaction with the bacteria. The atmospheric nitrogen reduced into ammonium through the action of the nitrogenase under microaerobic conditions at low nitrogen. The level of nitrogen fixation is determined by several factors, including soil temperature (*Azospirillum* species thrive in more temperate and/or tropical environments), low oxygen environment provided by the host, photosynthates (C) that are available to the bacteria, and the nitrogenase enzyme efficiency [24].

2.1.3 Symbiotic Nitrogen fixation

The host plant form symbiosis with many microorganisms that fix nitrogen symbiotically. The host plant provides photosynthates (sugars) from photosynthesis that are required for the nitrogen-fixing microorganism. These sugars are used for the energy required to fix atmospheric nitrogen. In return to theses sugars, N-fixing bacteria provides fixed nitrogen to the host plant for its growth and development [25].

The most important nitrogen-fixing symbiotic associations are the relationships between *Rhizobium* and *Bradyrhizobium* bacteria which colonize most of the leguminous plants. In agricultural systems, alfalfa, beans, clover, cowpeas, lupines, peanut, and soybean are economically very important and form symbiosis with rhizobia Beringer, Brewin [26]. In this relationship, rhizobia convert the atmospheric nitrogen into ammonia which is available to the plants and in return bacteria gets organic acids from plants.

However, in some plants such as bayber and sweet fern, the symbiont for nitrogen fixation is not rhizobia but *Frankia* [27], and water fern Azolla's colonized by a cyanobacterium *Anabaena azolla* for N- fixation.

2.2 Legume-Rhizobia symbiosis

Mutually beneficial interaction of two different organisms from distinct species is termed as 'symbioses. The bacteria which form nitrogen-fixing symbiosis with legume plants belonging to diverse groups of α- and β-proteobacteria are collectively called rhizobia [28, 29]. When two partners are compatible to each other during the symbiotic development that led to establishment of a successful symbiosis. However, incompatibility frequently occurs, a bacterial strain could not nodulate or forms nodule that are incapable of fixing nitrogen in a particular host. Legumes being in second largest food and feed crops cultivated globally and contribute to more than 25% of world food production (European Association for Grain Legume Research, 2007). The legume-rhizobia symbiosis itself provides around 200 million tons of nitrogen annually [30]. Therefore, leguminous crops have a special advantage in sustainably meeting agricultural nitrogen needs.

The Rhizobium or *Bradyrhizobium* bacteria colonize the host plant's root system and cause the roots to form nodules to house the bacteria. The nodulation process illustrates an orchestrated interaction between the bacteria and host plant [31]. At the onset of nodule organogenesis, the rhizodeposition of flavonoid compounds from legume seed coats or root exudates induce the synthesis of Nod proteins/Nod Factors (NFs) which activate the transcription of genes needed to produce rhizobial lipo-chito oligosaccharide Nod factors [32]. The NFs, belonged to the MAMPs, an elicitor molecules, such as chitin and chitooligosaccharides in fungi, peptidoglycan, flagellin epitope and lipopolysaccharides

(LPS) in bacteria and are conserved [33, 34]. Recognition of Nod factors by very specific plant receptors result in bacteria getting attached to tip of the root hair. This attachment led to deformation of root hair of host plants and the activation of specific plant pathways and cause cortical cell division and formation of nodule primordia. This deformation results curling of root hair that give rise “shepherd’s crook” shape which is an unusual root hair shape [25]. Then, the rhizobia bacteria enter the plant cortical cells via infection thread. This infection thread is a passage for entering host cells and forms a peri bacteroid membrane by a process called endocytosis. This special structure formed is called as symbiosome [2, 35]. Bacteria can divide within the symbiosome and whole symbiosomes can also divide inside the host cell, both these types of division being carried out synchronously or not. In symbiosome, the nitrogen fixed by bacteroid is exported as ammonium to the host plant cytoplasm and distributed toward the other parts of the plant. The Soybean which forms a determinate-nodule primarily transport ureides as fixed-N compounds, while the Medicago that forms indeterminate nodule assimilate asparagine (Asn) and glutamine (Gln) as a fixed- compounds [36, 37]. On the other hand, reduced carbon compounds (photosynthates) and metabolites transported towards the nodule from host plants [2, 35]. Nitrogenase enzyme has important roles for all the process mentioned above. The bacteroides obtain energy for the N-fixation process from the host cell. At the same time, nitrogenase being an oxygen sensitive, leghemoglobin (provide pink color to effective nodules), a heme protein produced by the legume and bacterium seems to transfer oxygen to rhizobia for cellular respiration but not too much to alter the action of nitrogenase [38].

2.3 Arbuscular mycorrhizal fungi

Symbiosis refers to any coexistence of interactions that ranges from mutualistic, commensalistic and parasitic. Among different symbioses, mycorrhizal symbiosis is very common [39]. The fungal association with plants named as “mycorrhiza”, is a combination of the Greek words mykes, meaning fungus, and rhiza, meaning root [40]. The origin of AM symbiosis is thought to be in the approximately 400 million years ago. Thus, AM symbiosis is also called the mother of plant root endosymbiosis [41].

Ubiquitous group of arbuscular mycorrhizal fungi (AMF) form widespread endomycorrhizal symbiosis. As an obligate biotrophs, AMF are completely dependent on the photoautotrophic host to complete their life cycle and to reproduce. AMF are a member of the phylum, *Glomeromycota* that form symbiosis in 65% of all terrestrial plant species including many agroeconomically important crops like maize, rice, soybean, *Medicago*, wheat etc. [5]. This interaction between plant and microbe lead to benefits for both organisms where AM fungi will obtain a fraction of the plant’s carbon supplies and in return, the plant will receive numerous benefits that can improve the growth and development of host plants and eventually the environmental sustainability. Arbuscular mycorrhizal fungi (AMF) facilitate host plants to grow vigorously under stressful conditions by mediating a series of complex communication events between the plant and the fungus. Uptake of nutrients by plants is limited by the insufficiency of biologically available forms of these compounds in the environment. Because of selection pressure for survival, plants have broadly evolved beneficial symbiotic interactions with commensal microbes. AMF have developed a symbiotic relationship with most land plants, which is highly beneficial for the uptake of minerals and water from the soil [41].

The host plant colonized with mycorrhizal fungi has two pathways for nutrient absorption: A direct pathway where root epidermis and root hairs help to uptake nutrients and mycorrhizal pathway where the available nutrients ger transported indirectly via AM fungal hyphae from external mycorrhizal hyphae into internal mycorrhizal hyphae and then root cortex where arbuscules provide the symbiotic interfaces [42].

Nutrients	**Transporter's name**	**Species**	**References**
Sugar	SUTs, SWEETs, MST,	*Medicago truncatula Pisum sativum*	[43-45]
Lipid	*STR, STR2*	*Medicago truncatula*	[46, 47]
Phosphorus	*MtPT4, 8*	*Medicago truncatula*	[48, 49]
	LjPT4	*Lotus japonicum*	[50]
Potassium	Cation/H+ exchanger	*Medicago truncatula*	[51]
Nitrogen	GmAMT4.1	*Glycine max*	[52]
	AMT2;3	*M. truncatula*	[49]
	LjAMT2;2	*L. japonicum*	[53]
Sulphate	*MtSultr1;2*	*M. truncatula*	[54]
	LjSultr1;2	*L. japonicum*	[55, 56]

Table 1. 1 A list of transporters from different species of host plants and symbiotic Fungi

2.3.1 Common mycorrhizal networks

Mutualistic symbiosis between mycorrhizal fungi and plants are very common. When two or more plants of the same or different species are connected via a mycorrhizal network consisting of one or more different fungal species, they are connected via the so-called common mycorrhizal network (CMN). There is growing evidence that plant growth [57], physiology [58], survival and fitness [59], behavior, competitiveness and soil properties [60] are improved via these networks. Not only the soluble nutrients, but also C can be transported through this CMNs from one plant to another neighborhood plants [61, 62]. It has been found that supply of photosynthates (C) from host plants increased when fungus increased the transfer of N and P [63, 64]. Another study showed that AM fungi allocated more nutrients to non-shaded host plants which were also able to transfer more C to the mycorrhizal fungi [65]. How exactly C is transferred via the mycorrhizal network has not been fully uncovered. It is possible that C atoms are transferred in the form of amino acids such as glutamate and glutamine by which also N can be transferred [66]. It has also been reported that belowground community composition has substantial influence on aboveground species diversity [67]. Growth competition experiments revealed that not every plant benefit from a common mycorrhizal network to the same extent. Depending on the fungal network and plant community composition, some plants show reduced competitiveness in terms of biomass production compared to being grown with the fungus alone [66]

To understand and locate the belowground mycorrhizal networks, various tools and techniques are applied for example, microscopic analysis and nutrient tracer techniques. CMNs mediated transfer of ^{32}P from the source plant to the neighboring via CMN mediated

transfer, [65] ^{15}N isotopic tracer technique has provided strong evidence of nutrient absorption in the host plants and the neighboring plants by CMNs [68] interplant transfer of carbon by providing $^{14}CO2$ [69]. Therefore, studies of these below-ground mycorrhizal networks can provide the bases for nutrients sharing between the interconnected plants. CMNs also play an important role in the plant-to-plant' communication 'by transferring info chemicals and warning signals between plants. Plants that are attacked by herbivores produce volatile organic compounds that act as a repellent for aphids but attract the natural enemies of aphids to the infested leaves. These volatiles are only produced by non-infested plants when they share a CMN with infested plants. These warning signals between plants within one CMN are transmitted rapidly, and non-infested plants up-regulated genes of the jasmonate defense pathway shortly after plants within their CMN were attacked by herbivores [70].

2.3.2 Development of mycorrhizal symbiosis

2.3.2.1 Establishing connections

The colonization process of AMF fungal to the roots of host plant is categorized by different stages involving a series of complex morphogenetic changes in the fungus as well. Those include spore germination, hyphal differentiation, appressorium formation, root penetration, intercellular growth, arbuscule formation, and nutrient transport [71-73]. The development of symbiosis between host roots and AM fungi is based on signal exchange between both partners which establish a symbiotic state by triggering the coordinated differentiation leading interaction between them. Strigolactones and other root exudates stimulate the fungal spore germination that later forms an extensive hyphal branching near host roots [74]. At the root surface, the fungal hypha differentiates into a hyphopodium and

enters the rhizodermis. The fungus progresses through the outer into the inner root cortex and spreads intercellularly along the longitudinal axis of the root, forming highly ramified structures, termed arbuscules, inside cortex [75]. At the whole root level, development of the AM symbiosis is asynchronous, with various stages of colonization being present simultaneously. The lipochitooligosaccharides, or Myc factors released 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 rhizobia Nod factors [76]. In *M. truncatula*, these signals help to stimulate lateral root formation and to induce the expression of a gene (*MtENOD11*) also induced by Nod factors [77]. Myc or Nod factors by the rhizodermis plays a important role in the membrane-bound receptor-like kinase SYMRK led activation of HMGR1(3-hydroxy-3-methylglutaryl CoA reductase 1), a mevalonate (MVA) biosynthetic enzyme. Shortly after Myc perception, A second set of CSSP protein that includes three nucleoporins NUP133, NUP85, and NENA, Ca^{2+} pump MCA8 and CASTOR and POLLUX (cation channels) involved in Ca^{2+} oscillation in nucleus of rhizodermal cells. Another set of proteins which decodes Ca^{2+} signals [41, 78] resides in nucleoplasm and then phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase (CCaMK) occurs by the help of calmodulin CYCLOPS. This led to gene expression regulation either directly, or through NSP1, NSP2, and RAM1 (GRAS transcription factors) [41, 78, 79]. The proteins involved in the CSSP is highly conserved even in the plants that are not colonized by AMF. Moreover, defects in CCamK found to have an impact on arbuscule development. Based on these it is assumed that the interaction

between plants and AM fungi led to the formation of a symbiotic signaling pathway which set the origin for the rhizobium-legume symbioses [41, 80, 81].

2.3.2.2 Arbuscule formation and development

The arbuscles are the interface between the mycorrhiza and roots and are responsible for the exchange of carbon that the fungi require for energy. Once the root contact is established, hyphopodium (Figure 1.1) is formed from hyphae. A hollow tube is formed from which fungal hyphae can grow on it. This led to enable symbiotic colonization of root cortex including development of dichotomously branched hyphae, called arbuscules [41, 81]. Arbuscules are terminally differentiated, and they develop on side branches that arise from the long intercellular hyphae (Figure 1.1). These elaborate structures form inside the plant cell but remain separated from the plant cell cytoplasm by an extension of the plant plasma membrane that surrounds the fungus and follows the contours of the hyphal branches [82]. These also include formation of vesicle and spores. The developing arbuscule is surrounded by the plant plasma membrane called as periarbuscular membrane (PAM). Whereas the space between the PAM and the fungal plasma membrane is the periarbuscular space (PAS). The exchange of nutrients and carbohydrates takes place in PAS.

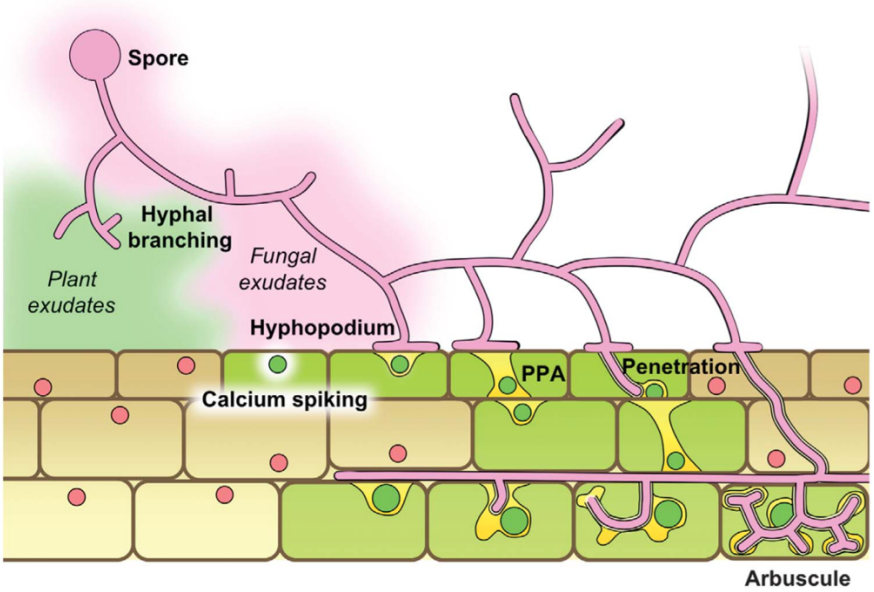

Figure 1.1 Plant exudates attracted the AMF fungal spore and they are germinating and growing towards plants roots [83]

3 Tripartite symbioses with arbuscular mycorrhizal fungi and rhizobia

Most of the Leguminous plants like soybean, *Medicago*, cowpea, etc. can form tripartite symbiotic associations with not only nodule-inducing rhizobia but also AMF simultaneously, which may provide both P and N benefits [84]. But depending on the crop, uneven effects of co-inoculation with rhizobia and AM fungi have been observed [84, 85]. Synergistic effects on fababean were found by co-inoculation with rhizobia and AM fungi [86]. In contrast, none or negative responses to co-inoculation have been reported in green gram and pea [87, 88]. Depending on the environmental condition, AM fungi and rhizobia interact antagonistically or synergistically [89]. And the compatibility between symbiotic partners play role on the host plant response [90, 91]. For example, STM 7183 a rhizobia strain co-inoculated with AM fungus *Rhizophagus clarus*, resulted higher biomass, nodulation, nitrogenase activities compared to the plants that were inoculated only with STM 7282 [90]. Likewise, co-inoculation with AM fungus *Rhizophagus irregularis* and

rhizobia led to increase in plant productivity and seed yields than with other AM fungi *Gigaspora gigantea* or *Acaulospora tuberculata* [91]. Not only the root symbiont strain, the plant host (for example, soybean) cultivars affect the benefits that are provided by microbial interaction [92]. An increase in the nutrient efficiency and production of legumes via symbiosis therefore represents an urgent research priority to integrate in soybean breeding programs, and AM fungi and N-fixing bacteria with high compatibility should be identified that results cost-effective stress resistance and environmentally sustainable crop production in the future.

For host plant, both symbiosis are costly allocating up to 20% of its photosynthates (C) to its fungal partner [93, 94], and up to 30% to its N-fixing symbionts (Figure 1.2) [95]. A reduction in BNF by rhizobia inoculated roots [96], and P and N uptake and transport by AM fungi [97-99] was found when C fluxes decreases in symbionts. This indicates an important role of C in symbiosis.

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 the control mechanisms are poorly understood. How the host plant mechanisms to allocate its C under selective pressure are not well known. Resource exchange between host and AM fungi are controlled by a reciprocal reward mechanism that is driven by biological market dynamics [100]. Nutrient demand by host plant and fungal access to exogenous nutrients can play an important role for the carbon transport to different root symbionts of tripartite systems. It has been found that plant allocated more carbon to rhizobia under nitrogen demand, but access to N allocated more carbon to the fungal partner [101] confirming AMF as a strong competitor for C resources [102]. Based on different nutrient supply conditions, host plant

changes its carbon allocation to different root symbionts to maximize its benefits. For N, P and sugar transport from host to the root symbionts, multiple transporters play role. Some of them are highlighted below (Figure 1.2).

3.1 Legume rhizobia carbon transfer

Sucrose gets divided into glucose and fructose in plant cytosol by Alkaline invertase or Uridine diphosphate (UDP) glucose and fructose via sugar synthase, which is catabolized via glycolysis and forms Phosphoenolpyruvate (PEP). Carbon from Phosphoenol Pyruvate (PEP) and carbonic acid diverted to Oxaloacetic acid (OAA) and then malate by the enzyme called PEP carboxylase and the Malate dehydrogenase (MDH), respectively. OAA may be converted to succinate or fumarate. Carbon sources (OAA, fumarate, succinate) are now transported across the peri-bacteroid and bacteroid membranes and go into the Tricarboxylic acid (TCA) cycle in the bacteroid where metabolism takes place. On the other hand, atmospheric di-nitrogen is converted to ammonia (NH_3) and subsequently ammonium (NH_4^+) through symbiotic nitrogen fixation. Amino acids and/or ureides are then produced and distributed from the legume nodules to the shoots. The determinate-nodule legumes primarily transport ureides as fixed-N compounds, while the indeterminate nodules assimilate asparagine (Asn) and glutamine (Gln) [36, 37]. Several sugar transporters are also likely to be involved in the sugar transport between legume plants and nitrogen-fixing Rhizobia (e.g., *SWEET15, SWEET11, LjSWEET3* in *L. japonicum*, vacuolar SUT4-type sucrose transporters, and monosaccharide STP transporters) (Figure 1.2).

3.2 Legume-AMF carbon transfer

Arbuscular mycorrhiza (AM) symbioses contribute to global carbon cycles. Conversion of atmospheric CO_2 into organic carbon in presence of sunlight takes place in photosynthetic leaves. As mentioned earlier, host plants provide to AMF which fluxes toward the colonized roots and is tightly controlled by both host plant and fungus. From endodermis, photosynthates carbon (sucrose) is delivered to arbuscule containing cells simplistically. In the cytoplasm, Sucrose can be broken down by sucrose synthase (SS) or invertase to glucose (Glc) and fructose (Fru). In most plants, sucrose is translocated from the sources to sinks through the sieve element/companion cell complex of the phloem [48]. To maintain the optimum concentration gradient, hexoses can translocate into the vacuole via tonoplast located SWEETs or other transporters. Alternatively, a direct export of sucrose into apoplast or periarbuscular membrane (PAM) via transporters like *MtSWEET12a*. From apoplast hexose is taken up by either of plant monosaccharide transporter (*MST1*) or via sucrose transporter SUT2 (shown for *S. lycopersicum*-SlSUT2). In addition to this vacuolar sugar transporters (*SUT4*-type and *StSWEET2c*) are also activated in AM colonized cells, sugar now exported towards the symbiotic interface, across the PAM, possibly via sucrose effluxers of the SWEET family (for example *MtSWEET1b in M truncatula, StSWEET7a* and *StSWEET12a* in *Solanum tuberosum*) [103, 104]. Mycorrhizal colonization of host plant roots increases the sink strength to unload more sucrose from phloem and is associated with increased expression of several sucrose transporters (SUTs/SWEETs) in leaves and in colonized roots of *M. truncatula* and *Pisum sativum* [43, 44]. The carbon obtained by AM fungi from host plants must be transported from the intraradical mycelium (IRM) to the extraradical mycelium (ERM) to support the

development of hyphae and production of spores. Not only the sugars but also the fatty acids synthesized in the host plants are transferred to the fungus to sustain mycorrhizal colonization [47].

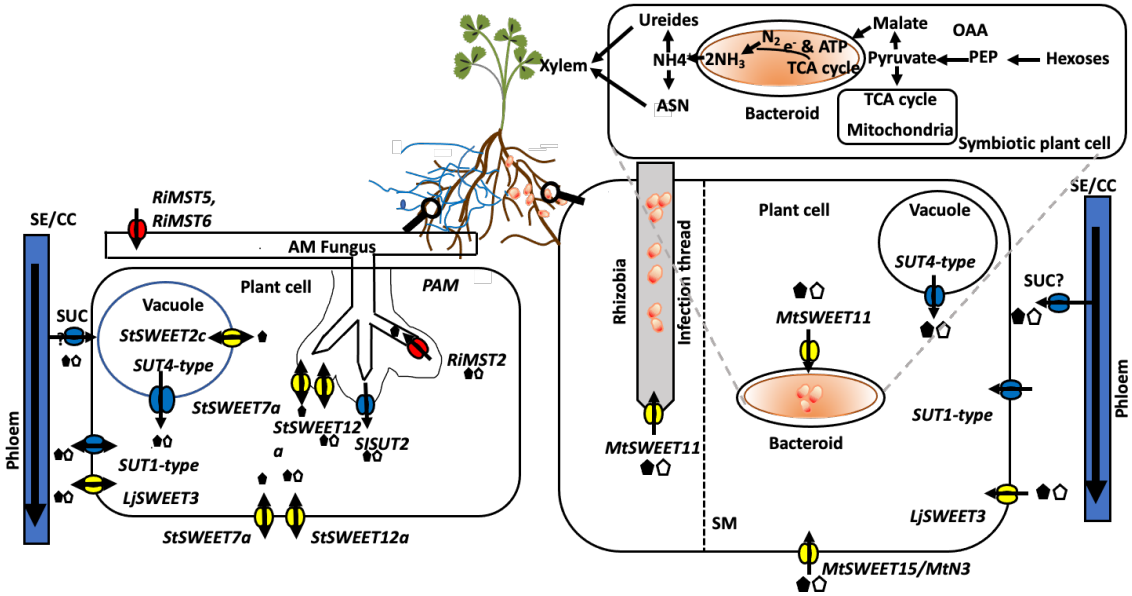

Figure 1.2 Molecular mechanism involving transport of sugar in legume- rhizobia (Right) and legume -AMF symbiosis (Left) (modified from Hennion et al (2019) and Liu et al (2018) During symbioses with mycorrhiza and rhizobia, plants deliver nutrients (N, P) to the host plants, and host plants allocate source photosynthates (sugars, organic acids, and lipids) to root symbionts. PAM: Peri-arbuscular membrane, SM: Symbiosome membrane, SUC: Sucrose, OAA: Oxaloacetic acid, TCA: Tricarboxylic acid cycle, SE: Sieve element, CC: companion cells [105].

4 Role of AMF to biotic stress tolerance

Pest and disease cause serious losses of crop plants. Plant diseases can be managed in various ways, including cultural controls, chemical controls such as fungicides, bactericides, insecticides, nematicides and herbicides, and biological controls [106]. Most prominently farmers use chemical controls to alleviate the pathogen effect. These chemicals can have very adverse in environment for example ground water pollution. Use of Microbes for controlling plant diseases are safer and maintains environmental

sustainability compared to chemical pesticides; however, this might be less effective [107, 108].

Plants colonized with AMF present enhanced resistance to several soilborne pathogens [107, 109]. Previous studies have demonstrated reduced disease incidence of various pathogens such as *Fusarium*, Rhizoctonia, pathogenic bacteria, *Phytophthora*, *Pythium,* and parasitic nematodes. The presence of mycorrhizal fungi in the roots results in the establishment of a mycorrhizal-induced resistance (MIR), a type of priming that could explain why colonized plants are more resistant to this large set of soil pathogens. There are several ways that AMF may reduce the incidences disease, including enhanced nutrition, competition for nutrients and infection sites, change in the root morphology and architecture, chemical changes in the plant for example, root exudates, easing the plant stress and change in microbial community in rhizosphere [108, 110]. However, there are few evidence of aboveground pathogen altered by AMF colonization. Inconsistencies were found in disease severity that might be due to the lifestyle of foliar pathogens.

5 Important research gaps and future challenges

Beneficial plant microbe interactions with AM fungi and rhizobia have enormous potential to improve plant growth and nutrient uptake in stressful environments and to increase the environmental sustainability of soybean agriculture. However, limited information is known about nutrient exchange during tripartite interactions.

AMF being obligate biotrophs, production of fungal inoculum in large scale is challenging. But the advancement of sterile transgenic root organ cultures has mitigated this problem and it has been increased commercialization of AM fungal inocula for utilization in agroecosystems [111]. Furthermore, asymbiotic growth of AMF has been

accomplished recently. External supply of strigolactones, 2-hydroxy fatty acids, palmitoleic acid, myristate and branched fatty acids [112-115] help generate a pure culture of biotrophic AM fungi and also produce spores capable of infecting plants. Although increases in yield and biomass have been reported in different crops (cotton, potatoes) [116, 117], by AMF inoculation, some reports also observed inconsistent or neutral effects [118]. So, mycorrhizal growth responses are highly context-dependent [119]. The compatibility between host plant and fungi, microbial population/community that fungi need to compete with and also the inoculation time significantly affect fungal performance [120]. Currently our understanding of the effect of beneficial plant microbes on soybeans and other legume crops is mainly based on studies with single root symbiont, but in natural condition plants interact with a diverse group of microorganisms where microbes themselves interact with each other.

Previous research on molecular mechanisms responsible for compatible plant microbe interactions has allowed us to harness and utilize beneficial symbiotic microbes in agroecosystems. Most of the research is focused on model legumes, such as *Medicago truncatula*, but the information about soybeans and other legume crop is limited. However, the accumulation of genomic and transcriptomic data , along with the development of molecular tools such as development of transgenic lines [e.g. 121], CRISPR-Cas9 system for gene editing [122], or knock out/down mutant populations will provide us with a better understanding of these interactions in soybeans. This information will allow us to conduct a good farming practice to maximize benefits from symbiotic microorganism and this led reduction on our growing dependence on synthetic/chemical fertilize

CHAPTER 2: NUTRIENT AND CARBON ALLOCATION STRATEGIES IN TRIPARTITE INTERACTIONS OF *MEDICAGO TRUNCATULA*

2.1 Abstract

Leguminous plants maintain root symbioses with two nutritional mutualists: rhizobia that fix atmospheric nitrogen and arbuscular mycorrhizal fungi (AMF) that enhance uptake of mainly phosphorus(P) and Nitrogen (N) from the soil and delivers it to host plants. In return, both root symbionts receive plant carbon. We hypothesized that when AMF interacts with the rhizobia that lack BNF capability, a significantly higher amount of carbon will be delivered to mycorrhizal roots, and that AM fungal access to exogenous N increases plant C allocation towards mycorrhizal roots and change in expression of key transporters. We conducted a customized pot split-root experiment to test the effect of an arbuscular mycorrhizal fungus (*Rhizophagus irregularis*), on host plant's (*Medicago truncatula*) carbon allocation pattern, N acquisition, and gene expression dynamics when the host plant was also colonized with rhizobia (*Ensifer meliloti*) that have capability to fix atmospheric N (Fix+) and a mutant rhizobium of same strain (FixJ2. 3::Tn5233) which lacks N fixing capability (Fix-). Dual inoculation with N fixing rhizobia (Fix+) and AMF showed synergistic beneficial effects on shoot biomass, enhanced nitrogen, and phosphate uptake in the sink but unable to deliver it toward source for further development of host plants. On the other hand, tripartite interactions of Fix- rhizobia that lacks biological N fixation activity and AMF lead to significant increase in the N uptake and delivery towards the roots whereas carbon allocation on Fix- rhizobia was significantly lower. Consistent with these changes in C allocations, we found several SUTs and SWEETs expression shifting their expression pattern. SUTs like *MtSUT1-1*, *MtSUT2* and *MtSUT4-1* primarily uptake

sucrose from phloem and unloads towards the root as well as root symbionts providing benefit to the host plants. *MtSWEET1b, MtSWEET12* and *MtSWEET13* involved in allocating sugars to mycorrhizal roots whereas *MtSWEET3c*, *MtSWEET12* in rhizobia root of tripartite interactions. We also found *MtSWEET1b* gene upregulation in case of rhizobia symbiosis. Our results demonstrate substantial information about how host plants control its carbon allocations under different status of N demand in terms of rhizobia and AMF. The change in N allocation to different root symbionts increase the symbiotic benefits.

Keywords *Medicago truncatula, Rhizophagus irregularis, Ensifer meliloti*, phosphate, nitrogen, carbon

2.2 Introduction

Legumes (Fabaceae) are the third largest family of angiosperms with 750 genera and around 19,500 species, including important crops such as soybean, faba bean, cowpea, and *Medicago* [123]. Many legumes can form root endosymbiosis with rhizobia and arbuscular mycorrhizal fungi (AMF). Rhizobia are diazotrophic bacteria capable of fixing atmospheric nitrogen in nodules that they induce on the roots of legumes. Within these root nodules, the host plant provides rhizobia with carbon (C) in the form of dicarboxylates in exchange for the fixed nitrogen (N) that rhizobia provide to the host as NH_4^+ and amino acids [124]. Rhizobia can vary dramatically in the benefits they provide to their host plant. For example, some naturally occurring strains of rhizobia fix little or no N even though they can successfully induce nodule formation [125, 126]. Rhizobia strains that do not perform Biological Nitrogen Fixation (BNF) are sanctioned by the host and receive less C [127]; however, those that do can receive up to 30% of the host plant's photosynthate to support BNF [128]

Legumes are among the 72% of land plants that form a mutualism with AMF [129]. AMF form extensive hyphal networks in the soil and forage for nutrients beyond the rhizosphere [39, 130]. Thus, they improve the host plant's ability to acquire phosphorus (P) and N from the soil [39, 65, 131]. As an obligate biotroph, AMF rely exclusively on their plant partners to meet their C requirements and, as such, may exert significant C demands on the host, which allocates up to 30% of its photosynthetically-derived C to the fungus [132-134] as lipids and/or sugars [47, 135, 136]. Evidence suggests that the amount of plant C transferred to the root symbiont may be positively correlated with the host assimilation of fungal acquired nutrients [101]. The amount of C the host transfers to AMF

is regulated by the host based on the amount of mineral nutrients the fungus provides from the soil. In fact, plants can discriminate between AMF species and provide more cooperative partners with more C [64]. In the past, detailed ^{13}C-labeled studies demonstrated that hexose sugars are a main vehicle for C transfer from plants to fungi [137]. The roots of legumes can be simultaneously colonized by rhizobia and AMF, resulting in a tripartite interaction that provides synergistic benefits for the host and both microbial partners; however, the degree of the benefits that each symbiont provides depends on the nutrient status of the plant [138].

Sucrose is the primary substrate that plants translocate via phloem from source to sink organs [44]. It is also one of the substrates involved in C flux towards rhizobia and AMF [43, 44, 124, 139]. Both *MtSWEET11* and *LjSWEET3* seem to be involved in sucrose distribution within nodules in *M. truncatula*; however, knock-down/out studies revealed that neither one is required for the success of the rhizobia-legume symbiosis, most likely because their function can be compensated for by other SWEET transporters [140, 141]. One sucrose is delivered to infected cells in nodules, it is metabolized into malate in the cytoplasm and is then translocated across the symbiosomal membrane and taken up by the bacteria to use as a C source for fueling BNF. In the AM symbiosis, sucrose is delivered to arbuscule-containing root cortical cells symplastically through the endodermis. Here, various sugar transporters appear to be involved in the export of sugars to the fungus. Sugar Will Eventually be Exported transporters (SWEETs) are involved in both the efflux and influx of sugars and are the most likely candidates for sugar efflux to AMF [104, 142, 143]. Transcriptomic and promoter-GUS expression analyses revealed that *GmSWEET6, GmSWEET15d* and *MtSWEET1b* in soybean and *Medicago* roots, respectively, are induced

by AMF colonization [104, 144]. This implies that SWEET transports likely shuttle sucrose to the apoplast of cortical cells where it is taken up by the intraradical hyphae, or sugar is sent across the peri-arbuscular membrane for uptake by the arbuscule. Plants also provide AMF with lipids [47, 136, 145].

The C cost of colonization by rhizobia or AMF is high, and yet, the roots of legumes can associate with both endosymbionts simultaneously. It is therefore important to understand how legumes manage these tripartite interactions to ensure that they result in a net yield increase rather than a major C loss. A few studies report a synergistic effect of dual symbiont inoculation in legumes. However, these studies were conducted with high-benefit symbionts, but low benefit rhizobia and AMF do exist in nature as strains of Rhizobium present in soils may range from highly efficient symbionts to those that are capable of nodule formation but are unable to reduce atmospheric N [146]. 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 with plants associated with a single symbiont. We predicted that in association of AMF and inefficient rhizobia, AM behave as a strong role for plant growth and development, but degree of benefit may vary with the nutrient availability to the hyphae of AMF. The access of N to the AMF led to changes in the cost and benefit by both partners and host strategies towards carbon allocation on these root symbionts. In this ecological relevant tripartite system (Figure S2.1) because AMF and efficient rhizobia each provide plants with essential soil nutrients, we expected that co-inoculation would result in the strongest synergistic effects in conditions to improve plant performance. This knowledge is critical to improve the nutrient efficiency and symbiotic benefits in agriculturally important legumes. In this

sense, we hypothesized that host plant allocates significant amount of carbon toward the root symbionts which helps to fulfill the demand of nutrients to the plants. When any of the symbionts fail to deliver nutrients, the host plant punishes the inefficient root symbiont. The sugar transporters involved in the phenomenon are context dependent.

2.3 Materials and method

2.3.1 Plant culture

Medicago truncatula A17 seeds were acid scarified using 36N H_2SO_4 for 8 min, rinsed several times with tap water, and sterilized with 8% household bleach for 2 min. Sterilized seeds were thoroughly rinsed with sterile deionized water. The seeds were then incubated in water with 1 mM gibberellic acid (GA_3) at 4°C for 2 d and then transferred onto sterile, moist germination paper in square Petri dishes (23cm x 23cm), wrapped in aluminum foil, and incubated in the dark for 4 d. Then, the seedlings were removed from the dark and placed on a benchtop with ambient light for 4 d.

To accelerate lateral root development, the tip of the primary root of the germinated seedlings was excised using a sterile scalpel. The seedlings were then transferred into a hydroponic tank measuring 54.3 cm × 43.5 cm × 13 cm (L × W × H) filled with sterile low nitrogen (N) and phosphate (P) modified Ingestad's solution ((0.05 mM KH_2PO_4, 0.125 mM NH_4NO_3, 0.30 mM KCl, 0.5 mM $CaCl_2 \cdot 2H_2O$, 0.312 mM $MgSO_4 \cdot 2H_2O$, 6.8 µM Fe-EDTA, 1.50 µM $MnCl_2 \cdot 2H_2O$, 8.08 µM H_3BO_3, 0.05 µM Zn-EDTA, 0.14 µM $CuCl_2 \cdot 2H_2O$, and 0.01 µM $Na_2MoO_4 \cdot 2H_2O$[147]. The plants in the hydroponic system were incubated in a growth chamber (TC30, Conviron, Winnipeg, MB, Canada) with a photosynthetic photon flux of ~225 µmol m^{-2} s^{-1}, a 16 h photoperiod, 25 °C / 20 °C day/night temperatures, and a relative humidity of 60%. Twice daily the nutrient solution

was agitated with a sterile rod to homogenize nutrient and oxygen levels. The nutrient solution was replaced once after 7 d.

After 16 d in the hydroponic system, we transferred the plants into sterile, custom-made, three-compartment pots (12 cm x 8 cm x 8 cm, L x W x H) comparable to those used previously (Figure S2.1) [101]. Briefly, two root compartments (RC 1 and RC 2) were separated by a 0.1 cm thick plastic divider. The hyphal compartment (HC) was separated from RC 2 by a divider with a center hole measuring ~3.12 cm in diameter. The hole was covered on both sides with 50 µm nylon mesh and a coarse nylon mesh with a pore size of 1000 µm was placed in between to provide an air gap and prevent mass flow while still allowing fungal crossover from RC 2 to the HC. Before transferring the plants, all the pots were sterilized by keeping them in 3% bleach solution for 2 hours and dried overnight in oven at 70^0C. All compartments were filled with ~200 ml of sterile soil substrate consisting of 60% sand, 20% perlite, and 20% organic soil (v:v:v). We divided the root system of each plant into two nearly equal halves, and each root half was placed into one of the RCs. After transplanting, the plants were grown in the same growth chamber and watered with sterile DI water as needed.

2.3.2 Fungal and bacterial inoculum

The fungal inoculum was derived from Ri T-DNA carrot (*Daucus carota* clone DCI) root organ cultures colonized by *Rhizophagus irregularis* DAOM197198 and grown on minimal medium [148]. After approximately 12 weeks of growth, the spores were isolated by blending the medium in 10 mM sodium citrate buffer (pH 6.0). One-week post-transplanting, nearly 450 *R. irregularis* spores and ~0.1 g of carrot roots was deposited in

the mycorrhizal root compartment (RC 2) in a ~5 cm hole in the soil substrate adjacent to the root.

The bacterial inoculum was prepared by growing either *Ensifer meliloti* Dangeard (1021) (Fix +) or the *E. meliloti* FixJ mutant (V02675 - *E. meliloti* 1021 (FixJ2. 3::Tn5233) in tryptone yeast (TY) broth on a rotatory shaker at 220 rpm at 30 °C for 24 h. The *E. meliloti* Fix J mutant is compromised in a promotor fused to the bacA gene, which causes stops bacteroid differentiation in the nodule and prevents nitrogen fixation [149-152]. Before inoculation, the bacteria were pelleted by centrifugation and resuspended in autoclaved Ingestad's nutrient solution without N and P [147] to an OD_{600} of 0.1. Six weeks after the AM inoculation, 10 ml of bacterial suspension was added to the rhizobial root compartment (RC 1).

2.3.3 Experimental design and stable isotope labeling

The root halves were separately inoculated in the following combinations: an AM inoculated root half in RC 2 combined with either *E. meliloti* Fix+ or Fix- inoculated root half in RC1 (Fix+/AM or Fix-/AM), an uninoculated root half in RC 1 combined with an AM inoculated root half in RC 2 (C/AM), an uninoculated root half in RC2 combined with a *E. meliloti* Fix+ or Fix- root half in RC 1 (Fix+/C or Fix-/C), and uninoculated root halves in both RC 1 and 2 (C/C). There were three to six biological replicates for each treatment. To induce nutrient demand and ensure nodulation and mycorrhizal colonization, all plants in each treatment were fertilized two times with low N and P (250 μM NH_4NO_3, 50 μM KH_2PO_4) modified Ingestad's nutrient solution throughout the experiment [147].

Three weeks post-inoculation with the rhizobia, several extra plants were destructively harvested to confirm both AM and rhizobia colonization of the roots and

hyphal crossover from the AM colonized RC 2 into the HC. After confirming colonization, we added a low P Ingestad solution spiked with 4 mM $^{15}NH_4Cl$ (Sigma Aldrich, St. Louis, USA; $+^{15}N$) to the HC of the growth chamber systems with AM colonized root halves in RC 2 (C/AM, Fix+/AM, and Fix-/AM). To confirm no mass flow occurred between the HC and RC 2 in the absence of hyphae, we also added 4 mM $^{15}NH_4Cl$ to the HC of C/C growth chamber systems. Since none of these control plants showed any significant ^{15}N labeling above natural ^{15}N abundance, these plants were later treated as non-labeled controls. To the HCs of all other plant systems, an equal volume of sterile DI water was added. Two weeks after ^{15}N labeling, all experimental plants were labeled with $^{13}CO_2$ by placing them in an airtight chamber (76 × 61 × 15.6 cm, L × W × H), in which 69 µL mL^{-1} $^{13}CO_2$ was released. A battery-powered fan was placed in the center of the container to promote a homogenous distribution of the $^{13}CO_2$ within the chamber. After 2 h, the plants were removed from the sealed containers and were allowed to allocate fixed ^{13}C throughout their tissues for 24 h.

2.3.4 Plant harvest

All plants were destructively harvested 13 weeks post-transplanting. The fresh weight of shoots and roots were taken and both tissues were divided into different subsamples aliquots. From both shoot and root tissues, 0.1 to 0.3 g subsamples were flash-frozen in liquid nitrogen and stored at -80 °C for future RNA extraction and gene expression analysis. Additional fresh root subsamples were taken to evaluate the AM colonization and to conduct acetylene reduction assays (ARA) as described below. The root subsample for evaluating AM colonization were stored in 50% ethanol (v:v) at 4 °C.

Residual shoot and root tissues were dried at 70 °C to evaluate dry mass, stable isotope enrichment (^{15}N and ^{13}C), and both N and P contents.

2.3.5 AM colonization and acetylene reduction assays

Subsamples of roots colonized with AM fungi were cleared by incubation in 10% KOH at 90°C for 2 h, rinsed with water several times, and stained by incubation in 5% Sheaffer ink - vinegar (v/v) at 90°C for 30 min [153]. At least 80 stained roots segments per plant were then examined for AM colonization using the gridline intersection method [154].

We examined the nodulated and control roots for their nitrogenase activity using the acetylene reduction assay as described previously [155]. At plant harvest, the root aliquots were carefully placed on sterile moist filter paper in 30 ml glass tubes. All tubes containing root samples were sealed with a rubber septum at the same time and 3 ml (10 %, v:v) of acetylene gas was immediately injected into each tube using a syringe. After 24 h, we measured the production of ethylene using an Agilent Technologies 7890A Gas Chromatography System (Santa Clara, CA, USA). Multiple standards of ethylene gas were used to generate a calibration curve and sample peaks were then fitted to the curve. We normalized the ethylene production by the total number of nodules of each root aliquot and calculated the amount of acetylene reduced per nodule. The root subsamples used for ARA were also used for nodulation assay. After counting the total nodule number on root subsample, we normalized them according to total biomass for each root halves.

2.3.6 Phosphate and stable isotope (^{13}C and ^{15}N) analysis in plant tissues

After drying, shoot and root aliquots were pulverized with a tissue homogenizer (Precellys 24, Cayman Chemical Company, Ann Arbor, MI, USA). To determine the P

content, the homogenized plant tissue was first digested with 1 ml of 2N HCl for 2 h at 95 °C, and P was measured spectrophotometrically at 436 nm after adding ammonium molybdate vanadate (AMV; (Fisher Scientific, Pittsburgh, USA). Both ^{13}C and ^{15}N enrichment in shoot and root tissue samples 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 $\delta^{13}C$ into the ^{13}C contents in plant biomass was conducted as reported previously [156]. The percentage of ^{13}C and ^{15}N allocation was calculated based on the total recovered $\delta^{13}C$ and $\delta^{13}N$ from the plant tissues after ^{13}C and ^{15}N labeling.

2.3.7 Gene expression analysis

We analyzed the relative expression of three Sucrose Uptake Transporters (*MtSUT1-1, MtSUT2*, and *MtSUT4-1*; [primers acccording to 43], seven Sucrose Will Eventually be Exported Transporters (*MtSWEET1b, MtSWEET3c, MtSWEET9, MtSWEET11, MtSWEET12, MtSWEET13*, and *MtSWEET15c*), and two putative lipid transporters *STR* and *STR2* [46, 140]. As a control, we also evaluated the relative expression of two AM-induced genes, the P transporter *MtPT4* [48, 157] and the ammonium transporter *MtAMT2;3* [49, 158]. All steps for the DNase treatment of RNA, cDNA synthesis, and qPCR amplifications were performed according to the manufacturer's instructions. For the RNA extraction, the root samples were pulverized in a pre-chilled mortar and pestle with liquid nitrogen. Total RNA of frozen root tissue at -80 $^{\circ}C$ were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), Chloroform RNA extraction method. Isolated RNA was digested with TURBO™ DNase (Thermo Fisher Scientific, Waltham, MA, USA) following the manufactures instruction. The quantity and

quality of RNA was determined using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific), and RNA integrity was tested by using native agarose gel electrophoresis. Between 400 to 800 ng of DNase-treated RNA was used for the synthesis of cDNA using the Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific). All cDNA samples were diluted to a final concentration of 20 ng μl^{-1} and used for qPCR with the iTaq™ Universal SYBR® Green Supermix kit (Bio-Rad, Hercules, CA, USA) in a 20 µl reaction. The qPCR reaction was run in a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) using the following cycle: 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 with fluorescent signal recording at the end of each cycle; dissociation at 95°C for 15 s; 60°C for 15 s; and 95°C for 15 s. For all reactions, *Mtef1α* was used as a reference gene (primes list in supplementary data Table1). No template control reactions were performed for all sets of primers used in this experiment. The gene expression coefficients were calculated using the $2^{-\Delta Ct}$ method. The results are based on three to five biological replicates with three technical replicates.

2.3.8 Statistical analysis

All results were analyzed using a one-way ANOVA after fulfilling the assumptions by Levene's test of homogeneity of variance and Shapiro–Wilk normality test with model residuals. Data that did not meet the normality test were log-transformed before analysis (Table S2.2). Least significant difference (LSD) test was performed for multiple group comparisons. T-tests were performed for pairwise comparisons of certain groups. All statistical tests were performed at the significance level of $p \leq 0.05$.

2.4 Results

2.4.1 Degree of arbuscular mycorrhizal colonization, rhizobia-induced nodulation, and rates of nitrogen fixation

We examined the roots from all *Medicago truncatula* split-root systems inoculated with AM fungi and/or rhizobia, respectively. No cross-contamination was observed on adjacent root halves and uninoculated control roots remained uncolonized (Figure S2.2A, B. Both Fix+ and Fix- strains of *E. meliloti* colonized roots equally well, and the number of nodules present on each root half was not affected by the absence (Fix+/C, Fix-/C) or presence of AM fungi (Fix+/AM, Fix-/AM) on the adjacent root half (Figure S2.2A). Similarly, AM colonization did not differ among plants that were only inoculated with AM fungi on one root half, or with the Fix+ or Fix- strain of *E. meliloti* on the adjacent root half (Figure S2.2B). As expected, only Fix+ occupied nodules exhibited biological N fixation, and colonization of the adjacent root half with AM fungi (Fix+/AM) did not affect the rate of N fixation (Figure S2.2C).

2.4.2 Effect of tripartite interactions on plant biomass and both P and N content

Root and shoot dry weight were strongly dependent on the type of root symbiont colonizing each root half (Figure 2.1A, B). Root halves colonized by AM fungi consistently weighed significantly more than adjacent root halves colonized by either Fix+ or Fix- *E. meliloti* and uninoculated control roots, especially when the fungus had access to $^{15}N\text{-}NH_4^+$ in the hyphal compartment (Figure 2.1A). In contrast, the dry weight of roots colonized by Fix+ *E. meliloti* was significantly lower than that of the adjacent non-inoculated root halves. Finally, root dry weight was similar between roots colonized by Fix- *E. meliloti* and adjacent non-inoculated roots.

Shoot dry weight was highest in plants with one non-inoculated root half and the other colonized by Fix+ *E. meliloti* (Fix+/C; Figure 2.1B). Replacing the non-inoculated root half with roots colonized by AM fungi (Fix+/AM) caused a significant decrease in shoot biomass except when the fungus had access to $^{15}N\text{-}NH_4^+$ in the hyphal compartment. The shoot biomass of Fix-/AM plants followed this same pattern. Two of the control treatments, Fix-/C and C/AM, had shoot dry weights comparable to that of the uninoculated control (C/C). These results indicate that plant growth was particularly limited by N deficiency and that both root symbionts improved the supply of N to their host plant—Fix+ *E. meliloti* through biological N fixation, and *R. irregularis* through the transfer of $^{15}N\text{-}NH_4^+$ to the plant from the hyphal compartment.

The P content of adjacent root halves in the same split-root system was not significantly different for any of the colonization regimes (Figure 2.1C); however, some differences were observed for P concentration (Figure S2.3A). In the C/AM and Fix-/AM colonization regimes, roots colonized by AM fungi had a significantly higher P concentration, especially when the fungus had access to $^{15}N\text{-}NH_4^+$. For plants that were colonized on one root half by Fix+ or Fix- *E. meliloti* and on the adjacent root half by AM fungi, fungal access to $^{15}N\text{-}NH_4^+$ led to a significant increase in both the shoot P content (Figure 2.1D and P concentration (Figure S2.3B). However, the highest shoot P concentration was observed in the non-inoculated control plants, indicating that the P supply was not growth-limiting. The lower P concentration in the Fix+/C, Fix-/AM, and Fix+/AM colonization regimes without $^{15}N\text{-}NH_4^+$ addition to the HC is likely the result of a dilution effect due to the higher shoot biomass of these plants.

We consistently observed significantly higher N content in root halves colonized by AM fungi when the fungus had access to $^{15}N\text{-}NH_4^+$ as compared to all other root halves (Figure 2.1E). However, N concentration was only significantly elevated in the AM and Fix+ root halves in the C/AM and Fix+/C colonization regimes (Figure S2.3C) and both N content and N concentration in roots from the Fix-/C colonization regime were either significantly lower or equal to that of the non-inoculated control roots (C/C; Figure 2. 1E and Figure S2.3C). The delivery of total N to the shoot was consistently higher in plants from the Fix+ rhizobia inoculated plants (Fix+/C and Fix+/AM) regardless of whether the fungus had access to $^{15}N\text{-}NH_4^+$ or not in Fix+/AM systems over Fix- rhizobia inculcated or only AM inoculated systems (Figure 2.1F and Figure S2.3D). Conversely, fungal access to $^{15}N\text{-}NH_4^+$ in the Fix-/AM and C/AM inoculation regimes significantly increased shoot N content and concentration compared to tripartite system of Fix-/AM that do not had access to $^{15}N\text{-}NH_4^+$ uninoculated and Fix-/C control plants. As expected, shoots from plants in the C/C and Fix-/C inoculation regimes had lower N content and concentration. In summary, fungal access to N leads to an increase in shoot N content and concentration, particularly when the plant is not colonized by Fix+ *E. meliloti.* Thus, AM fungi can play a significant role in delivering N to their host plant even though their contribution is not as significant as that of Fix+ *E. meliloti*.

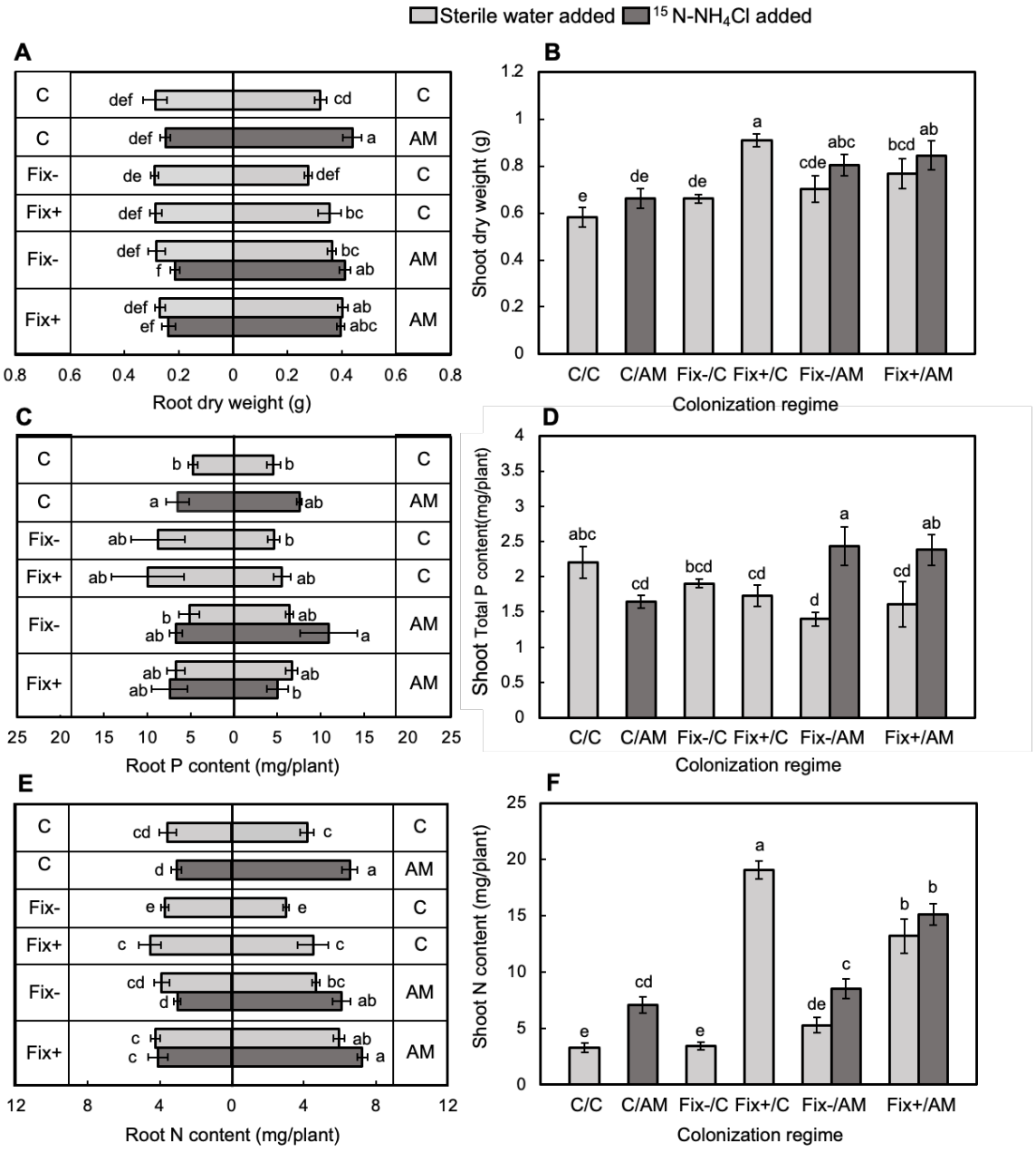

Figure 2. 1: Root (A) and Shoot (B) dry weights; P content in roots (C) and shoot (D); N content in roots (E) and shoot (F) of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions for the fungal partner (dark grey bars indicates addition of ^{15} N-NH_4Cl to the hyphal compartment; and light grey bar represents control plants (addition of sterile DI water to the hyphal compartment). Root colonization abbreviations: C/C: controls, both root halves non-inoculated; C/AM: one root half colonized by *Rhizophagus irregularis*, one root half non-inoculated; Fix-/AM: one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti* (fixJ mutant that lack biological N-fixation); Fix+/AM: one root half colonized by *R. irregularis*, one root half colonized by wild type *E. meliloti*. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$, n = 3 to 6).

2.4.3 Competition with rhizobia does not affect N uptake from the soil but reduces N transport across the mycorrhizal interface

We observed that δ ^{15}N enrichment and total ^{15}N content were significantly higher in the roots and shoots of plants colonized by AM fungi with access to ^{15}N-NH_4^+ (Fig 2.2A, B and Fig S2.4A, B, Figure S2.5A, B). In the AM roots, the levels of δ ^{15}N and total ^{15}N content for the C/AM, Fix-/AM, and Fix+/AM colonization regimes were the same; however, in the shoots, δ ^{15}N enrichment and total ^{15}N content were consistently significantly higher in the C/AM and Fix-/AM colonization regimes compared to the Fix+/AM regime. In the roots and shoots of plants without fungal access to ^{15}N-NH_4^+, δ ^{15}N enrichment and total ^{15}N content was very low; however, it appears that in the C/C regime, some ^{15}N-NH_4^+ in the hyphal compartment moved across the mesh barrier and into the root compartment where it was taken up directly by the plant. Thus, root and shoot δ ^{15}N enrichment and total ^{15}N content were slightly higher than expected, but not beyond the value of natural ^{15}N abundance and still significantly lower than plants colonized by AM fungi with fungal access to ^{15}N-NH_4, thus confirming that massive flow from the hyphal compartment to the second root compartment system was minimal. The pronounced differences in δ ^{15}N (Figure 2.2 B) and total ^{15}N (Fig S2.4B, Fig S2.5B) between the shoots of Fix+/AM and Fix-/AM colonization regimes can partially be explained by a dilution effect caused by an increase in shoot dry weight in the Fix+/AM regime. But it is also further explained by the ability of the AM fungus to take up nitrogen from the soil but not necessarily deliver it to the plant when Fix+ rhizobia are already fulfilling the plant nitrogen demand; thus, N transport across the mycorrhizal interface is not limited. Finally, it is also important to point out that mycorrhizal N acquisition was not

determined by the degree to which plant root systems were colonized by the AM fungus because all AM-inoculated roots were colonized equally (Fig S2.2c).

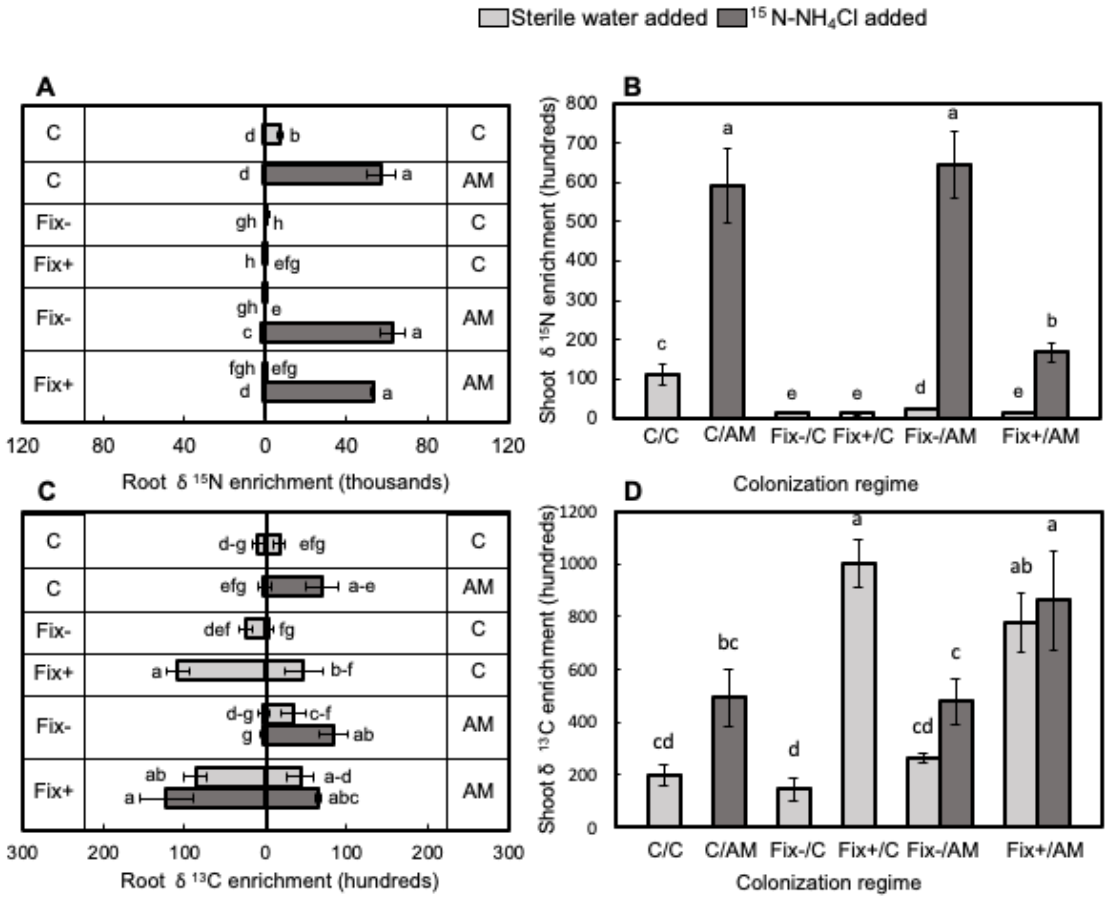

Figure 2.2: δ ^{15}N enrichment in root (A) and shoot (B); δ ^{13}C enrichment in root (C) and shoot (D) of Medicago truncatula depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions for the fungal partner (Dark grey bars indicates addition of ^{15}N-NH_4Cl to the hyphal compartment; and light grey bar represents control plants (addition of sterile DI water to the hyphal compartment). Root colonization abbreviations: C/C: controls, both root halves non-inoculated; C/AM: one root half colonized by Rhizophagus irregularis, one root half non-inoculated; Fix-/AM: one root half colonized by R. irregularis, one root half colonized by E. meliloti (fixJ mutant that lack biological N-fixation); Fix+/AM: one root half colonized by R. irregularis, one root half colonized by wild type E. meliloti. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$, n = 3 to 6).

2.4.4 Carbon allocation to root symbionts during tripartite interactions is dependent on their ability to provide the host with nitrogen

Overall, we observed that δ ^{13}C enrichment and total ^{13}C content were greatest in roots colonized by symbionts that were actively providing the host plant with N (Figure 2.2C, D**, F**igure. S2.4C, D). These included AM-colonized roots with access to ^{15}N-NH_4^+ in the C/AM, Fix-/AM, and Fix+/AM colonization regimes, and roots with nodules occupied by Fix+ *E. meliloti* in the Fix+/C and Fix+/AM colonization regimes. In the Fix-/AM colonization regime, while δ ^{13}C enrichment remained constant in roots with nodules occupied by Fix- rhizobia, it increased significantly in adjacent mycorrhizal roots when the fungus gained access to ^{15}N-NH_4^+ (Figure S2.4C). This trend did not occur in the Fix+/AM regime, rather δ ^{13}C enrichment slightly increased in both Fix+ and AM-colonized roots when the fungus gained access to ^{15}N-NH_4^+. Similarly, δ ^{13}C enrichment (but not total ^{13}C content) increased substantially in roots with nodules occupied by Fix+ *E. meliloti* in the Fix+/C regime compared to roots with nodules occupied by Fix- *E. meliloti* in the Fix-/C regime (Figure 2.2C; Figure S2.4C and Figure S2.5C).

In the shoots, total ^{13}C content and δ ^{13}C enrichment were highest in plants from the Fix+/C colonization regime, followed by the Fix+/AM regime when, first, the fungus had access to ^{15}N-NH_4^+ and second, when it did not (Figure 2.2D Figure S2.4D and Figure S2.5D). In the absence of Fix+ *E. meliloti*, shoot total ^{13}C content and δ ^{13}C enrichment dropped significantly in the Fix-/AM and C/AM colonization regimes, particularly when the fungus did not have access to ^{15}N-NH_4^+. Similarly, in the absence of AM fungi as well, shoot ^{13}C content and δ ^{13}C enrichment dropped even further in the Fix-/C and C/C colonization regimes, which were also comparable to one another.

The increase in δ ^{13}C enrichment in roots with nodules occupied by Fix+ *E. meliloti* was strongly negatively correlated with the decrease in δ ^{15}N enrichment observed in the

shoots of the same plants (r = -0.73, p = 0.017; Fig 2.3A; however, no similar correlation existed with root δ ^{15}N enrichment (r = -0.44, p = 0.2; Figure 2.3B). In contrast, while a nearly significant positive correlation existed between δ ^{13}C enrichment in mycorrhizal roots and shoot δ ^{15}N enrichment (r = 0.42, p = 0.12; Figure 2.3C), an even stronger positive correlation was observed between δ ^{13}C and δ ^{15}N enrichment in mycorrhizal roots (r = 0.72, p = 0.0027; Figure 2.3D). This suggests that the more N the fungus provides to the host plant, the more C the host plant will allocate to mycorrhizal roots.

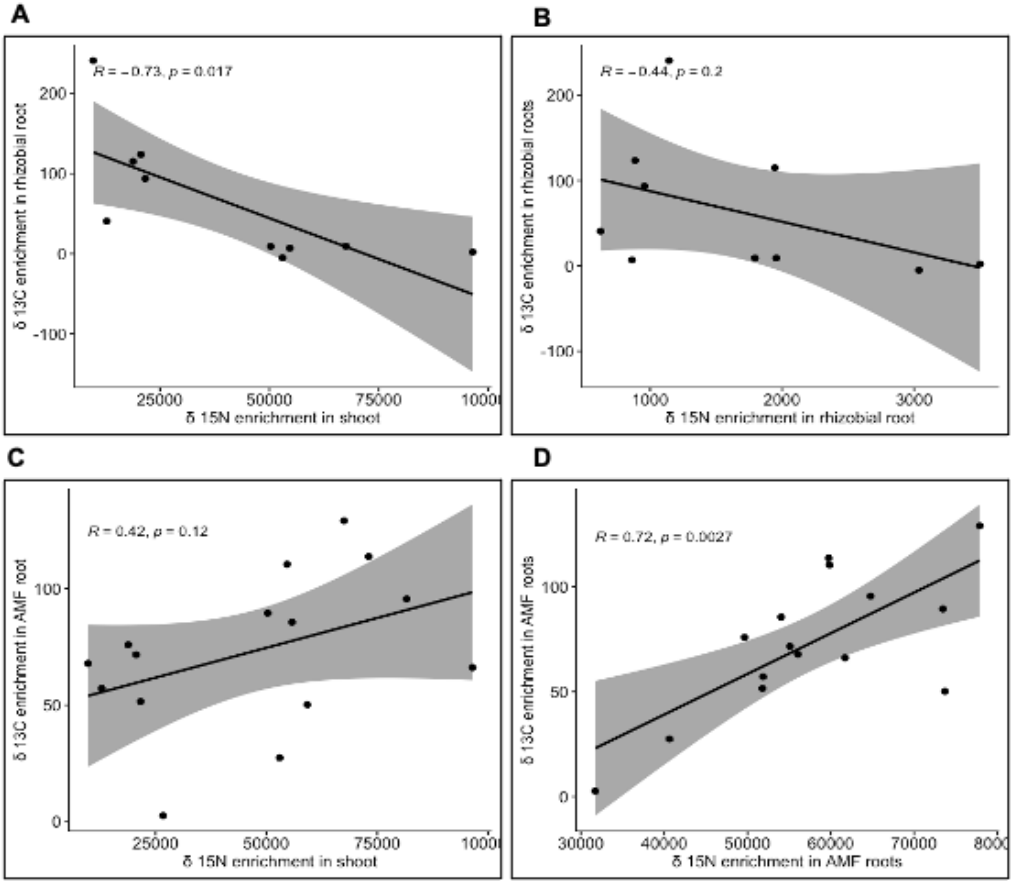

Figure 2.3: Co-relation between δ ^{15}N and δ ^{13}C enrichment in rhizobia root (A, B) and mycorrhizal root (B, C). (A) Co- relation between δ ^{13}C enrichment in the rhizobia root and δ ^{15}N enrichment in same root system (B) Co- relation between δ ^{13}C enrichment in the shoot and δ ^{15}N enrichment in rhizobia root system (C) Co- relation between δ ^{13}C

enrichment in the mycorrhizal root and δ ^{15}N enrichment in same root system (D) Co-relation between δ ^{13}C enrichment in the shoot and δ ^{15}N enrichment in mycorrhizal root system

2.4.5 N fixing capability of rhizobia and AMF access to exogenous N affects the gene expression pattern of sugar transporters in the tripartite interactions

To identify the putative molecular mechanisms regulating host-determined carbon allocation to root symbionts, we evaluated the expression of candidate sucrose transporters from two different transporter families, including SUTs (Sucrose Uptake Transporters) and SWEETs (Sugar Will Eventually be Exported Transporters). We analyzed the expression of *MtSUT1-1*, *MtSUT2*, and *MtSUT4-1* which belong to the fabacean family-like SUT1, SUT2, and SUT4 clades respectively, based on phylogentic analysis [44, 159, 160]. Each of these transporters showed different expression patterns in each of the colonization regimes used in this study (Figure 2.4 and Figure S2.6).

The expression of *MtSUT1-1* was highest in AM-colonized roots in the Fix-/AM regime when the fungus did not have access to ^{15}N-NH_4^+ (Figure 2.4A). But when it did, *MtSUT1-1* expression dropped significantly and was comparable in both root halves. In contrast, the expression of *MtSUT1-1* was initially equivalent in both root halves in the Fix+/AM colonization regime, but when the fungus had access to ^{15}N-NH_4^+, *MtSUT1-1* expression in AM-colonized roots dropped below that of the roots colonized by Fix+ *E. meliloti*. These results indicate that this transporter could play an important role in C transport towards both AM and rhizobia-colonized roots independent of N-fixing ability. However, the expression in AM-colonized roots tended to be lower when fugus had access to ^{15}N-NH_4^+.

The expression of *MtSUT2* was highest in roots colonized by Fix+ *E. meliloti* in the Fix+/AM colonization regime when the fungus did not have access to ^{15}N-NH_4^+ (Figure

2.4B). When the fungus did have access to $^{15}N\text{-}NH_4^+$, *MtSUT2* expression remained unchanged in the mycorrhizal roots but dropped slightly in roots colonized by Fix+ *E. meliloti*. In the Fix-/AM colonization regime, although *MtSUT2* expression was again consistent in mycorrhizal roots with or without access to $^{15}N\text{-}NH_4^+$, it increased slightly in roots colonized by Fix- *E. meliloti* (Fig 2.4B). Despite consistently low *MtSUT2* expression levels in mycorrhizal roots from tripartite colonization regimes, *MtSUT2* expression was significantly higher in AM-colonized roots compared to the uninoculated root half in the C/AM colonization regime and in roots colonized by Fix+ *E. meliloti* compared to the uninoculated root half in the Fix+/C colonization regime (Fig S2.6B). Thus, when one root in a spit-root system is colonized by either AM fungi or rhizobia but the adjacent root half is non-inoculated, it appears that the plant may upregulate *MtSUT2* to potentially allocate more carbon to the colonized root.

MtSUT4-1 expression was highest in roots colonized by Fix- *E. meliloti* from the Fix-/AM colonization regime (Figure 2.4C). In the adjacent mycorrhizal root half which had access to $^{15}N\text{-}NH_4^+$, *MtSUT4-1* was expressed at a similar level. However, when the fungus did not have access to access to $^{15}N\text{-}NH_4^+$, *MtSUT4-1* expression dropped dramatically. In the Fix+/AM colonization regime, *MtSUT4-1* expression was comparable in both root halves whether the fungus had access to access to $^{15}N\text{-}NH_4^+$ or not. Like *MtSUT2*, the expression of *MtSUT4-1* was significantly higher in roots colonized by Fix+ *E. meliloti* in the Fix+/C colonization regime (Figure S2.6C) suggesting that *MtSUT4-1* is not specifically expressed in roots colonized by one symbiont over the other. However, nitrogen delivery by any root symbiont increased *MtSUT4-1* expression except in roots colonized by Fix- *E. meliloti* from the Fix-/AM colonization regime when the fungus had

no access to N. This could be because the Fix- *E. meliloti* required sugar for its growth and development as root nodule not only provide shelter but also organic carbon and other essential nutrients [161].

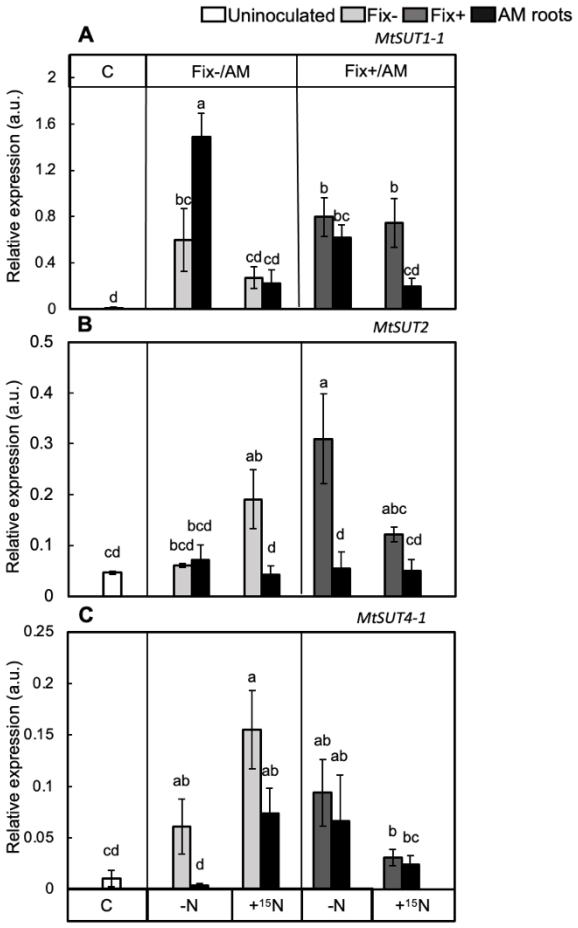

Figure 2.4: Relative expression of *MtSUT1-1* (A), *MtSUT2* (B) and *MtSUT4-1* (C) assessed by quantitative RT-PCR in *M. truncatula* roots after mycorrhization by *R. irregularis* and nodulation by *E. meliloti* (Fix+ and Fix-). The C_t values (threshold cycles) of the samples are corrected against the C_t values of the housekeeping gene *Mtef1α*. Data for each condition are presented as mean + S.E. and were obtained from 3-5 biological and three technical replicates. White bar represents control plants (non-inoculated); black bars indicates mycorrhizal root halves; light grey bars indicates mutant FixJ *E. meliloti* (Fix-) inoculated root halves; dark gray bar indicates N-fixing (Fix+) inoculated root halves. C: Control; -N: water added; $+^{15}N$: ^{15}N-NH_4Cl to hyphal compartment. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$).

We also evaluated a subset of Clade I (*MtSWEET1b* and *MtSWEET3c*) and Clade III (*MtSWEET11, MtSWEET12, MtSWEET13* and *MtSWEET15c*) SWEET transporters that are at least partially characterized as playing a role in the allocation of carbon to symbionts [44]. *MtSWEET1b* expression was consistently higher in mycorrhizal roots compared to nodulated roots and most significantly in the Fix-/AM colonization regime when the fungus

had access to ^{15}N (Figure 2.5A). Interestingly, although *SWEET1b* is considered to be an AM-induced SWEET transporter [104], in single inoculation regimes with one uninoculated root half, *SWEET1b* expression was upregulated in roots inoculated either AM fungi or rhizobia (Figure S2.7A). Similarly, both *MtSWEET3c* and *MtSWEET11*, which are characterized as rhizobia-induced SWEET transporters [140, 141], were primarily expressed in nodulated roots regardless of the fixation ability of the Fix+ and Fix-strains of *E. meliloti* (Fig 2.5B, C); however, comparable AM-induced expression was observed in both mycorrhizal roots from the Fix+/AM colonization regime without fungal access to N, and in the C/AM colonization regime (Figs. 2.5B, C and S2.7B, D. The expression pattern of *MtSWEET13*, which is from a different clade than *MtSWEET1b*, followed a very similar expression pattern to *MtSWEET1b* (Fig 2.5E, Fig S2.7E), particularly when the fungus had access to N in the Fix-/AM and Fix+/AM colonization regimes. Unlike the other four SWEET transporters, the expression of *MtSWEET12* and *MtSWEET15c* was not significantly different in mycorrhizal and nodulated roots on adjacent root halves in the same split-root system (Figure 2.5D, F). Nor were they different in the single inoculation regimes except that *MtSWEET12* was more highly expressed in mycorrhizal roots in the C/AM regime, and *MtSWEET15c* more highly expressed in nodulated roots from the Fix+/C regime (Figure S2.7F). Collectively, these results indicate that during tripartite interactions with AM fungi and rhizobia, *MtSWEET1b* and *MtSWEET13* typically function in translocating sugars to AM fungi, while *MtSWEET3c* and *MtSWEET11* most likely function in translocating sugars to rhizobia, with the caveat that each of these SWEET transporters can function in the opposite role under certain conditions, including single inoculations with only one symbiont. Recent finding suggested

that sources of carbon for AM symbiosis include not only sugar but also, fatty acids that are exported from the host plant. To see that, we determined two lipid transporters, i.e, *STR* and *STR2* (Fig 2.6A, 6B; Fig S2.7A, S2.7B). The exclusive expression of these transporters (*STR* and *STR2*) in mycorrhizal root in which STR gene expression was slightly lower when fungus had access to N. the nodulated and non-inoculated roots had very low expression. Furthermore, we evaluated other AM specific P (*MtPT4*) and ammonium transporters (*AMt2;3*) (Fig 2.6C D and Fig S2.8C, D) As expected, we found exclusive expression of these transporters only in AM roots but not in the rhizobia and non-inoculated control roots. Expression of these two transporters did not vary based on the presence of rhizobia (Fix- and Fix+) on the adjacent root half or by fungal access to ^{15}N. This also confirm that no mycorrhizal cross contamination occurred in any of the split-root systems.

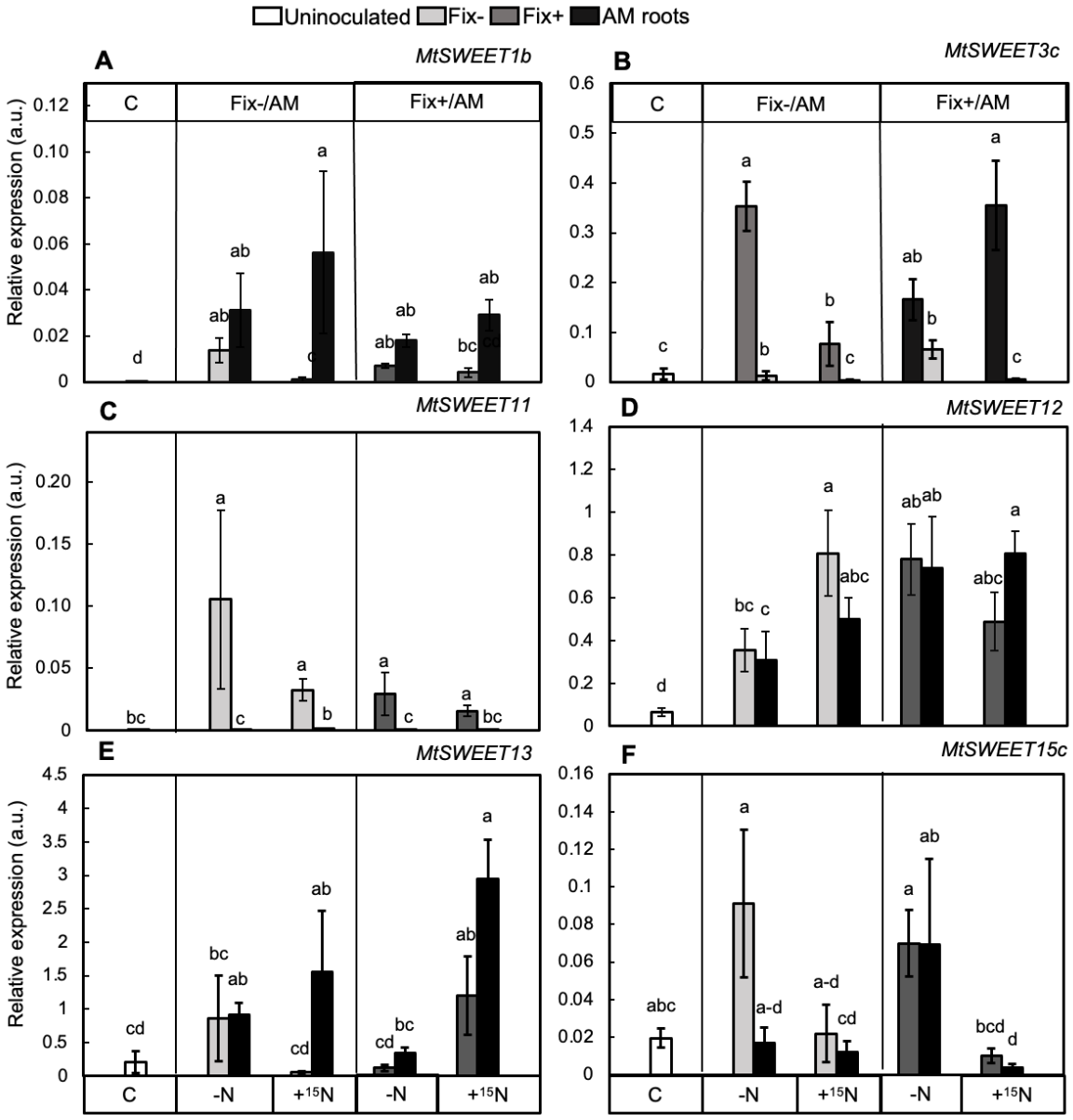

Figure 2.5: Relative expression of *MtSWEET1b* (A), *MtSWEET3c* (B) and *MtSWEET11* (C), *MtSWEET12* (D), *MtSWEET13* (E), and *MtSWEET15c* (F) assessed by quantitative RT-PCR in *M. truncatula* roots after mycorrhization by *R. irregularis* and nodulation by *E. meliloti* (Fix+ and Fix-). The C_t values (threshold cycles) of the samples are corrected against the C_t values of the housekeeping gene *Mtef1α*. Data for each condition are presented as mean + S.E. and were obtained from 3-5 biological and three technical replicates. White bar represents control plants (non-inoculated); black bars indicates mycorrhizal root halves; light grey bars indicates mutant FixJ *E. meliloti* (Fix-) inoculated root halves; dark gray bar indicates N-fixing (Fix+) inoculated root halves. C: Control; -N: water added; $+^{15}N$: ^{15}N-NH_4Cl to hyphal the compartment. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$).

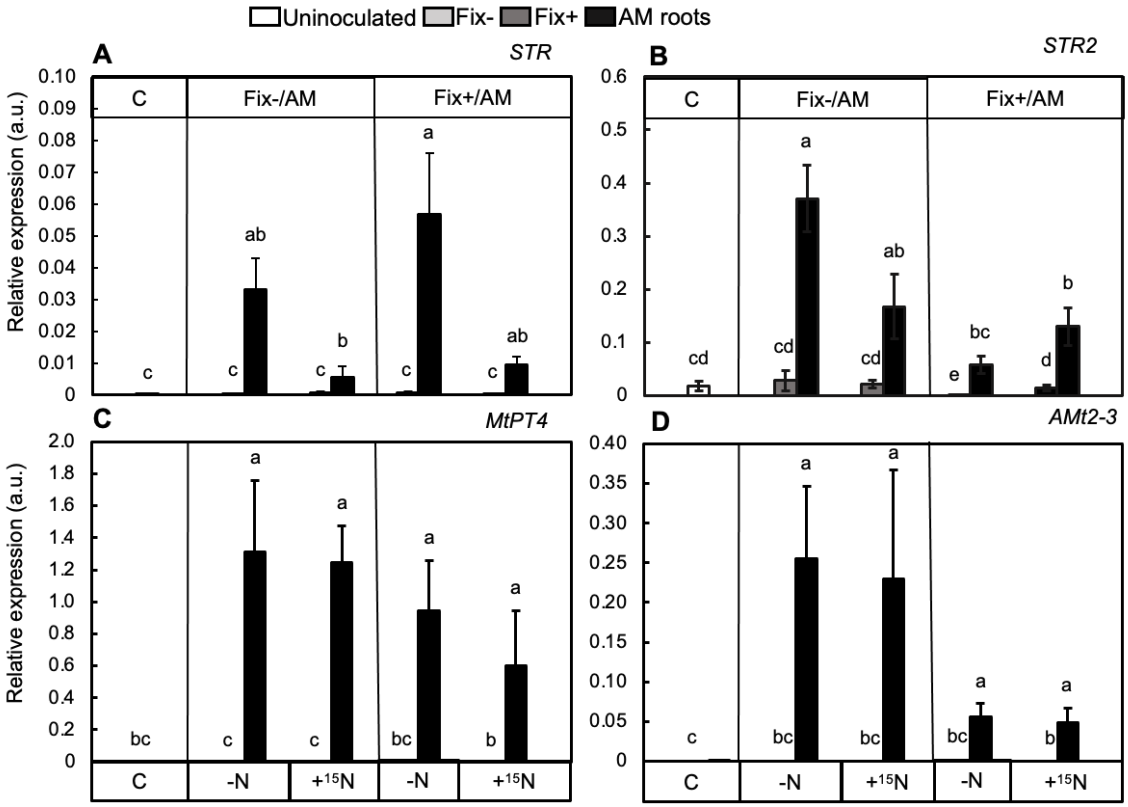

Figure 2. 6: Relative expression of *STR* (A), *STR2* (B), *AMT2;3* (C), and *PT4* (D) assessed by quantitative RT-PCR in *M. truncatula* roots after mycorrhization by *R. irregularis* and nodulation by *E. meliloti* (Fix+ and Fix-). The C_t values (threshold cycles) of the samples are corrected against the C_t values of the housekeeping gene *MtTef1α.* Data for each condition are presented as mean + S.E. and were obtained from 3-5 biological and three technical replicates. White bar represents control plants (non-inoculated); black bars indicates mycorrhizal root halves; light grey bars indicates mutant FixJ *E. meliloti* (Fix-) inoculated root halves; dark gray bar indicates N-fixing (Fix+) inoculated root halves. C: Control; -N: water added; $+^{15}N$: ^{15}N-NH_4Cl to hyphal the compartment. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$)

2.5 Discussions

AMF and rhizobia play a crucial role in nutrient exchange in legumes, an agronomically important group of plants. The legumes signaling pathways that control the initiation, maintenance and number of infections are known to be shared by both symbioses. The functional understanding of these complex symbiotic interactions is primarily focused on single inoculation studies with either AMF or rhizobia. Thus, limited

information exists on how host plant function in carbon allocation when both symbioses happen. In this study, we evaluated tripartite interactions using a split-root system with one root half colonized by AMF and the other colonized by either N-fixing rhizobia (Fix+) non-N-fixing rhizobia (Fix-). In addition, we varied the ability of the fungus to access ^{15}N or not, which allowed us to determine how the host allocates C to both symbionts simultaneously based on them to provide the host with N.

Both AMF and rhizobia colonization were uniform among all colonization regimes, thus allowing us to attribute the differences we observed to the setup of the experiment and not to experimental error. We observed a consistent trend in higher root dry weight of the mycorrhizal root halves than the rhizobia or non-inoculated root halves (Figure 2.1A) and pronounced when the fungus had access to ^{15}N-NH_4^+. Despite the N fixing capability of rhizobia bacteria, we found higher growth responses in tripartite interactions when fungus had N access. However, the response was pronounced when Fix- rhizobia were inoculated with AMF *R. irregularis*. The positive biomass response was observed in other reports as well [101, 162, 163] in low P and N supply conditions. In Fix- dual system, AMF was the potential source for the N that led to overall increase in shoot dry weight. The impact on plant benefit remains in comparable level even host plant gets colonized by Fix- rhizobia.

In earlier reports, the improved AMF acquisition of P help to improve biological N fixation that led plant benefit overall [164]. Since we did not find the differences in the P concentration (Fig 2.1C, 2.1D, Fig S2.3B, S2.3C) in the shoot of mycorrhized and non-inoculated roots might be due to available P in the growing medium. This also suggests that P was not the growth limiting factor for this experiment. In addition to this, AMF are

rarely responsible for the entirely of plant P assimilation. Plants engage in their own direct P uptake via the root epidermis and root hairs, along with indirect mycorrhizal P pathway [5]. In our experiment, we only quantified the total P content in the plants. Thus, no differences at total P in plant tissues of AMF-inoculated and non-inoculated plants. It is important to note that total P quantification represents P accumulation over the lifetime of the plant. It is possible that AMF contribute most to plant P assimilation in the earlier stages of plant development, which were not measured during this experiment [165, 166]. Isotopic tracking of P is required for actual P assimilation by AMF.

The consistent high N root content in mycorrhizal root when fungus had access to $^{15}N\text{-}NH_4^+$ (Fig 2.1E) and lower N content in Fix+ inoculated roots might be resulted from N access to fungus and dilution effect by total root biomass respectively. However, the delivery of N to the shoot was consistently higher in plants with Fix+ rhizobia regardless of whether the fungus had access to $^{15}N\text{-}NH_4^+$ or not (Figure 2.1F and Figure S2.3D). Evidence suggested that inoculation of Fix+ rhizobia can serve as 65% to 95% of total nitrogen available to the host plants [167]. Fungus with no access of N and inoculated with Fix- rhizobia bacteria had very low (almost equal to non-inoculated control plants). These results confirms that fungal access to N leads to an increase in shoot N content and concentration, particularly when the plant is not colonized by Fix+ rhizobia. Thus, AM fungi can play a significant role in delivering N to their host plant.

We found that delivery of ^{15}N in AMF inoculated plants were pronounced on all mycorrhizal roots independent of any colonization regime when fungus had access to external $^{15}N\text{-}NH_4^+$ (Figure 2.2A, 2.2B and Fig S2.4A, 2.4B). Interestingly, delivery of this ^{15}N to the shoot were higher in mycorrhizal plants that were inoculated with Fix- *E. meliloti*

and when fungus was inoculated alone. Multiple evidence support that AM hyphae can transport significant amounts of N to their host plant [168, 169]. However, Fix+/AM system had significantly lower delivery of ^{15}N in plant shoot but not in the roots. This reduction only in shoot of Fix+/AM systems can be further explained as AM fungus was able to uptake nitrogen from the soil, but rhizobia are fulfilling plant nitrogen demand which reduces the transport across the mycorrhizal interface. Mycorrhizal N acquisition was not determined by the degree to which plant root systems were colonized by the AM fungus (Fig S2.2B). Slight increase of ^{15}N in the non-inoculated control plant than other water added root halves of system but are not beyond the value of natural abundance, confirms no massive flow from the hyphal compartment to the second root compartment system.

As AMF and rhizobia bacteria provide N to host plant, significant amount of carbon or photosynthates get allocated towards both root symbionts in the tripartite interaction. Biological nitrogen fixation being a very energy driven phenomenon (16ATP required per mole of N) [170] also confirmed by highest $\delta^{13}C$ in Fix+ rhizobia roots among all. However, mycorrhizal roots also have comparable amount of $\delta^{13}C$ but higher ^{13}C content was due to total biomass effect in the AM inoculated roots (Figure 2.2C and Figure S2.4C). Fix- nodulated root get very small amount of carbon that is almost like non-inoculated roots. On the other hand, mycorrhizal roots of same system (Fix-/AM) had higher C allocation and when fungus able to deliver N to host, the difference in C allocation between Fix- and AMF roots was pronounced. This suggests that host plant punish the rhizobia if they unable to fix N [127]. The non- inoculated roots for all the system receive very low C from the host plant. These finding were also supported by experiment on RNA based

stable isotope probing in plants where they found an elevated CO_2 can increase plant C allocation to mycorrhizal symbionts by up to 25% [171] which is likely due to increased photosynthesis and availability of plant C [172] and upto 30% in N-fixing root nodule [128]. The negative co relation graph between the $\delta^{15}N$ in shoot and $\delta^{13}C$ in the rhizobial root (Figure 2.3A, 2.3B) also supported the fact that plant allocate less C (sugar) to the root symbionts if the root symbionts unable to provide nutrients to the host plants. On the other hand, if AMF inoculated roots consists higher amount of $\delta^{15}N$ then ^{13}C allocated on those roots (Figure 3C, D) also increased following the biological market theory [173]. The C sink strength of rhizobial symbioses is mainly related to the respiration associated with rates of N_2-fixation, whereas the C sink strength of AM symbioses is mostly associated with the growth respiration of mycelium. The C sink strength of both symbioses is regulated according to the nutritional demand of the plant [174, 175]. We tried to measure the microbial respiration but due to our customized pot system, which we tried making airtight by using a plasticine, but they were not airtight and could not collect the gas. However, ^{13}C recovered content in the roots give us an idea about much photosynthates were able to allocate to the respective root halves colonized with different root symbionts.

To reveal the molecular mechanisms for sugar transport to the root symbionts, we analyzed *MtSUT1-1, MtSUT2* and *MtSUT4-1* (sucrose uptake transporters) and found none of them are specific to individual root symbiont. The increased *MtSUT1-1* expression in mycorrhizal roots when fungus had no access to N and nodulated roots indicates their possible role in sugar transport towards both root symbionts. The N delivery by fungus led decrease in expression level indicates sugar transport by *MtSUT1-1* is not related to the N status of the host plants. It has been known that *SUT1-1* considered as apo-plastic phloem

loaders comprise a single protein member. For instance *StSUT1* mutant in potato (*Solanum tuberosum*) led decrease in tuber production suggesting this transporter play important role in efflux toward the sink organs [176, 177]. Whereas ZmSUT1 involves in both efflux and influx for phloem loading and efflux toward sink [43]. When one root half were inoculated with Fix+ rhizobia and other root half were non inoculated, this transporter was exclusively higher in rhizobia inoculated roots which can be also supported by the higher $\delta^{13}C$ enrichment on those roots (Figure 2.2C).

However, *MtSUT4-1* expression level in mycorrhizal roots increased when AMF interact with Fix- and non-inoculated AM control (C/AM) plants but had access to N however, there were no change in expression level in Fix+/AM system (Fig 2.4C and Fig S2.5C). In Fix-/AM C/AM system, AMF considered as sole N source to host plant. This led to statistically higher *MtSUT4-1* expression on those roots as AMF are getting more carbon in this condition (Fig S2.4C). The level of expression in Fix- and Fix+ *E.melliloti* in tripartite system followed the same pattern as adjacent mycorrhizal root and the similar level of Fix- root expression like its adjacent control root (Fix-/C) furthermore supports the N status dependency of *MtSUT4-1* expression. Number of reports also suggested that expression of *SUT4* transporter increased symbiotic root sink [43] and arbusculated cells [45] from vacuole. Higher level of expression of *MtSUT4-1* in other roots might be due to its role in symbiotic carbon flux.

The N access to plants either from fungi or from Fix + *E. meliloti* increase the *MtSUT2* expression level in rhizobia roots (Fig 4B). Conversely, no N supply to HC or both root symbiont providing N then the expression remains same. Because at this condition both root symbionts have equal priority based in N supply. However, when AMF

and Fix+ rhizobia were inoculated alone (C/AM and Fix+/C) then the expression were exclusively higher on mycorrhizal, and rhizobia roots respectively. But same level of expression was found in between Fix- and non-inoculated root (Fix-/C) (Fig S2.6B). A tomato plants *SISUT2* localized to the peri-arbuscular membrane (PAM) indicates a role in back transport of sucrose from the PAM into the plant cell thereby affecting hyphal development and up regulation of *SISUT1, SISUT2* and *SISUT4* provide transport capacities in mycorrhizal root as significant amount of sugars could be used by AMF fungus [178, 179].

We also determined the expression of several SWEET transporters responsible for sugar transport in the tripartite interaction. The recent knowledge about the SWEET transporters that can facilitate both influx and efflux of sugars [142, 143]. For example *PsSWEET12* and *AtSWEET12* of same cluster found to be responsible for the efflux of sucrose into apoplasm [180]. Couple of research confirmed that SWEET transporters are involved in transfer of sugar in rhizobia and AMF symbiosis [43, 104, 140, 141]. In this study, we found that *MtSWEET1b, MtSWEET12, MtSWEET13* are strongly upregulated in the mycorrhizal roots than non-inoculated roots. In rhizobia roots, expression of *MtSWEET1b* remains slightly lower, same in *MtSWEET12* and lower in *MtSWEET13* (Fig 2.5A, 2.5D, 2.5E and Fig S2.7A, S2.7D, S2.7E).

Molecular characterization by Kryvurichko *et. al.* revealed that in *M. truncatula* root nodule colonized with rhizobia, sucrose distribution happened by the help of *MtSWEET11*. Similarly, *LjSWEET3 were expressed* in *Lotus japonicum root nodule*, however both of these transporter seems to be not crucial for biological nitrogen fixation may be because of its redundant function with other sugar transporters that can fulfill its

role (s) [140, 141]. Consistent with these e findings, we observed upregulation of *MtSWEET3c, MtSWEET11, MtSWEET12, MtSWEET13* and, *MtSWEET15c* genes in not only Fix+ rhizobia bot also in Fix- nodulated root [101]. The transcriptomic analysis of *M. truncatula* show *MtSWEET6* and *MtSWEET1b* were highly expressed in arbusculated cells, *GmSWEET6 GmSWEET15* and sugar invertase (*Glyma.17G227900*) were exclusively upregulated when the roots get colonized with more beneficial AMF *R. ireegularis* [144] in soybean root and *StSWEET1a*, *StSWEET1b*, and *StSWEET7a* in potato [103]. *An J et. al*. functionally characterized *MtSWEET1b* that strongly upregulated in arbuscule-containing cells compared to non-mycorrhized roots and localizes to the peri-arbuscular membrane, across which nutrient exchange takes place [104]. We found same trend on upregulation of *MtSWEET1b* in AM colonized *Medicago* roots (Figure S2.7A). The consistent upregulation of *MtSWEET1b, MtSWEET12*, and *MtSWEET13* (both from clade III) in mycorrhizal root also suggest C transfer via these sugar transporters in AMF symbiosis. However, *MtSWEET12* was also upregulated in nodule forming rhizobia root which is supported by our previous findings as well [101]. The redundancy function may also impact in upregulation of these transporters [140, 141]. *STR and STR2 lipid* transporters all displayed enhanced expression in AMF inoculated roots indicating that not only sugar, host plant also allocate lipid towards arbusculated roots [47, 136]. In addition, we also quantified AMF specific P and ammonium transporter. *MtPT4* a low affinity P transporter has role in acquisition of P released by fungus in the AM symbiosis [48]. We did not find any differences *MtPT4* in N starved and N supplied condition at HC which might be due to equal colonization rate in the AMF inoculated roots. This is supported by other evidence that *NtPT5* a

mycorrhiza-specific phosphate transporter during arbuscular mycorrhizal symbiosis in tobacco roots, and the induction was tightly correlated with the degree of root colonization by *Glomus etunicatum* [181]. *AMT2;3,* AM symbiosis specific ammonium transporter exclusively expressed in mycorrhizal roots but not in non-inoculated and rhizobia root [49]. *AMT2;3* expression trended to be higher in Fix-/AM system than Fix+/AM could be due to N status of the plant. As Fix+ rhizobia provide significant amount of N to host cell, the role of AMF for transferring exogenous N via *AMT2;3* is minimal.

2.6 Supplementary Information

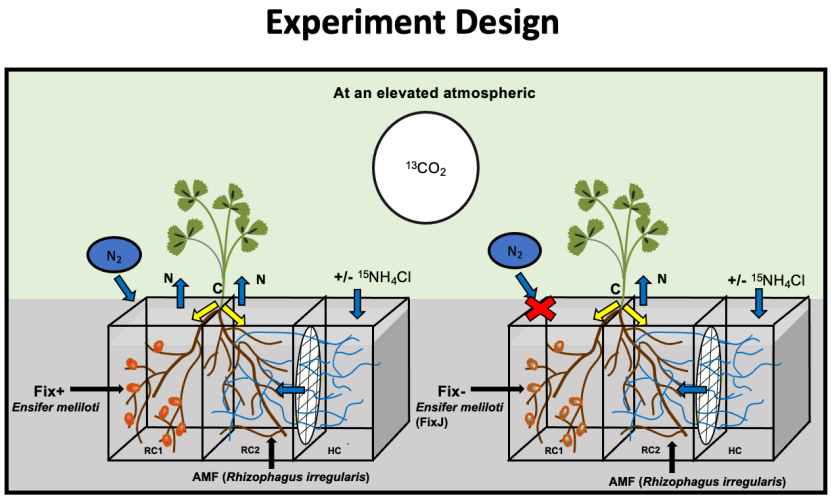

Figure S2. 1: Schematic model of the experimental pot system used for both Fix+ and Fix- rhizobia bacteria in combination with AM or uninoculated roots. Abbreviations of the root chamber systems used in experiment AMF: root half inoculated with *Rhizophagus irregularis*, Fix+: root half inoculated with *Ensifer meliloti*, Fix-: root half inoculated with mutant *Ensifer meliloti* that are unable to fix atmospheric N. RC1: root compartment 1, RC2: root compartment 2 and HC: hyphal compartment

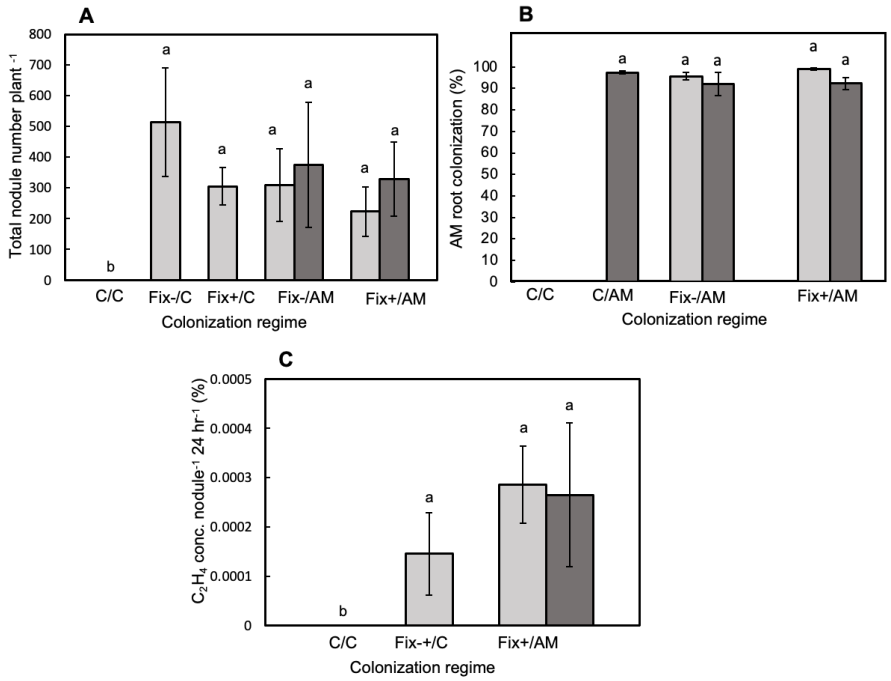

Figure S2. 2: Number of nodules per plant (A) Total number of nodule plant -1 ; Acetylene reduction assay (B) and root colonization (C) *Medicago truncatula* in symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and the nitrogen-fixing diazotroph *Ensifer meliloti* and mutant (*FixJ*) *E. meliloti* under N supply conditions for the fungal partner (dark grey bars indicates addition of ^{15} N-NH4Cl to the hyphal compartment; and Light dark grey bar represents control plants (addition of sterile DI water to the hyphal compartment). Root colonization abbreviations: C/C: controls, both root halves non-inoculated; C/AM: one root half colonized by *Rhizophagus irregularis*, one root half non-inoculated; Fix-/AM: one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti* (*FixJ* mutant that lack biological N-fixation). Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$, n – 3 to 6).

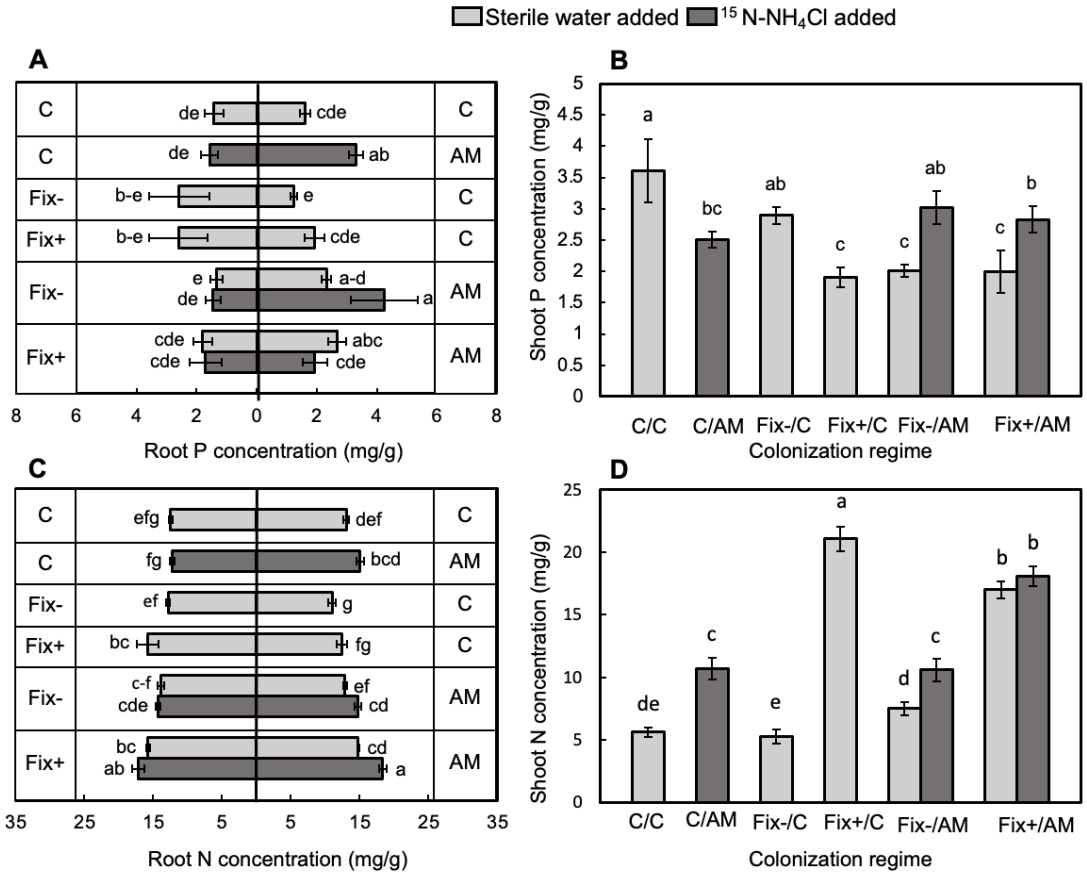

Figure S2. 3 Root (A) and Shoot (B) P concentration Root (C) and Shoot (D) N concentration of *Medicago truncatula* in symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and the nitrogen-fixing diazotroph *Ensifer meliloti* and mutant (*FixJ*) *E. meliloti* under N supply conditions for the fungal partner (dark grey bars indicates addition of ^{15} N-NH4Cl to the hyphal compartment; and Light dark grey bar represents control plants (addition of sterile DI water to the hyphal compartment). Root colonization abbreviations: C/C: controls, both root halves non-inoculated; C/AM: one root half colonized by *Rhizophagus irregularis*, one root half non-inoculated; Fix-/AM: one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti* (*FixJ* mutant that lack biological N-fixation). Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$, n – 3 to 6).

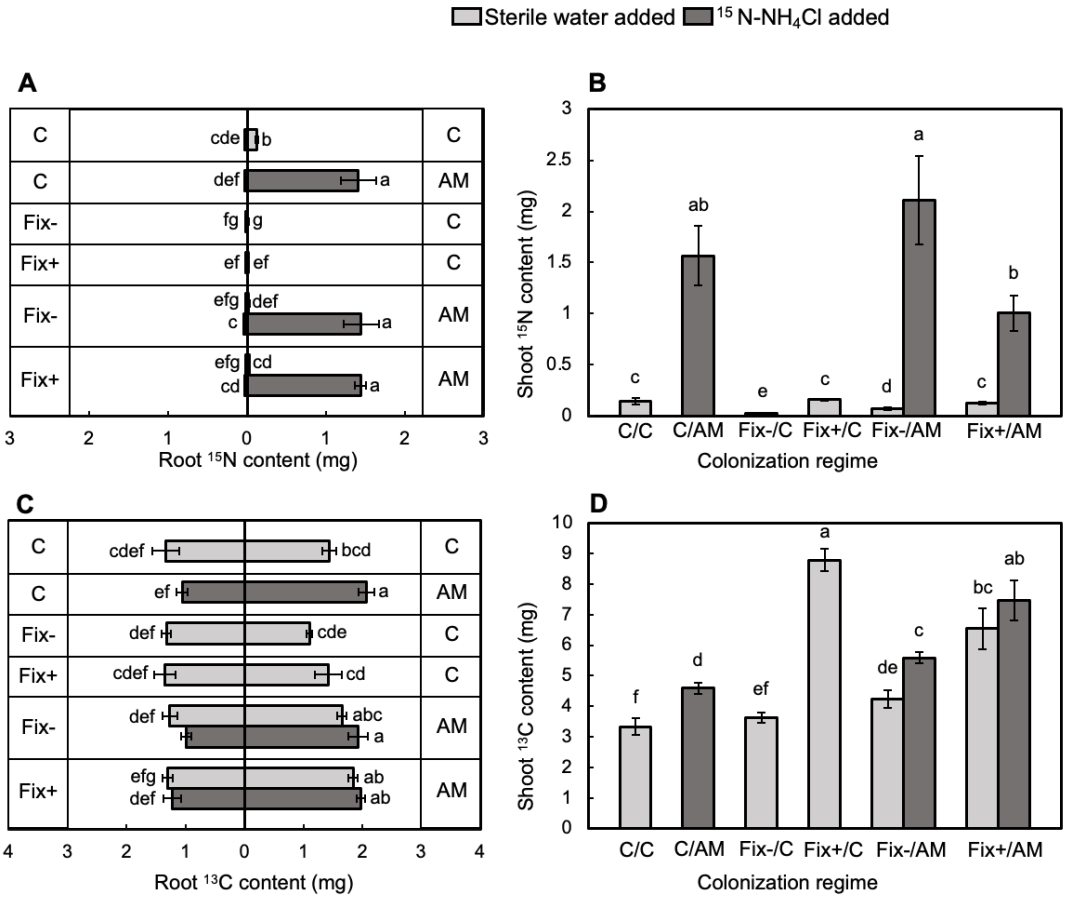

Figure S2. 4 Total ^{15}N content (mg) in root (A) and shoot (B); recovered ^{13}C contents in different root (C) and shoots (D) of *Medicago truncatula* in symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and the nitrogen-fixing diazotroph *Ensifer meliloti* and mutant (*FixJ*) *E. meliloti* under N supply conditions for the fungal partner (dark grey bars indicates addition of ^{15} N-NH4Cl to the hyphal compartment; and Light dark grey bar represents control plants (addition of sterile DI water to the hyphal compartment). Root colonization abbreviations: C/C: controls, both root halves non-inoculated; C/AM: one root half colonized by *Rhizophagus irregularis*, one root half non-inoculated; Fix-/AM: one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti* (*FixJ* mutant that lack biological N-fixation). Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$, n – 3 to 6).

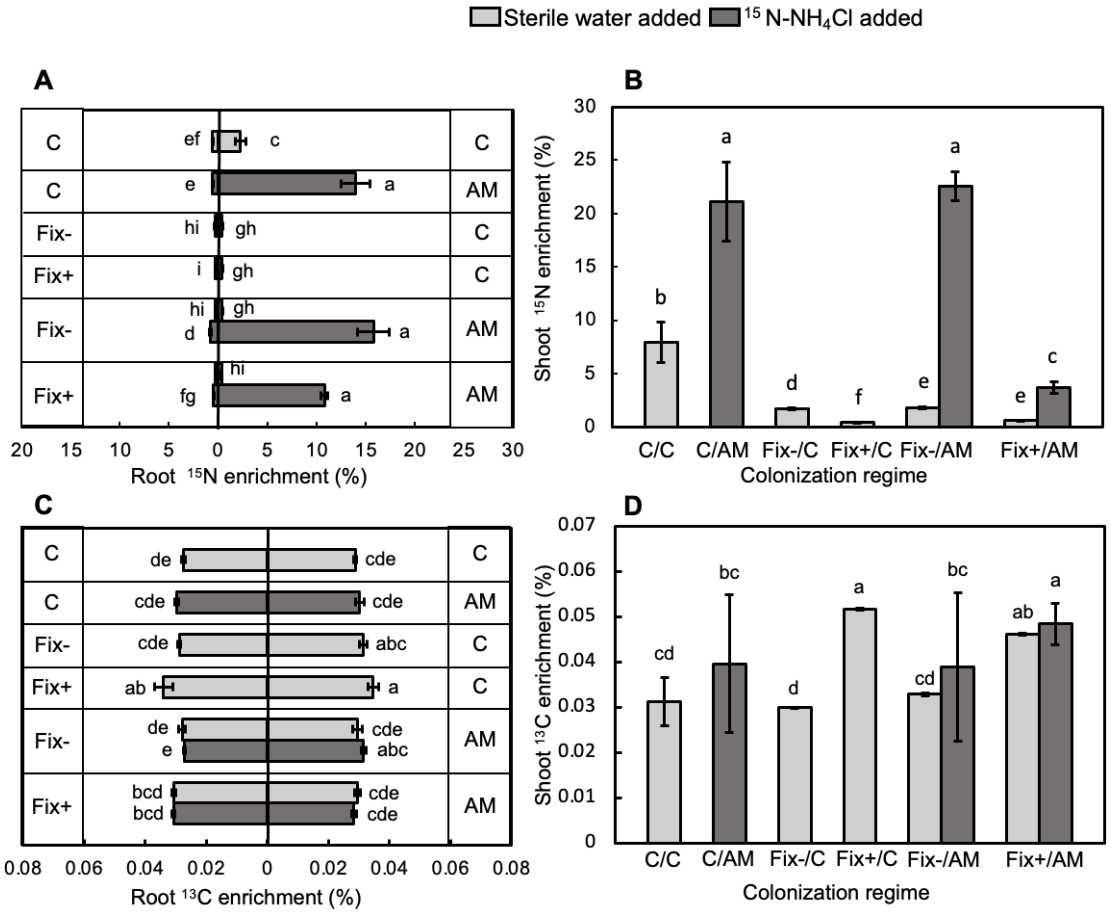

Figure S2. 5 ^{15}N enrichment (%) in root (A) shoot (B) 13C enrichment in root (C) and shoot (D) of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions for the fungal partner (dark grey bars indicates addition of ^{15}N-NH_4Cl to the hyphal compartment; and light grey bar represents control plants (addition of sterile DI water to the hyphal compartment). Root colonization abbreviations: C/C: controls, both root halves non-inoculated; C/AM: one root half colonized by *Rhizophagus irregularis*, one root half non-inoculated; Fix-/AM: one root half colonized by *R. irregularis*, one root half colonized by *E. meliloti* (fixJ mutant that lack biological N-fixation); Fix+/AM: one root half colonized by *R. irregularis*, one root half colonized by wild type *E. meliloti*. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$, n = 3 to 6).

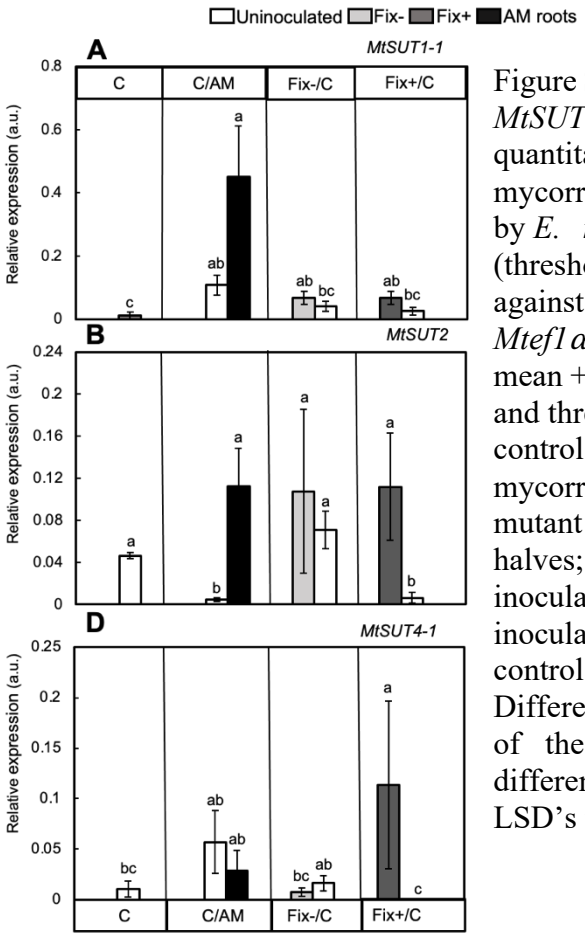

Figure S2. 6: Relative expression of *MtSUT1-1* (A), *MtSUT2* (B) and *MtSUT4-1* (C) assessed by quantitative RT-PCR in *M. truncatula* roots after mycorrhization by *R. irregularis* and nodulation by *E. meliloti* (Fix+ and Fix-). The C_t values (threshold cycles) of the samples are corrected against the C_t values of the housekeeping gene *Mtef1α*. Data for each condition are presented as mean + S.E. and were obtained from 3-5 biological and three technical replicates. White bar represents control plants (non-inoculated); black bars indicate mycorrhizal root halves; light grey bars indicate mutant *FixJ E. meliloti* (Fix-) inoculated root halves; dark gray bar indicates N-fixing (Fix+) inoculated root halves. C/C: Control roots (non-inoculated); Fix-/C: Fix- Control; Fix+/C: Fix+ control; and C/AM: mycorrhizal control plants. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$).

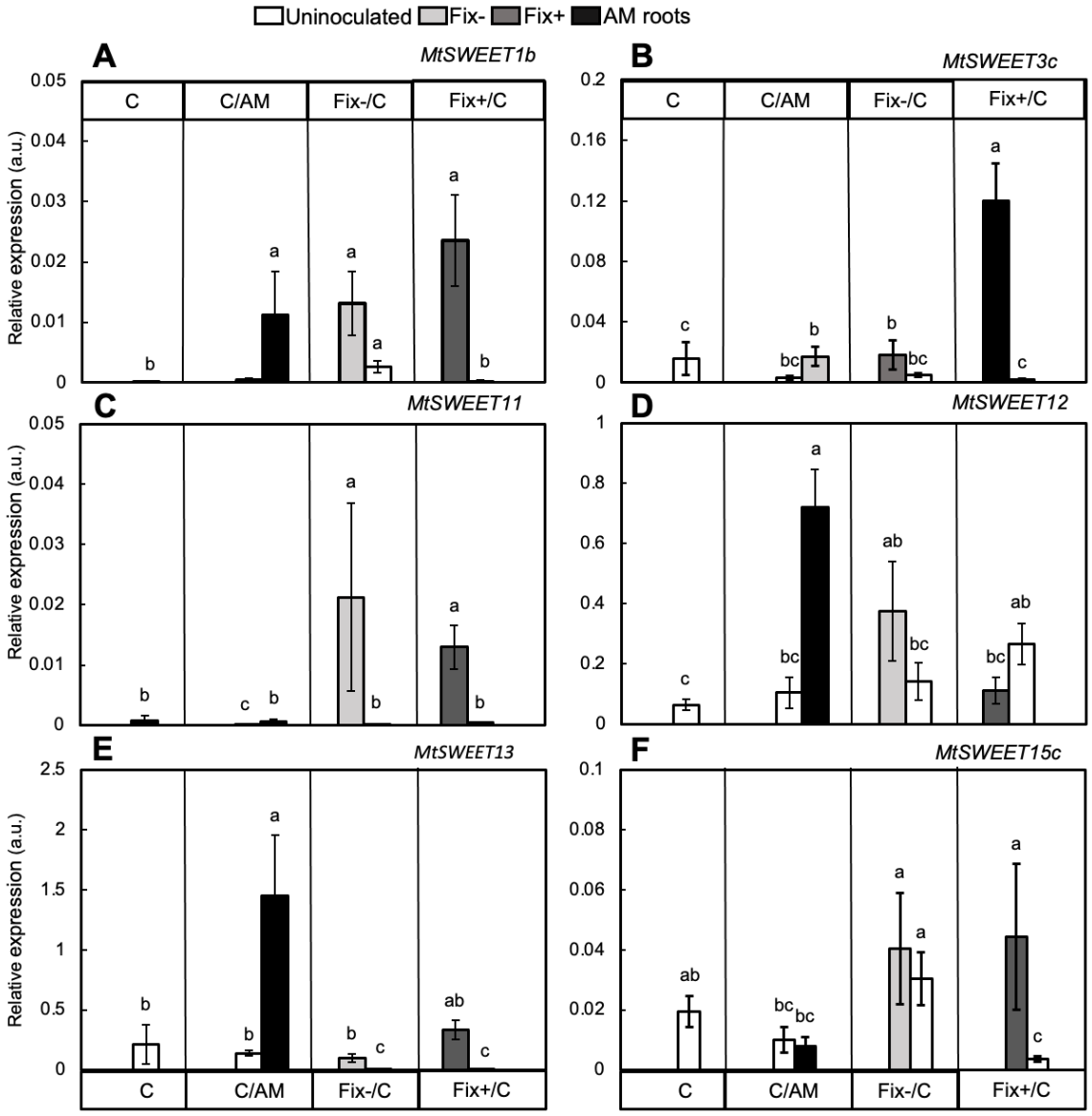

Figure S2. 7 Relative expression of *MtSWEET1b* (A), *MtSWEET3c* (B), *MtSWEET13* (C), *MtSWEET11* (D), *MtSWEET12* (E) *MtSWEET15c* (F) assessed by quantitative RT-PCR in *M. truncatula* roots after mycorrhization by *R. irregularis* and nodulation by *E. meliloti* (Fix+ and Fix-). The C_t values (threshold cycles) of the samples are corrected against the C_t values of the housekeeping gene *MtTef1α*. Data for each condition are presented as mean + S.E. and were obtained from 3-5 biological and three technical replicates. White bar represents control plants (non-inoculated); black bars indicates mycorrhizal root halves; light grey bars indicates mutant *FixJ E. meliloti* (Fix-) inoculated root halves; dark gray bar indicates N-fixing (Fix+) inoculated root halves. C/C: Control roots (non- inoculated); Fix-/C: Fix- Control; Fix+/C: Fix+ control; and C/AM: mycorrhizal control plants. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$).

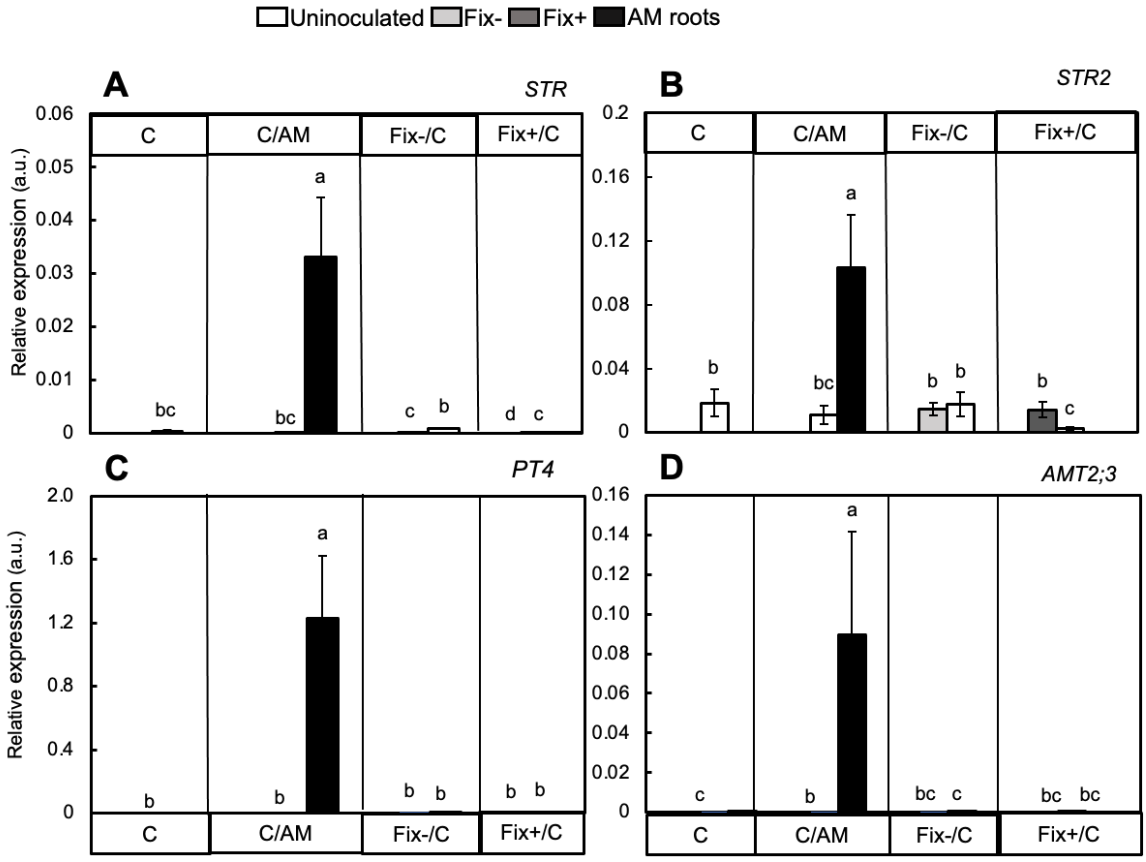

Figure S2. 8 Relative expression of *STR* (A), *STR2* (B), *MtPT4* (C), *AMT2;3* (D) assessed by quantitative RT-PCR in *M. truncatula* roots after mycorrhization by *R. irregularis* and nodulation by *E. meliloti* (Fix+ and Fix-). The C_t values (threshold cycles) of the samples are corrected against the C_t values of the housekeeping gene *MtTef1α*. Data for each condition are presented as mean + S.E. and were obtained from 3-5 biological and three technical replicates. White bar represents control plants (non-inoculated); black bars indicate mycorrhizal root halves; light grey bars indicate mutant *FixJ E. meliloti* (Fix-) inoculated root halves; dark gray bar indicates N-fixing (Fix+) inoculated root halves. C/C: Control roots (non- inoculated); Fix-/C: Fix- Control; Fix+/C: Fix+ control; and C/AM: mycorrhizal control plants. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the LSD's test ($P \leq 0.05$).

Gene	Primer sequence	Reference
MtTef1a F	ACTGTGCAGTAGTACTTGGTG	Doidy et al. (2012)
MtTef1a R	AAGCTAGGAGGTATTGACAAG	
MtSUT1-1 F	AGTGGCATATTATCCGTAGTC	Doidy et al. (2012)
MtSUT1-1 R	TGAAGAAAATGTTCCACACTG	
MtSUT2 F	AACTGCCAAACCTTTCTAGC	Doidy et al. (2012)
MtSUT2 R	CACAATCAACGTGCCTACTC	
MtSUT4-1 F	GCAGATTGTGGTATCACTG	Doidy et al. (2012)
MtSUT4-1 R	TAAGTGCCAAAAGAAAACAGC	
MtSWEET1b F	GTGTTTCTTTGCGGCAGTTC	Kryovoruchko et al. (2016)
MtSWEET1b R	ACCATTAGGTACAGCAACAAATAGG	
MtSWEET3c F	TGTGCCATTGCTCTTGCATC	Hu. B et al (2019)
MtSWEET3c R	ACTTGGTCCCGCAACGAATA	
MtSWEET11 F	TATCGACGACTTTTATATCGCTATACC	Kryovoruchko et al. (2016)
MtSWEET11 R	GTTGACTCTAGTGGAATGGCATC	
MtSWEET12 F	GTGGTGGTCATATAATTGATGTTGTG	Kryovoruchko et al. (2016)
MtSWEET12 R	CTACCAGCACCTCCACCTG	
MtSWEET13 F	TGTGGGATTTGGCACGATTG	Hu. B et al (2019)
MtSWEET13 R	TGTGGGATTTGGCACGATTG	
MtSWEET15c F	CGTATTGGGGTTACTTCAGATGC	Kryovoruchko et al. (2016)
MtSWEET15c R	TTTGGGTGGCTCAATTGGTG	
STR F	ATGAAGAAGCACGGTAAGCATTG	Zhang et. al. (2010)
STR R	ACTTCACACTCACCTTTCGGG	
STR2 F	CCTGTTAGTTTCACTGGAGGACTTG	Zhang et. al. (2010)
STR2 R	GCCCTAATCTGAAATCAGCAGCA	
MtPT4 F	GACACGAGGCGCTTTCATAGCAGC	Doidy et al. (2012)
MtPT4 R	GTCATCGCAGCTGGAACAGCACCG	
MtAMT2;3 F	TGTCCGGTTCAATTCCATGG	Breuillin-Sessoms et.al. (2015)
MtAMT2;3 R	TGGCAAACACACCAGAAAGG	

Table S2. 1: The list of primer sets used in qPCR

Table S2. 2: ANOVA and normality test (Shapiro-Wilk) results.

Response variable	**F value**	**df**	**P value**	**Shapiro-Wilk**
Root weight (Figure 2. 1A)	8.4411	15, 62	4.76E-10	0.4573
Shoot weight (Figure 2.1B)	4.8557	7,31	0.0008775	0.9566
Root P content (Figgure 2.1C)	0.9759	15,62	0.4905	0.006716 *
Shoot P content (Figure 2.1D)	3.9049	7,31	0.003682	0.2116
Root N content (Figure 2.1E)	8.9058	15,62	1.73E-10	0.06961 *
Shoot N content (Figure 2.1F)	42.135	7,31	4.27E-14	0.47
Root δ 15N enrichment (Figure 2.2A)	198.82	15,62	2.20E-16	0.3945 *
Shoot δ 15N enrichment (Figure 2.2B)	186.7	7,31	2.20E-16	0.32
Root δ 13 C enrichment (Figure 2.2C)	6.9483	15,62	1.58E-08	0.0007477 *
Shoot δ 13 C enrichment (Figure 2.2D)	9.8301	7,31	2.11E-06	0.2242
Relative expression *MtSUT1-1* (Figure 2.4A)	8.5815	11,25	4.91E-06	0.3579
Relative expression *MtSUT2-1* (Figure 2.4B)	6.813	11,26	2.96E-05	0.1052 *
Relative expression *MtSUT4-1* (Figure 2.4C)	4.1804	11,24	0.001647	0.2457 *
Relative expression *MtSWEET1b* (Figure 2.5A)	11.463	11,26	2.25E-07	0.1615 *
Relative expression *MtSWEET3c* (Figure 2.5B)	11.006	11,26	3.39E-07	0.06225 *
Relative expression *MtSWEET11* (Figure 2.5C)	16.69	11,24	1.17E-08	0.4257 *
Relative expression *MtSWEET12* (Figure 2.5D)	11.6	11,24	4.1643-07	0.4945 *

Relative expression *MtSWEET13* (Figure 2.5E)	5.7627	11,27	0.0001013	0.5442 *
Relative expression *MtSWEET15* (Figure 2.5F)	2.8884	11,25	0.01357	0.3295 *
Relative expression *STR* (Figure 2.6A)	14.955	11,23	5.65E-08	0.2777 *
Relative expression *STR2* (Figure 2.6B)	15.287	11,25	1.78E-08	0.6409 *
Relative expression *MtPT4* (Figure 2.6C)	20.025	15,33	2.28E-12	0.1621 *
Relative expression *MtAMT2;3* (Figure 2.6D)	32.084	11,24	1.16E-11	0.9222 *
Total nodule number/plant (Figure S2.2A)	0.75	5,24	0.59	0.17
AM root colonization (Figure S2.2B)	0.8	4,17	0.53	0.0002 *
Acetylene reduction assay/nodule (Figure S2.2C)	0.69	4,20	0.6	0.91 *
Root P concentration (Figure S2.3A)	2.6045	15,62	0.004296	0.05611 *
Shoot P concentration (Figure S2.3B)	6.1036	7,31	0.0001561	0.4173
Root N concentration (Figure S2.3C)	9.0986	15,62	1.15E-10	0.000005871*
Shoot N concentration (Figure S2.3D)	60.308	7,31	2.77E-16	0.5359
Total15N content in Roots (Figure S2.4A)	93.35	7,31	2.20E-11	0.59 *
Total 15N content in Shoots (Figure S2.4B)	100	15,62	2.20E-11	0.34 *
Total 13C content in Roots (Figure S2.4C)	7.3851	15,62	5.48E-09	0.27 *
Total 13C content in Shoots (Figure S2.4D)	28.1	7,31	9.89E-12	0.23 *
Root 15N enrichment (%) (Figure S2.5A)	305.75	15,62	2.26e-16	0.00615
Shoot 15N enrichment (%) (Figure S2.5B)	175.22	7,31	2.2e-16	0.044
Root 13 C enrichment (%) (Figure S2.5C)	3.21	15,62	0.0006*	0.17*

Shoot 13 C enrichment (%) (Figure 2.5D)	9.326	7,31	3.55e-06	0.304*
Relative expression *MtSUT1-1* (Figure S2.6A)	2.633	5,12	0.07867	0.3589
Relative expression *MtSUT2* (Figure S2.6B)	4.3152	5,12	0.01768	0.2149
Relative expression *MtSUT4-1* (Figure S2.6C)	4.0816	5,12	0.02135	0.1404
Relative expression *MtSWEET1b* (Figure S2.7A)	0.8756	5,13	0.002016	0.4144
Relative expression *MtSWEET3c* (Figure S2.7B)	12.06	5,13	0.0001654	0.9071
Relative expression *MtSWEET11* (Figure S2.7C)	7.2228	5,13	0.001941	0.0508
Relative expression *MtSWEET13* (Figure S2.7D)	33.38	5,14	2.67E-07	0.0508
Relative expression *MtSWEET12* (Figure S2.7E)	0.1246	5,12	0.1084	0.1246
Relative expression *MtSWEET15c* (Figure S2.7F)	5.6516	5,13	0.005524	0.3282
Relative expression *STR* (Figure S2.8A)	36.445	5,11	1.75E-06	0.5319
Relative expression *STR2* (Figure S2.8B)	5.3061	5,12	0.008407	0.0852
Relative expression *MtPT4* (Figure S2.8C)	16.585	5,12	5.04E-06	0.5681
Relative expression *MtAMT2;3* (Figure S2.8D)	17.247	5,12	4.13E-05	0.989

CHAPTER 3: NUTRIENT ALLOCATION STRATEGIES OF ARBUSCULAR MYCORRHIZAL FUNGI (AMF) IN COMMON MYCORRHIZAL NETWORKS WHEN THEY COMPETE WITH RHIZOBIA BACTERIA

3.1 Abstract

Legumes form a complex but extremely important tripartite interaction with both arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing rhizobia in which both root symbionts provide mineral nutrients to the host plant in exchange for fixed carbon. During tripartite interactions, rhizobia are restricted to occupying the nodules they induce on the roots of the host plant, but extraradical mycelia of AMF can simultaneously colonize the root system of additional compatible host plants. This results in the formation of a common mycelial network (CMN), thus creating a biological market for nutrient exchange. In this study, we asked whether the nitrogen-fixing efficiency of rhizobia would affect how AMF allocate nitrogen (N) to hosts plants connected by a CMN. We hypothesized that AMF would allocate more N to host plants colonized by Fix^- than by Fix^+ rhizobia. To test this, we provided AMF with the stable isotope ^{15}N to trace how much N the fungus would allocate to interconnected plants colonized by Fix^- or Fix^+ rhizobia. We found that tripartite interactions with Fix^+ rhizobia led to synergistic growth responses due to the host plant's increased access to fixed N. However, co-inoculation with Fix^- rhizobia and AMF or sole inoculation with AMF resulted in elevated ^{15}N enrichment in the shoot of the host plant. These results indicate that AMF do not exchange as much N with host plants colonized by Fix^+ rhizobia because their N demand is mostly fulfilled by the bacteria. Instead, they

allocate most of the N they take up from the soil to the host plant with a greater N demand due to the lack of access to fixed nitrogen. Our study provides important insights into how AMF control N allocation within their CMN under different inoculation regimes thereby ensuring that they deliver N to the host plant with the greatest N demand. In this way, AMF maximize both their carbon uptake from their host plant and the symbiotic benefits they provide to their hosts.

3.2 Introduction

Arbuscular mycorrhizal fungi (AMF) form an endosymbiosis with the roots of approximately three-fourths of all species of land plants [129]. All AMF are obligate biotrophs that obtain carbon (C) from their host plant in exchange for their ability to increase host nutrient capture. The extraradical mycelium (ERM) of AMF form an extensive network in the soil beyond the rhizosphere, thus allowing the plant to access slow-diffusing and poorly soluble nutrients, like phosphorus (P), as well as nitrogen (N), sulfur, and various trace elements. The fungus exchanges these mineral nutrients with the host plant through intraradical mycelia (IRM) that form nutrient exchange structures called arbuscules in root cortical cells [182-184].

In addition to AMF, most legumes can also associate with the diazotrophic soil bacteria rhizobia. These bacteria induce the formation of nodules, which are specialized root structures that provide rhizobia with an oxygen-reduced environment favoring biological N_2-fixation (BNF) [185, 186]. Within nodules, rhizobia differentiate into bacteroid and are able to reduce atmospheric N_2 to NH_3 using the nitrogenase enzyme complex [187]. This energetically costly process allows the bacteria to provide the host plant with N but requires a tremendous C cost—up to 30% of the host's fixed C [128]. AM fungi can receive up to 20% [134, 171, 188, 189] Although the combined C cost of legume-tripartite interactions with AMF and rhizobia is high (up to 50%), the C investment is worthwhile for the host plant because it results in synergistic benefits beyond the additive benefits of single inoculation with either symbiont [101, 163, 190, 191]. AMF colonization is increased following the addition of Nod factor produced by rhizobia [192, 193] Plants

can simultaneously benefit from N that is provided by both root symbionts. For example, nodulated *Phaseolus vulgaris* had a decreased dependency on BNF when colonized by AMF with access to NH_4^+ [138]. To ensure the success of tripartite interactions, plants must control the extent of root colonization by both symbionts, and they do so using auto-regulatory mechanisms [194, 195].

During tripartite interactions, rhizobia are restricted to occupying the nodules they induce on the roots of the host plant, but extraradical mycelia of AMF can simultaneously colonize the root system of additional compatible host plants. This results in the formation of a common mycelial network (CMN). The (CMNs) that AMF form can connect not only plants of the same, but also of different species at a variety of different developmental stages. There is growing evidence that CMNs affect the survival, fitness, behavior, and competitiveness of the plants and fungi interconnected by CMNs. In this study, we evaluated nutrient allocation strategies of AMF forming a CMN with plants colonized by rhizobia with different N-fixing abilities. We used a multi-compartment system in which two independently colonized plants shared one CMN. One Root compartment (RC) will contain a plant that is co-colonized with AM and Fix+. The *Medicago* plant in the other RC will co-colonized by either AM and Fix-or just AM. This will allow us to measure how the presence of a tripartite interaction influences the allocation of resources within a CMN. These systems will allow us for example to determine whether AM fungi in CMNs preferentially allocate N to a host, that is more dependent on the fungal N contribution for fungus or rhizobia bacteria. It is common in field condition; legumes reside not only nitrogen fixing rhizobia but also an inefficient rhizobium that could not fix atmospheric nitrogen. The role of CMNs on these tripartite interactions has received remarkably little

attention. In this study, the potential for exogenous N transfer by CMNs during tripartite interactions was examined.

3.3 Methods

3.3.1 Plant, fungal, and bacterial material

Medicago truncatula A17 seeds were acid scarified using 36N H_2SO_4 for 8 min, rinsed several times with tap water, and sterilized with 7.5 % household bleach for 2 min. After sterilization, the seeds were thoroughly rinsed with sterile deionized water. The seeds were kept in 1uM Gibberellic Acid (GA3) agar and incubated at 4°C for overnight and transferred to room temperature for one day. Germinating seeds were transferred onto Ingestad's square agar plates and incubated in the dark [147]. After 5 d, uniform seedling were transferred to a hydroponic system containing modified Ingestad's nutrient solution (250 μM NH_4NO_3, 50 μM KH_2PO_4) [147]. After 8 d, *Medicago* plants were transferred into custom-made multi compartment systems (Figure 1) filled with sterilized (2 h at 121°C) growth substrate containing 20% organic soil, 40% perlite, and 40% sand by volume. These systems were constructed using 4-way PVC pipe with an internal diameter of 4 cm where matching 50uM nylon mesh (BioDesign Inc., New York, NY, USA) were used (Figure 3.1). The mesh prevented root penetration to the hyphal compartment but allowed fungal hyphae to crossover and form a CMN traversing the hyphal compartment (HC) a modified version of our previous system [65]. HCs were made from a PVC pipe that exactly fits on both plant systems,17-cm-long and separated from the root compartments (RCs) by a double layer of a 50-um nylon mesh, which was divided by a 30-cm-long piece of wire (0.9 mm) wrapped into a spiral to prevent ion diffusion from the HC into the RCs.

Ten days after transplanting of *M. truncatula*, each *Medicago* plants received ~200 AMF spores and nearly 0.1 g of root material derived from the ~3-month-old. The fungal inoculum was produced in axenic Ri T-DNA transformed carrot (*Daucus carota* clone DCI) root organ cultures in Petri dishes filled with mineral medium [148]. AMF spores were isolated by blending the medium in 10 mM citrate buffer (pH 6.0) Five sets of plants were not inoculated and serve as a negative control treatment. At 21 d, no AMF colonization was observed, so we reinoculated each plant with an additional ~150 AMF spores. After an additional 3 w, experimentally designated plants were inoculated with either wild-type nitrogen fixing *Ensifer meliloti* Dangeard (1021) Fix^+ or a Fix^- strain compromised in a promotor fused to the bacA gene, which stops bacteroid differentiation in the nodule thus preventing nitrogen fixation [152] . The bacteria were grown in tryptone yeast (TY) broth on a rotatory shaker at 220 rpm at 30 °C for 24 h. For Fix- rhizobia growth we used TY broth/Agar with Neomycin (Nm200) and Spectinomycin (Sm50) antibiotics. Before inoculation, the bacteria were pelleted by centrifugation and resuspended in autoclaved Ingestad's nutrient solution without N and P to an OD_{600} of 0.1. Through all stages of the experiment, the plants were grown in a growth chamber (model TC30; Conviron, Winnipeg, MB, Canada) under photosynthetic photon flux of ~500 µmol m^{-2} s^{-1}, a 16 h photoperiod, 25°C Day/ 20°C night temperatures, and a relative humidity of 60%.

3.3.2 Experimental design and ^{15}N labeling

The inoculation regimes used in this study included the following a non-inoculated control system (C) where both plants remained uninoculated; a mycorrhizal control system (AM) where both plants were only inoculated with AMF ; a T^{Fix+}/AM system with one plant co-inoculated with Fix^+ rhizobia and AMF and the other only inoculated with AMF;

a T^{Fix+} system with both plants inoculated with Fix^{+} rhizobia and AMF; and a T^{Fix+}/T^{Fix-} system consisting of tripartite plants inoculated with Fix+ rhizobia and AMF and the other plant inoculated with Fix- rhizobia and AMF. Five biological replicates were established for each colonization regime, but one AM system as sacrificed to check for colonization. One system from each of T^{Fix+}/T^{Fix-} and AM only inoculated plant system were removed from the data analysis after outlier detected in the boxplot. For C, AM, and T^{Fix+} systems, both plants were evaluated the same since they were inoculated in similar way. To induce nutrient demand and ensure nodulation and mycorrhizal colonization, the plants were fertilized once halfway through the growing period with low N and P (250 μM NH_4NO_3, 50 μM KH_2PO_4) Ingestad's nutrient solution. The plants exhibited signs of nutrient stress, including stunted growth and yellowish leaves at the time of P and N addition.

After successful root colonization by both AMF and rhizobia and confirmation of hyphal crossover f the RC and HC, we added a low P Ingestad's solution spiked with 4 mM $^{15}NH_4Cl$ (Sigma Aldrich, St. Louis, USA; $+^{15}N$) to the HCs of all the plants, including C plant systems. This allowed us to confirm that no mass flow occurred between the HC and RC in the absence of hyphae, since none of the C plants showed any significant ^{15}N labeling above natural ^{15}N abundance; as such, these plants were later treated as non-labeled controls.

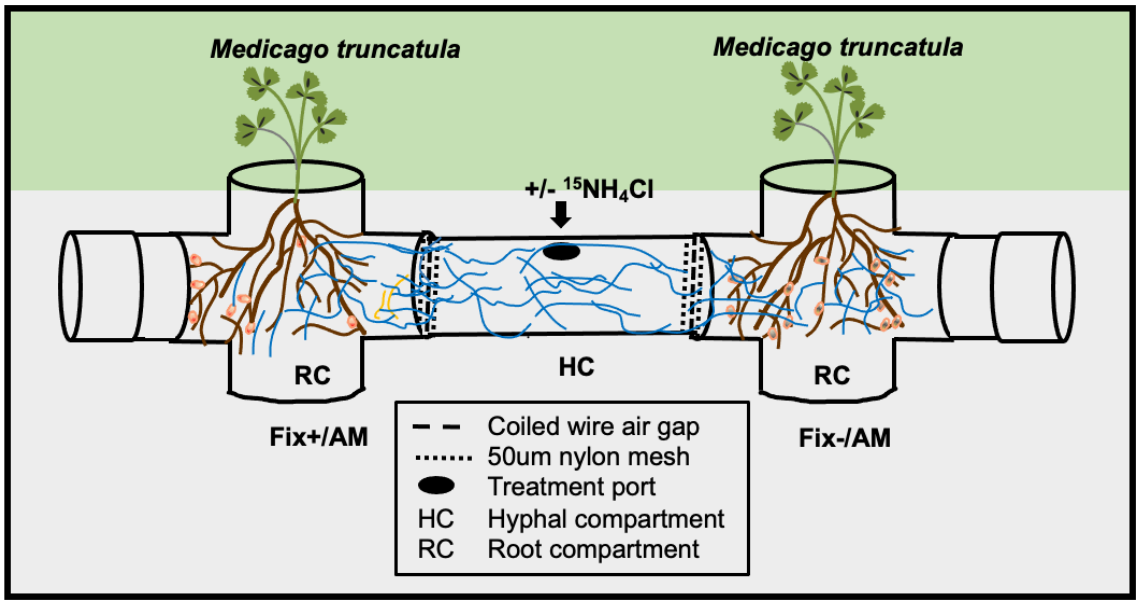

Figure 3. 1: The custom-made growth system. A double membrane with an airgap (two sheets of 50-um nylon mesh divided by a 35-cm-long wire (spiral) prevented the diffusion of nutrients from the hyphal compartment (HC) to the root compartment (RC) but allowed fungal hyphae to cross from the RCs into the HCs. In between the two plants system, 17 cm PVC pipe helps to connect via common mycelial networks.

3.3.3 Plant harvest and sampling

All plants were destructively harvested 10 d post-^{15}N labeling. The fresh weight of shoots and roots were taken and both tissues were divided into subsamples. From root tissues, 0.1 to 0.3 g subsamples were flash-frozen in liquid nitrogen and stored at -80°C for future gene expression analysis. Additional fresh root subsamples were taken to evaluate AMF root colonization and to conduct acetylene reduction assays (ARA) as described below. The root subsample for evaluating AM colonization were stored in 50% ethanol (v:v) at 4°C. Residual shoot and root tissues were dried at 70°C to evaluate dry mass, stable isotope enrichment (^{15}N), and both N and P content.

3.3.4 Mycorrhizal colonization and acetylene reduction assays

Subsamples of roots colonized with AMF were cleared by incubation in 10% KOH (v:v) at 90°C for 2 h, rinsed with tap water 5-6 times, and stained with a 5% Sheaffer ink-

vinegar (v:v) solution at 90°C for 30 min [153]. At least 75 stained roots segments per plant were then examined for AM colonization using the gridline intersection method [154]. We examined both nodulated (Fix^+ and Fix^-) and control roots for nitrogenase enzyme activity using the acetylene reduction assay as described previously [155]. At plant harvest, root subsamples were carefully placed on sterile moist filter paper in 30 ml glass tubes. All tubes containing root samples were sealed with a rubber septum at the same time and 3 ml (10 %, v:v) of acetylene gas was immediately injected into each tube using a syringe. After 24 h, we measured the ethylene production with an Agilent Technologies 7890A Gas Chromatography System (Santa Clara, CA, USA). Multiple standards of ethylene gas were used to generate a calibration curve and sample peaks were then fitted to the standard curve. We normalized the ethylene production by the total number of nodules of each root aliquot taken for ARA and calculated the amount of ethylene produced per nodule and per plant. The root subsamples used for ARA were also used for determining the total nodule number on subsamples. After counting the total nodule number on root subsample taken for ARA, we normalized them according to total dry weight of the roots.

3.3.5 Phosphate and ^{15}N analysis in plant tissues

A tissue homogenizer (Precellys 24, Cayman Chemical Company, Ann Arbor, MI, USA) was used to pulverize the dried shoot and root aliquots. To determine the P content, the homogenized plant tissue was first digested with 1 ml of 2N HCl for 2 h at 95°C, and P was measured spectrophotometrically at 436 nm after adding ammonium molybdate vanadate (AMV) (Fisher Scientific, Pittsburgh, USA). ^{15}N enrichment in shoot and root tissue samples 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 $\delta^{15}N$ to ^{15}N enrichment in plant biomass was calculated as reported previously [156]. The percentage of ^{15}N allocation was calculated based on the total recovered $\delta^{15}N$ from the plant tissues after ^{15}N labeling after considering total N present in the tissue. This was done by multiplying atom ^{15}N present in tissue (root or shoot) and percentage of nitrogen (N) as done by previous study [196].

3.3.6 Real-time quantitative PCR (RT-qPCR) of genes involved in nitrogen, phosphate, and sugar transport

Using RT-qPCR, we measured the transcript abundance of genes encoding the mycorrhiza-inducible plant P transporter *MtPT4* [46, 47] and the ammonium transporter *MtAMT2;3* [48, 49], both of which served as a control for AM specific P transporter and ammonium transporter. All steps for the DNase treatment of RNA, cDNA synthesis, and qPCR amplifications were performed as described previously (Yakha 2021 et al.). The RT-qPCR reaction was run in a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) using the same cycle of: 50°C for 2 min and 95°C for 15 min; 40 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s with fluorescent signal recording at the end of each cycle; and final dissociation at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. For all reactions, *Mtef1α* was used as a house keeping gene. The gene expression coefficients were calculated using the $2^{-\Delta Ct}$ method. The results are based on three to five biological replicates with three technical replicates. All the primers used for qPCR were found in Table S2.

3.3.7 Statistical treatment

All results were analyzed using a one-way ANOVA with colonization group with each response variable. Levene's test for homogeneity of variance and Shapiro–Wilk normality test with model residuals were fulfilled before conducting ANOVA. Data that were not normally distributed were log-transformed to fulfill the assumption. If p-value were significant, post hoc comparisons were done by least significant difference (LSD) test for multiple group comparisons. T-tests were performed for pairwise comparisons of certain groups as mentioned in the text. Correlations and computed P-values were analyzed using Pearson's correlation coefficient. All statistical tests were performed at the significance level of $p \leq 0.05$ and analysis conducted in R studio [197].

3.4 Results

3.4.1 Arbuscular mycorrhizal colonization and rhizobial nodulation in host plant roots

The average, percent AMF colonization in plants from the AM colonization regime was 33% (Fig 3.2A), but dual inoculation with Fix^+ rhizobia in the Fix^+/AM system led to a significant increase compared to both the AM and all other colonization regimes. However, this Fix^+-induced increase in percent AMF colonization did not hold true for the T^{Fix+}/AM colonization regime as T^{Fix+}/AM plants were not more colonized than AM only inoculated plants from the same system. Interestingly, % AMF colonization of T^{Fix-} plants was nearly significantly lower than that of T^{Fix+} plants in the same system ($p = 0.08$), which suggest that the nitrogen fixing status of rhizobia can alter the ability of AMF to colonize a nodulated host plant. As expected, no AMF colonization was observed in non-inoculated roots.

We also determined the nodule number on each plant per unit of root dry weight and found that plants from the T^{Fix+} colonization regime had the highest nodule number per gram of root dry weight (Fig S3.1A) and per plant (Fig 3.2B). Conversely, root nodule number was lowest in nodulated plants from the $T^{Fix+/}$AM colonization regime, but for the T^{Fix+}/ T^{Fix-} system, nodule numbers were equivalent on adjacent plants and not significantly different from nodulation rates observed in the other colonization regimes. No nodules were observed in non-inoculated control roots.

Based on the acetylene reduction assay, ethylene production per nodule was significantly higher for Fix^+ rhizobia from the T^{Fix+}/ T^{Fix-} system than from the T^{Fix+} system, but equivalent when compared to Fix^+ rhizobia from the T^{Fix+}/AM system (Fig S3.1B. However, there was no statistical differences in the ethylene production per plant by Fix^+ rhizobia from any of the colonization regimes (Fig 3.2C). As expected, roots with nodules occupied by Fix^- rhizobia as well as non-inoculated control and AM only inoculated roots did not show any ethylene production (Fig 3.2C and Figure S3.1B).

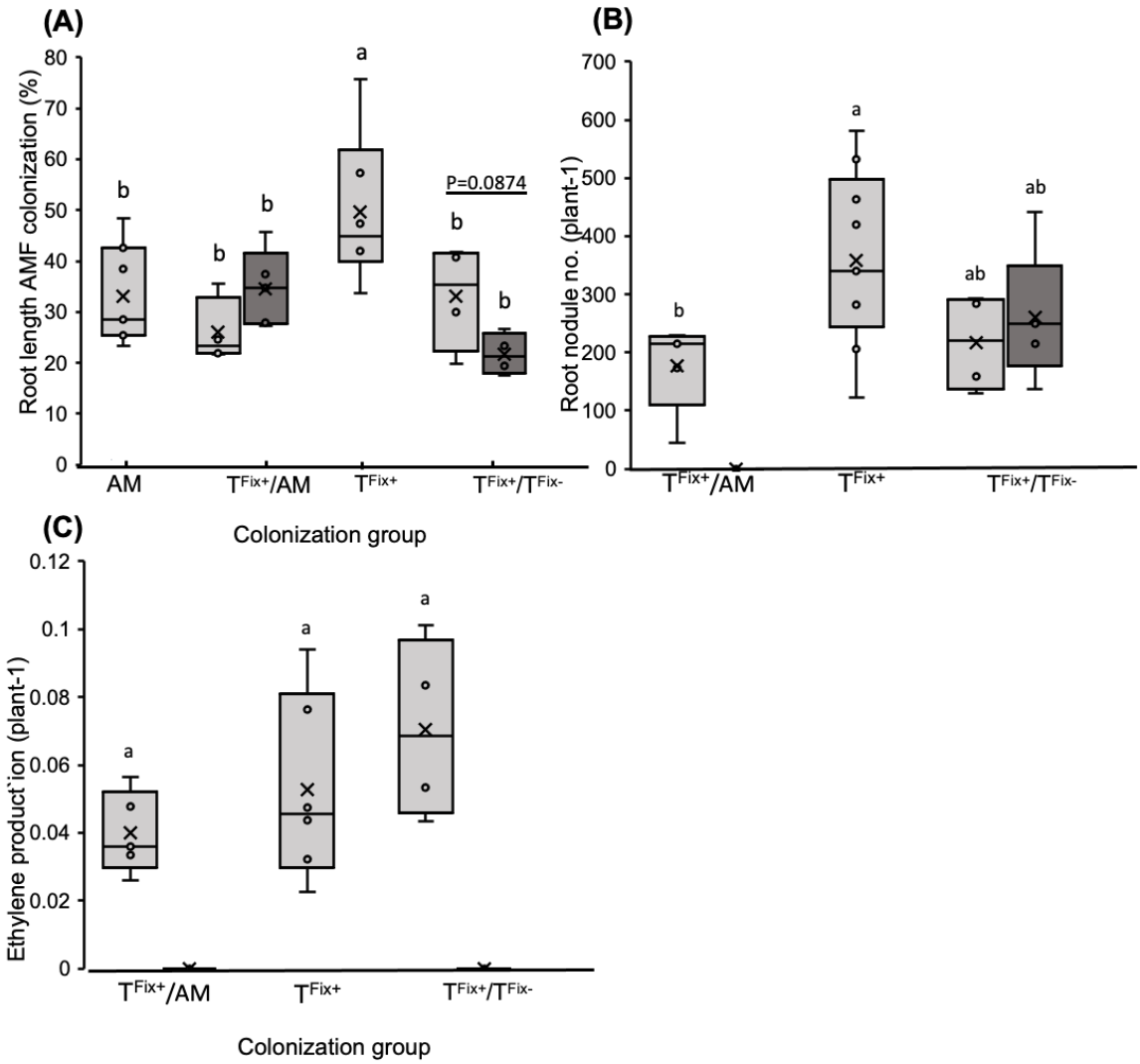

Figure 3.2: Arbuscular mycorrhiza fungi (AMF) colonization (% root length colonized) (A) Total number of nodule plant $^{-1}$; (B) and ethylene production plant $^{-1}$; of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions in system 1 (light grey bars); and system 2 (dark grey). Root colonization abbreviations: C: plants that were non inoculated; AM; plants were inoculated with AMF, *Rhizophagus irregularis*; T^{+}/AM: system 1 plants were inoculated with Fix+ rhizobia bacteria, *Ensifer meliloti* and AMF, and system 2 were inoculated with AMF only; T^{Fix+}: plants inoculated with Fix+ rhizobia and AMF; T^{Fix+}/T^{Fix-} : system 1 plants were inoculated with Fix+ rhizobia and AM while system 2 plants were inoculated with Fix- rhizobia, *E. meliloti* and AM in all the *Medicago truncatula* plants. Data are presented as mean ± SE (n = 3–5). Different letters on the bars indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$)

3.4.2 Plant growth response and N and P content

Inoculation with only AMF led to a significant increase in shoot dry weight compared to non-inoculated control plants (Figure 3.3A). Plants also inoculated with Fix^+ rhizobia experienced a further increase in shoot dry weight when comparing between the T^{Fix+}/AM inoculation regimes and within the T^{Fix} regime. In addition, within the inoculation regime of T^{Fix+}/ T^{Fix-} , the T^{Fix+} plants achieved a nearly significantly higher shoot dry mass than T^{Fix-} plants (t test, $p = 0.06$). We found similar effect of different colonization regime on root dry weight (Fig 3.3B).

We consistently observed significantly higher N concentrations in the shoots and roots of plants inoculated with Fix^+ rhizobia than in plants inoculated with either Fix^- rhizobia, only AMF, or nothing at all; among these three treatments, no significant differences in N concentration were observed (Fig 3.3C, 3.3D). Similar trends for Fix^+ inoculated plants were observed for shoot N content, but in addition, AM only inoculated plants had higher shoot N content than control plants (Figure S3.2A). For root N content the differences between plants inoculated with Fix^+ rhizobia and the other treatments diminished when compared to root N concentration, but the overall trend was similar (Fig S3.2B).

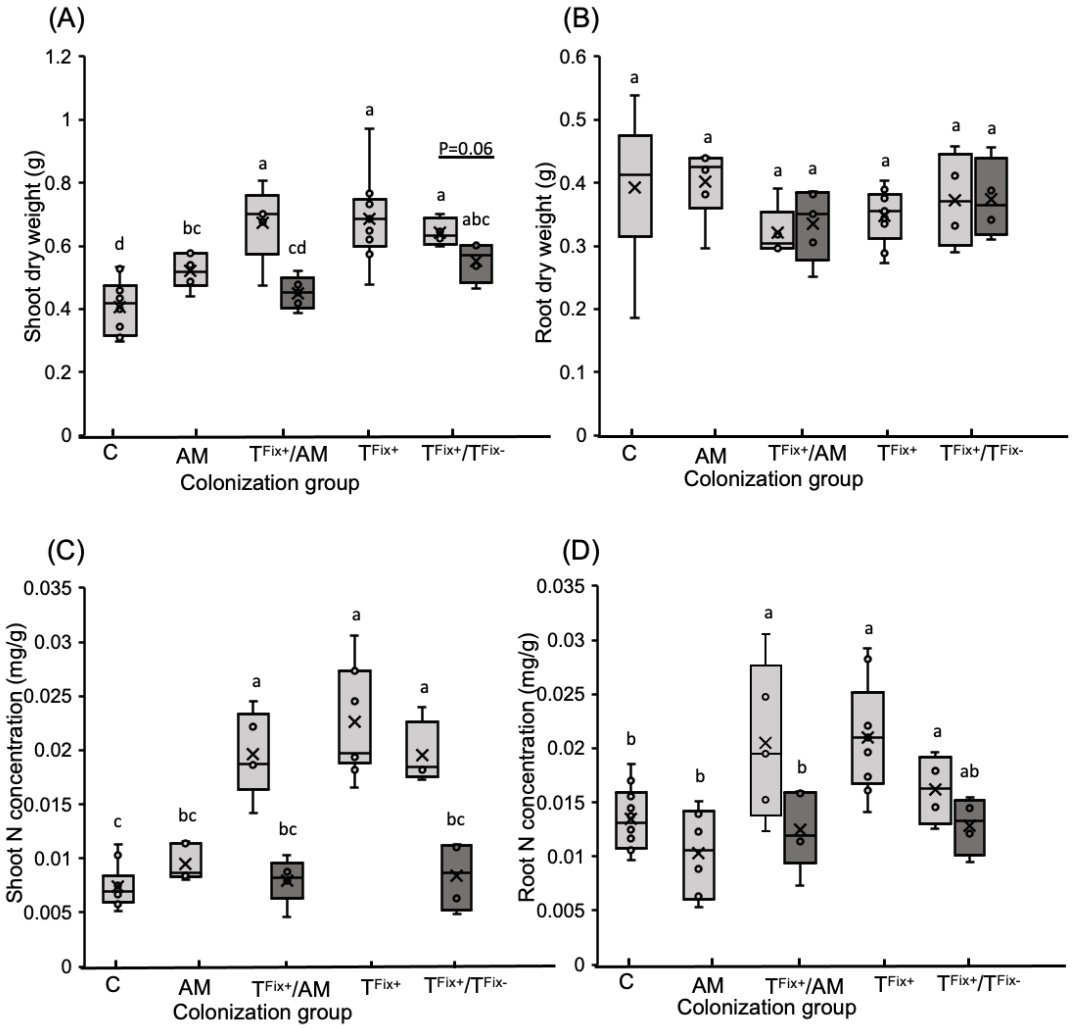

Figure 3.3: Shoot (A) and root (B) dry weights; N concentration in shoots (C) and root (D); of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions in system 1 (light grey bars); and system 2 (dark grey). Root colonization abbreviations: C: plants that were non inoculated; AM; plants were inoculated with AMF, *Rhizophagus irregularis*; T^{+}/AM: system 1 plants were inoculated with Fix+ rhizobia bacteria, *Ensifer meliloti* and AMF, and system 2 were inoculated with AMF only; T^{Fix+}: plants inoculated with Fix+ rhizobia and AMF; T^{Fix+}/T^{Fix-}: system 1 plants were inoculated with Fix+ rhizobia and AM while system 2 plants were inoculated with Fix- rhizobia, *E. meliloti* and AM in all the *Medicago truncatula* plants. Data are presented as mean ± SE (n = 3–5). Different letters on the bars indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$)

Few differences were observed between colonization regimes for shoot P concentration and P content, except in the T^{Fix+}/ T^{Fix-} regime in which T^{Fix+} plants had significantly more P than T^{Fix-} plants (Figure 3.4A and Fig S3.2C), even though there were

no differences in root P concentration and content (Figure 3.4b and S3.2d). Since we did not find the differences in the dry root of all colonization regime (Fig 3.3B), this led to no significant differences in the root P concentration (Fig 3.4B) and P content (Fig S3.2D) except T^{Fix+} root of T^{Fix+}/ T^{Fix-} system might be due to biomass effect on other root systems. For instance, the dry root weight of non- inoculated control plants (C) was comparable with all other AM or Fix+ rhizobia inoculated roots (Fig 3.3B). Based on these results, the low variability in shoot P concentration between non–inoculated and AM inoculated plants indicates that the P supply was not growth-limiting for the plants.

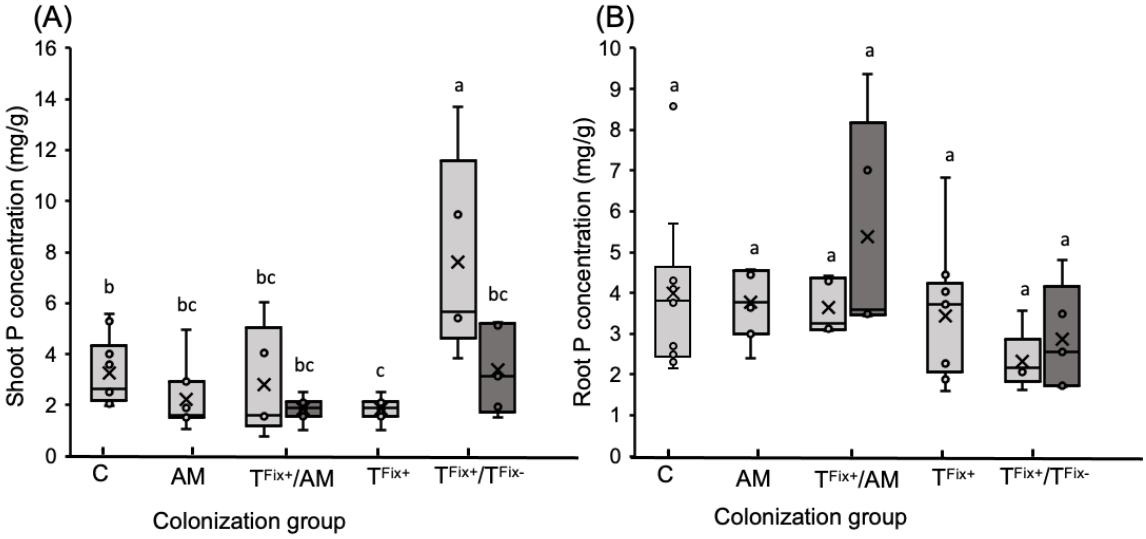

Figure 3.4: P concentration in shoots (a) and root (b); of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions in system 1 (light grey bars); and system 2 (dark grey). Root colonization abbreviations: C: plants that were non inoculated; AM; plants were inoculated with AMF, *Rhizophagus irregularis*; T^{+}/AM: system 1 plants were inoculated with Fix+ rhizobia bacteria, *Ensifer meliloti* and AMF, and system 2 were inoculated with AMF only; T^{Fix+}: plants inoculated with Fix+ rhizobia and AMF; T^{Fix+}/T^{Fix-} : system 1 plants were inoculated with Fix+ rhizobia and AM while system 2 plants were inoculated with Fix- rhizobia, *E. meliloti* and AM in all the *Medicago truncatula* plants. Data are presented as mean±SE (n=3–5). Different letters on the bars indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$)

3.4.3 ^{15}N delivery by common mycorrhizal networks

In all colonization regimes that included AMF inoculation, the addition of ^{15}N-NH_4Cl to the hyphal compartment resulted in ^{15}N transfer to specific host plants, but no ^{15}N transfer was observed in non-inoculated control plants (Figs. 3.5A and 3.5B and S3.3A and 3.3B). As hypothesized, the extent of ^{15}N transferred was highly dependent on the colonization regime. Shoot ^{15}N enrichment (%) was significantly higher in plants inoculated with AMF compared not only to uninoculated control plants, but also plants inoculated with Fix^+ rhizobia (Figure 3.5). The equivalent ^{15}N enrichment levels in uninoculated control and Fix^+-inoculated plants suggests that the N-fixing capacity of Fix^+ rhizobia almost entirely shut down AMF transfer of ^{15}N to the host. In turn, the AMF allocated significantly more ^{15}N to the other host plant in the same CMN, whether it was inoculated with Fix^- rhizobia or not. A similar trend was observed for root ^{15}N enrichment as well (Figure 3.5B), except that ^{15}N enrichment was equivalent in Fix^+/AM and Fix^-/AM roots in the same inoculation regime, both of which were higher than roots from non-inoculated control plants ($p=0.026$ and 0.020, respectively). Similar trends were found in $\delta^{15}N$ levels for both shoots and roots of the *Medicago* plants of all inoculation group of either of same or different systems (significant according to t test in shoot; between C and T^{Fix+}/AM; $p=0.026$, C and T^{Fix+}/ T^{Fix-}; $p=0.04$) (Fig S 3.3A and 3.3B). These findings were also supported by percentage distribution in shoot of single AMF inoculated was 64% while 36% were distributed towards Fix+/AM shoot. Whereas root had 59% and 41% respectively. Similarly, we found that 62% ^{15}N were allocated to shoot of Fix-/AM and 38% towards T^{Fix+}/AM colonized plants of same system with 54% and 46% respectively in the roots.

We also evaluated ^{15}N content in root and shoot tissues which takes tissue biomass into consideration. For this parameter, we observed that shoot and root ^{15}N content of all AMF inoculated plants was consistently significantly higher than non-inoculated control plants, suggesting that even plants inoculated with Fix^+ rhizobia received some ^{15}N from AMF (Fig 3.5C and 3.5D). However, shoot and root total ^{15}N content was still highest in AM-only inoculated plants.

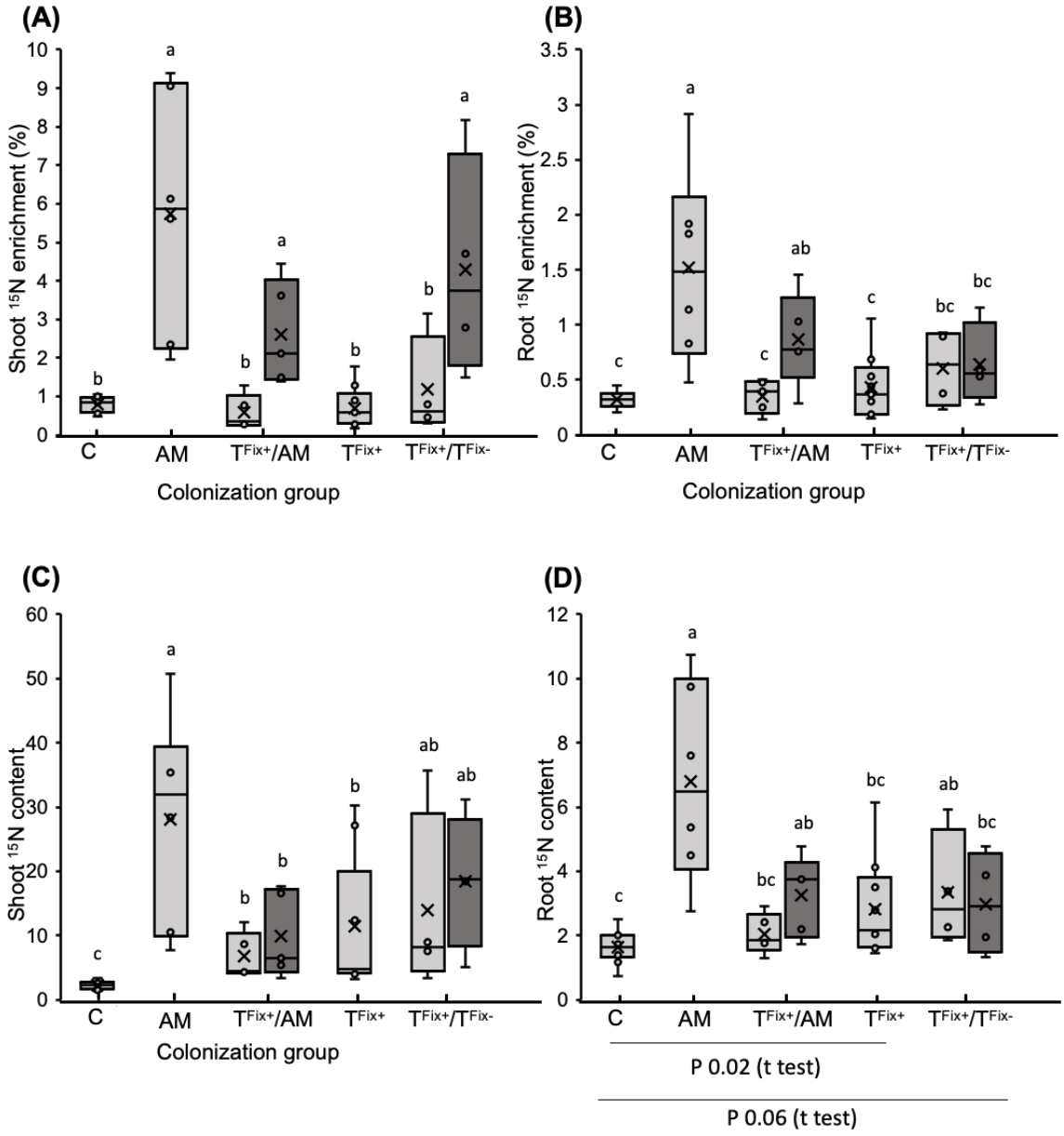

Figure 3.5: Shoot (a) and root (b) ^{15}N enrichment; and ^{15}N content in shoots (c) and root (d); of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions in system 1 (light grey bars); and system 2 (dark grey). Root colonization abbreviations: C: plants that were non inoculated; AM; plants were inoculated with AMF, *Rhizophagus irregularis*; T^{+}/AM: system 1 plants were inoculated with Fix+ rhizobia bacteria, *Ensifer meliloti* and AMF, and system 2 were inoculated with AMF only; T^{Fix+}: plants inoculated with Fix+ rhizobia and AMF; T^{Fix+}/T^{Fix-}: system 1 plants were inoculated with Fix+ rhizobia and AM while system 2 plants were inoculated with Fix- rhizobia, *E. meliloti* and AM in all the *Medicago truncatula* plants. Data are presented as mean ± SE (n = 3–5). Different letters on the bars indicate

statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$)

3.4.4 Expression of Plant P, N, and sugar transporters in roots

MtPT4 and *MtAMT2;3* expression was induced exclusively in roots colonized by AMF but not in non-inoculated control roots (Fig 3.6A and 3.6B). We observed that expression was not dependent on the colonization regime as Fix^-/AM plants had similar *MtPT4* expression compared to T^{Fix+}/AM plants from the same colonization regime. T We found that *MtAMT2;3* expression in mycorrhizal roots (Figure 3.6B) was not dependent on the type of co-inoculated rhizobia (Fix- and Fix+). However, there were some indications that system with T^{Fix+}/AM showed slightly lower expression than single inoculated AM plants of same system b which might be due to relatively high variability in fold expression within treatments.

To determine the role of *MtSWEET1b*, a known mycorrhiza-induced sucrose transporter, the expression was highest in Fix+ rhizobia and AM co-inoculated roots. And non-inoculated control plants were extremely low. And in the T^{Fix+}/ T^{Fix-} system, *MtSWEET1b* expression was significantly higher in the roots of Fix+/AM than in Fix^-/AM plants (Fig 3.6C). The exclusive expression of *MtPT4* and *MtAMT2;3* in the AMF inoculated plants but not in non-inoculated control plants indicates that there was no contamination.

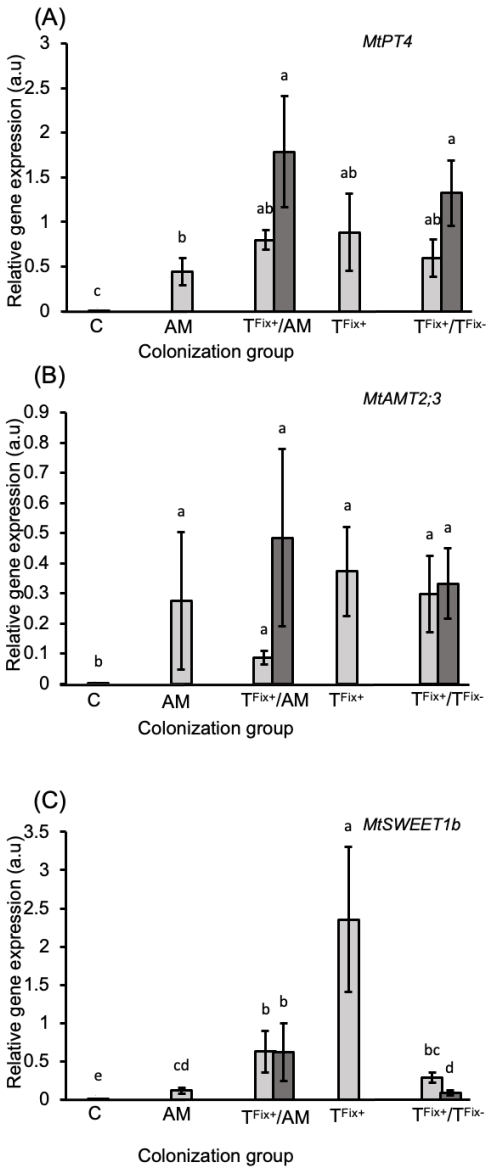

Figure 3.6: Relative expression of *MtPT4* (A) and *MtAMT2;3* (B) assessed by quantitative RT-PCR in *M. truncatula* roots after mycorrhization by *R. irregularis* and nodulation by *E. meliloti* (Fix+ and Fix-). The C_t values (threshold cycles) of the samples are corrected against the C_t values of the housekeeping gene *MtTef1α.* Data for each condition are presented as mean + S.E. and were obtained from 3-5 biological and three technical replicates. under nitrogen (N) supply conditions in system 1 (light grey bars); and system 2 (dark grey). Root colonization abbreviations: C: plants that were non inoculated; AM; plants were inoculated with AMF, *Rhizophagus irregularis*; T^{+}/AM: system 1 plants were inoculated with Fix+ rhizobia bacteria, *Ensifer meliloti* and AMF, and system 2 were inoculated with AMF only; T^{Fix+}: plants inoculated with Fix+ rhizobia and AMF; T^{Fix+}/T^{Fix-} : system 1 plants were inoculated with Fix+ rhizobia and AM while system 2 plants were inoculated with Fix- rhizobia, *E. meliloti* and AM in all the *Medicago truncatula* plants. Data are presented as mean ± SE (n = 3–5). Different letters on the bars indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$)

3.5 Discussion:

Underground, plants compete with other plants for nutrients provided by fungal CMNs, but the mechanisms that control the allocation patterns among plants are only poorly understood. The formation of CMNs is highly agriculturally relevant because legume crops are often used in intercropping management practices to increase yield and performance of non-nodulated crop species. One common intercropping scheme is to pair a cereal crop with a legume that can supply N through biological N_2 fixation. Yield advantages of intercropping legumes with non-N_2 fixing crops have been found in many intercropping systems, including wheat-faba bean [198], rapeseed-faba bean [199], and maize-soybean [200]. We examined how nutrients supplied to the CMNs were allocated between two host plants (model legume: *M. truncatula*) when fungus had competition with the rhizobia bacteria that fixes atmospheric N and inefficient rhizobia that lack N fixation capability. We found that, when fungi were given a choice, they consistently allocated a higher percentage N to hosts that had competition between inefficient Fix- rhizobia or when AMF were inoculated alone (Figure 3.4A and 3.4B). Host plants that have competition between Fix+ rhizobia and AMF seems to limit N allocation that were available from the CMNs.

For this study we did not find any transport of the rhizobia bacteria from one system to another as there were no visible cross contamination and no ethylene production on those root system that were not inoculated Fix+ rhizobia. However recent study done in -vitro and soil by Jiang *et. al.* (2021) found that phosphate solubilizing bacteria moved in a thick water film formed around fungal hyphae where carbon source provided to the media

containing petri dish which had 1cm bridge and air gap [201]. The distance to travel in the water film around soil particles may be too large for our system (17cm) as a result, no cross over of rhizobia were found in our experiment. We inoculated the host plants by different combination of rhizobia and AMF in plants of both systems. The systems with co-inoculation of Fix+ rhizobia and AMF on both systems, mycorrhizal colonization percentage and increase in the root nodule number per plant (Fig 3.2A and B). The enhancement of nodule number could be attributed to AMF facilitating the mobilization of certain elements such as N, P, K, and other minerals that involve in synthesis of nitrogenase and leghemoglobin [190]. However, contrary to this report, 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 [202-204]. This activity of suppression is known as autoregulation of nodulation (AON), a systemic mechanism that consists of a root-derived signal that is recognized by a CLAVATA1-like receptor kinase (*GmNARK; GsNARK; LjHAR1; MtSUNN; PsSYM29*) and activates the production of a CLE peptides signals in shoot for inhibition of further nodule formation [194, 205]. However, the ethylene production on this system were not statistically higher than other inoculation regime. Nodulation not only depends on the ethylene production, but also various hormones like auxin and cytokinin effects on the nodulation process [206-208]. In other systems of T^{Fix+}/AM, we found no differences in the AMF colonization as well as nodulation when the other plants of system were inoculated with only AMF as this colonization are context dependent. Higher AMF colonization in T^{Fix+} compared to T^{Fix-} connected with CMNs of two host plants also remained consistent with earlier mentioned statement.

When the fungus had access to an exogenous N source, N was delivered to the host by CMNs that led to increase in biomass and N concentrations in the shoots and roots (Figures 3.2A, 3.2C and 3.2D). Similar effects were found in other reports showing strong growth response in legumes by availability of N to the fungus [163, 190, 191, 209]. The dry root weight of non-inoculated control plants was quite high and not different than AM inoculated roots (Fig 3.3B) might be due to nutrient demand of the non-inoculated plants which resulted higher growth of root on them. And the size of the pot used for this experiment in some extent, limited the root growth of plants inoculated with different symbiont.

In earlier reports, the improved AMF acquition to P help to improve biological N fixation that led plant benefit overall [164]. Only on those plants that were colonized on one system with T^{Fix+}/AM and other with T^{Fix-}/AM, shoot P concentration and content on Fix+ mycorrhized plant was higher than Fix- mycorrhized plants indicating role of Fix+ rhizobia as nodules are known to be strong P sinks [210]. However, we did not find the differences in the shoots and roots P concentration (Figure 3.4A, B) and the acetylene reduction on each plant inoculated with Fix+ rhizobia (Figure 3.2C) that fixes atmospheric N_2. As we only fertilized the plants once with 50uM of P, similar level of P on mycorrhized, non-inoculated roots can be due to available P in the growing medium (P=5.91 ppm; Olsen's extraction, NH_4=6.4). An increase in P nutrition can improve nodule growth, metabolism and N2 fixation [211]. This suggest that P was not the growth limiting factor for this experiment. Furthermore, AMF are rarely responsible for the exclusively of plant P assimilation; plants engage in their own direct P uptake via the root epidermis and root hairs, in addition to the indirect mycorrhizal P pathway [5]. We only quantified the total P

content in the plants at harvesting time. The total P quantification represents P accumulation over the lifetime of the plant. It is possible that AMF contribute most to plant P assimilation in the earlier stages of plant development, which were not measured during this experiment [165, 166].

In previous experiment done in single tray system co-inoculated with AMF and rhizobia they found that legumes became less reliant on biological nitrogen fixation (BNF) when exposed to an external source of NH_4+ (unlabeled) [101, 212] . However same was not true when two host plants were connected by CMNs and colonized with T^{Fix-}/AM or just AM in another site of T^{Fix+}/AM systems. We found significant drop in the shoot and root ^{15}N enrichment in dual inoculated Fix+ rhizobia and AMF compared to its CMNs connected Fix-/AM or AM systems (Figure 3.5A, B, C, and D) but were higher than non-inoculated control plants. Depending on the N demand of host plant, AMF delivered more $^{15}NH_4$ towards shoots of them. The same level of enrichment on root but decrease in case of T^{Fix+}/AM shoot can be further explained as AMF was able to uptake nitrogen from the soil, but rhizobia are fulfilling plant nitrogen demand which reduces the transport from sink root to source leaves of host. This may also explain by significantly higher value for N concentration and content in the host plant (Figure 3.2C, D; Figure S3.2A, B).

We also analyzed the expression of the AM-inducible P transporter gene *MtPT4* in the roots. The peri arbuscular membrane localization of *MtPT4* involved in the P uptake from the mycorrhizal interface [213]. We found almost no differences at $p = 0.05$ in all the AM inoculated roots (Figure 3.6A), except non – inoculated control plants that show significantly low level of expression than any of the AM inoculated plants of all system. This is also supported by the P concentration and P content (Figure 3.4A, B and Figure S

3.2C and D) in the roots of *Medicago* as there was no differences in any of the root system. The present work and that of others [65, 101, 131, 214] demonstrate that AM fungi can contribute substantially to the N nutrition of plants. It is thought that the fungus transfers N in the form of ammonium across the mycorrhizal interface to the host [63, 215]. We found that fungal N transport was coupled to an induction of *MtAMT2;3* induced in roots that were colonized with AMF [216]. Similar expression in all colonization group inoculated with AMF could be due to the functional redundancy of mycorrhiza inducible AMT transporters. *MtAMT2;4* and *MtAMT2;5* was also up-regulated in mycorrhizal roots, in knock out mutants of *MtAMT2;3* but premature degeneration of arbuscules was observed, and *MtAMT2;4* was able to complement NH4+ uptake of yeast mutants in contrast to *MtAMT2;3* [49].

Sugars are essential carbon sources in plants and animals, as well as in microorganisms for their growth and development. Root symbionts compete for host plant C and trade off the nutrient resources [63, 65, 101, 132]. In this experiment we observed the expression of *MtSWEET1b* sugar transporter which belong to Clade I that help in sugar efflux [44]. Recent findings done by An, J et. al. (2019) in *M. truncatula*, *MtSWEET1b* is strongly upregulated in arbuscule-containing cells and localizes to the peri-arbuscular membrane and able to transfer glucose. Overexpression of this transporter in *M. truncatula* roots promoted the growth of intraradical mycelium (IRM) during AM symbiosis. However, the *MtSWEET1b* mutant where glucose transport was impaired, had no defects in AM symbiosis. Consistent with this finding, all the mycorrhized roots in our experiment showed higher level of *MtSWEET1b* expression suggesting the important role of it on sugar

transport in mycorrhizal symbiosis [64, 104]. Interestingly, we found higher expression in roots when fungus had competition with Fix+ rhizobia than fungus competing with Fix- rhizobia might be due to nitrogen fixing capability of Fix+ rhizobia. The host plant allocates more carbon on those plants colonized with N fixing tripartite system compared to inefficient rhizobia [104, 127, 144]. So, it is very important to understand how costs and benefits are monitored and modulated by both partners will be key to improve plant performance through tripartite interactions. However, neither of *SWEET* transporters function independently in delivering sugars to these microbial symbionts because, when they are knocked out, their activity seems to be compensated by other SWEET transporters [104, 140, 141].

3.6 Conclusions:

AMF and rhizobia symbiosis are a perfect mutualism that happens in leguminous plants. AMF form a CMNs and simultaneously colonize same or multiple host plants and species. AM fungi and their CMNs play a significant role in plant ecosystems and control the fitness and competitiveness of the plant individuals within their CMNs. Our current understanding about resource exchange in the AM symbiosis is primarily based on experiments with root organ cultures or with single plants that are colonized by one AM fungus [63, 64]. Very little is known about how AM fungi allocate nutrient resources or info chemicals within their CMN, or how host plants compete with other plants for nutrients that are available for their CMNs. More research is needed to better understand how fungal networks affect when it competes with rhizobia bacteria. when fungi were given a choice, they consistently allocated a higher percentage N to hosts that had competition between inefficient Fix- rhizobia or when AMF were inoculated alone. Host

plants that have competition between Fix+ rhizobia and AMF seems to limit N allocation that were available from the CMNs. This indicates allocation of exogenous N supply depends on demand conditions of host plants.

3.7 Supplementary Information

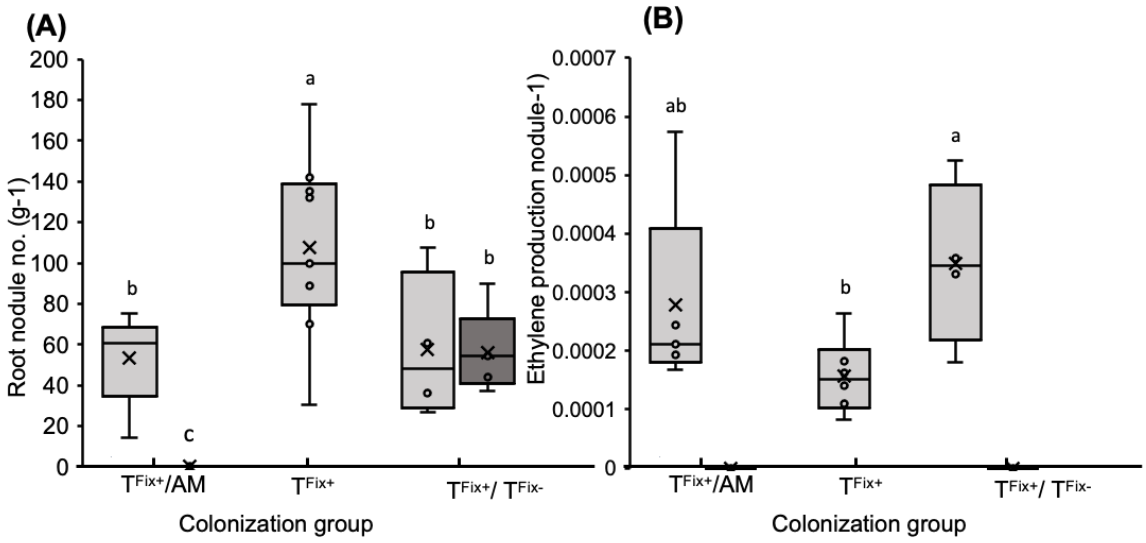

Figure S3. 1: Number of nodule gram^{-1} of dry root weight (A) and ethylene production nodule $^{-1}$ (B); of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions in system 1 (light grey bars); and system 2 (dark grey). Root colonization abbreviations: C: plants that were non inoculated; AM; plants were inoculated with AMF, *Rhizophagus irregularis*; T^{+}/AM: system 1 plants were inoculated with Fix+ rhizobia bacteria, *Ensifer meliloti* and AMF, and system 2 were inoculated with AMF only; T^{Fix+}: plants inoculated with Fix+ rhizobia and AMF; T^{Fix+}/T^{Fix-}

: system 1 plants were inoculated with Fix+ rhizobia and AM while system 2 plants were inoculated with Fix- rhizobia, *E. meliloti* and AM in all the *Medicago truncatula* plants. Data are presented as mean ± SE (n = 3–5). Different letters on the bars indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$)

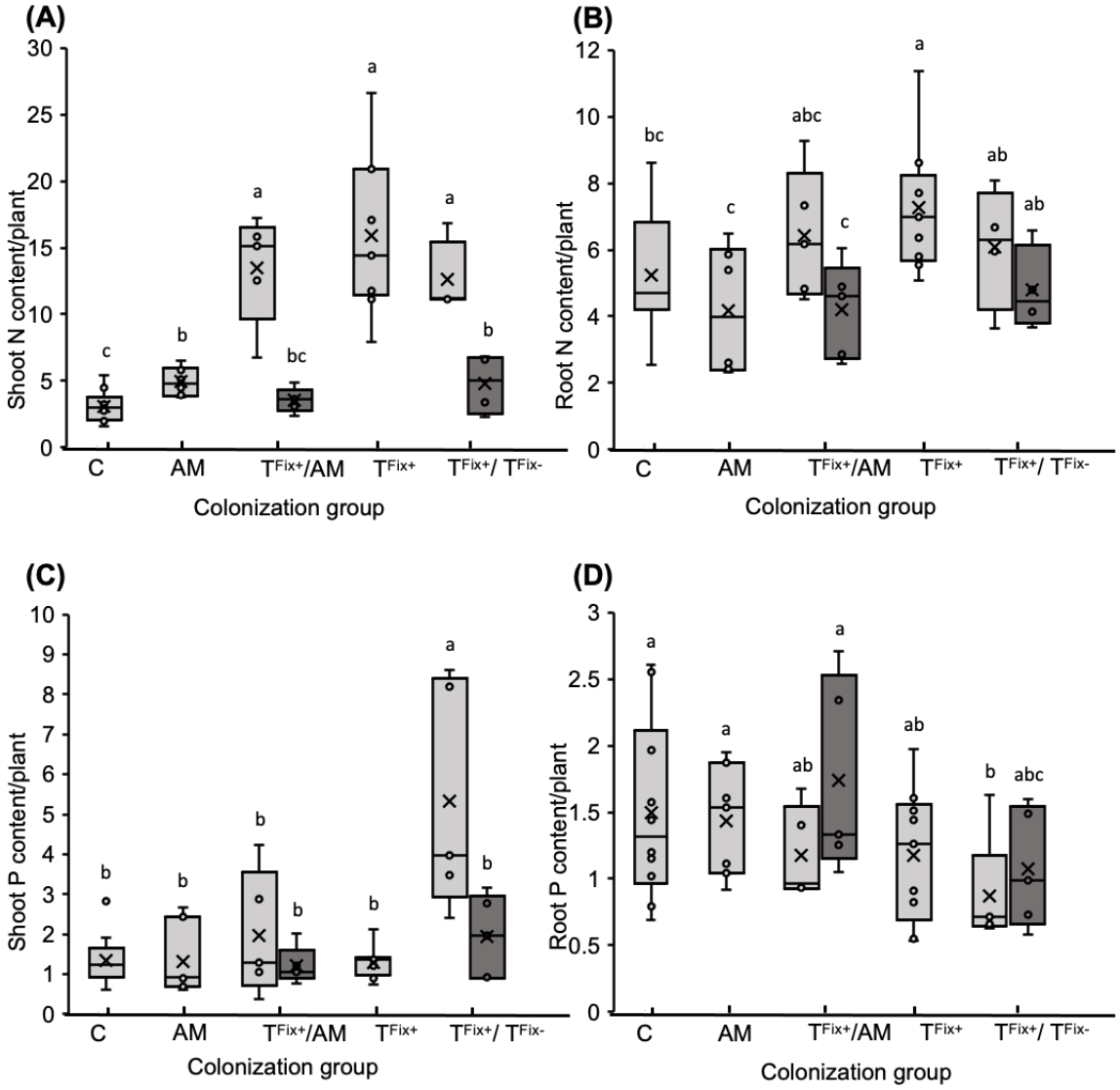

Figure S3. 2: N content in Shoot (A) and root (B) and P content in shoot (C) and root (D); of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions in system 1 (light grey bars); and system 2 (dark grey). Root colonization abbreviations: C: plants that were non inoculated; AM; plants were inoculated with AMF, *Rhizophagus irregularis*; T^{+}/AM: system 1 plants were inoculated with Fix+ rhizobia bacteria, *Ensifer meliloti* and AMF, and system 2 were

inoculated with AMF only; T^{Fix+}: plants inoculated with Fix+ rhizobia and AMF; T^{Fix+}/T^{Fix-}: system 1 plants were inoculated with Fix+ rhizobia and AM while system 2 plants were inoculated with Fix- rhizobia, *E. meliloti* and AM in all the *Medicago truncatula* plants. Data are presented as mean ± SE (n = 3–5). Different letters on the bars indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$)

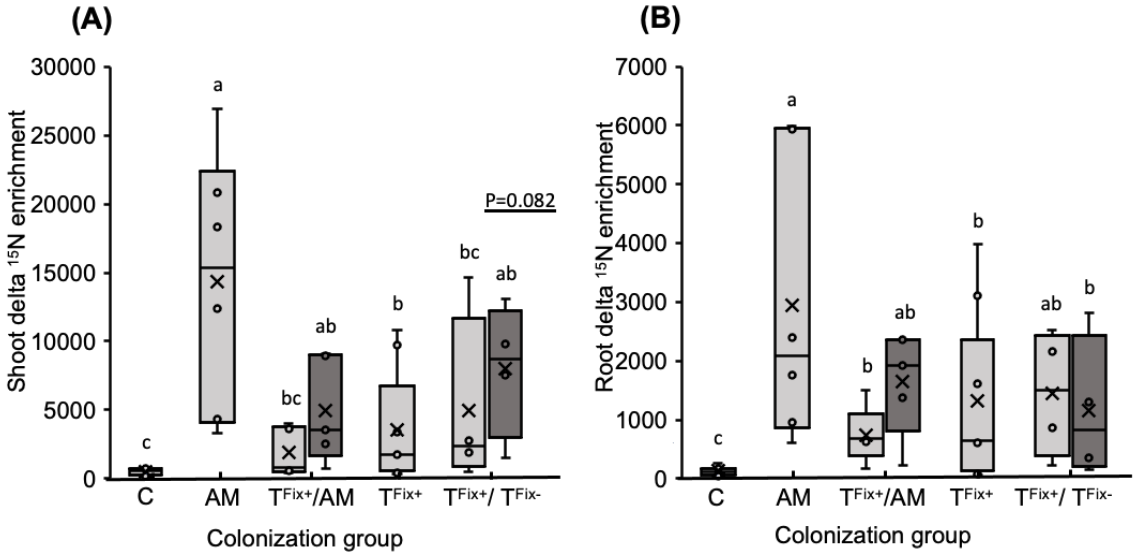

Figure S3. 3: Shoot (A) and root (B) delta ^{15}N enrichment in tissues of *Medicago truncatula* depending on the inoculation with different root symbionts and under nitrogen (N) supply conditions in system 1 (light grey bars); and system 2 (dark grey). Root colonization abbreviations: C: plants that were non inoculated; AM; plants were inoculated with AMF, *Rhizophagus irregularis*; T^{+}/AM: system 1 plants were inoculated with Fix+ rhizobia bacteria, *Ensifer meliloti* and AMF, and system 2 were inoculated with AMF only; T^{Fix+}: plants inoculated with Fix+ rhizobia and AMF; T^{Fix+}/T^{Fix-}: system 1 plants were inoculated with Fix+ rhizobia and AM while system 2 plants were inoculated with Fix- rhizobia, *E. meliloti* and AM in all the *Medicago truncatula* plants. Data are presented as mean ± SE (n = 3–5). Different letters on the bars indicate statistically significant differences among all the groups according to the LSD's test ($P \leq 0.05$)

Treatments	Shoot ^{15}N (%)	Root ^{15}N (%)
T^{Fix+}/AM		
T^{Fix+}	35.6428742022805±7.71	40.7482671122019±5.78
AM	64.3571257977195±7.71	59.2517328877981±5.78
T^{Fix+}/T^{Fix-}		
T^{Fix+}	38.4862283875234±12.50	53.6023942486504±11.76
T^{Fix-}	61.5137716124766±12.50	46.3976057513496±11.76

Table S3. 1: Shoot and root ^{15}N distribution. The calculation was done by total ^{15}N of shoot of same system was considered as 100% and calculated on each shoot of containing 15N. The root 15N distribution were also calculated same way.

Gene	**Primer sequence**	**Reference**
MtTef1a F *MtTef1a R*	ACTGTGCAGTAGTACTTGGTG AAGCTAGGAGGTATTGACAAG	Doidy et al. (2012)
MtPT4 F *MtPT4 R*	GACACGAGGCGCTTTCATAGCAGC GTCATCGCAGCTGGAACAGCACCG	Doidy et al. (2012)
MtAMT2;3 F *MtAMT2;3 R*	TGTCCGGTTCAATTCCATGG TGGCAAACACACCAGAAAGG	Breuillin-Sessoms et.al. (2015)
MtSWEET1b F *MtSWEET1b R*	GTGTTTCTTTGCGGCAGTTC ACCATTAGGTACAGCAACAAATAGG	Kryovoruchko et al. (2016)

Table S3.2: The list of primer sets used in qPCR

CHAPTER 4: PLANT-MICROBE INTERACTION TO REDUCE THE EFFECT ON SOYBEAN CYST NEMATODE INFESTATIONS

4.1 Abstract

Soybean cyst nematode (SCN: *Heterodera glycines)* is the most devastating pathogen for soybean productivity and is responsible for billions of dollars losses each year. Crop rotation with non-host crops, use of resistant varieties, and application of nematicide seed treatments in the field are widely used techniques for decreasing negative impact of SCN population in the soil. However, these strategies have become less efficient. An environmentally friendly biocontrol agent for example, Arbuscular mycorrhizal fungi (AMF) can be one of the alternatives to reduce negative effect of SCN. Through the AMF symbiosis with plant hosts, among other benefits, receive protection from pathogens. However, the mechanism for suppression of these pathogen is under researched. Here, we evaluated the effects of a commercially available arbuscular mycorrhizal fungi (AMF) (MycoApply) under greenhouse and field conditions on the reproduction of SCN and the soybean growth and yield increase. Mycoapply consists of equal combination of *Glomus mossaea, Rhizophagus irregulare, G. etunicatum, G. aggregatum* and applied in soil. Two soybean varieties: SCN susceptible and resistant, were used in these experiments. We observed increased shoot weight for AMF treated and SCN susceptible variety (*Williams-82*) infested with SCN but no effect on the resistant variety *Jack* (PI88788) in greenhouse. We found no statistical differences in the SCN egg population after application of Mycoapply® on both soybean varieties in greenhouse. However, soybean seed yield in mycorrhizal treated plots was higher than non-mycorrhizal treated plots in both year of 2018 and 2019 in Beresford by 27.91%, 24.26%, respectively. However, the AMF

colonization between Mycoapply treated and non-treated plots were not different. We found increase in soybean production in Brookings by 40.03% by application of Mycoapply. For all plots, consistently increase in the final SCN egg counts at the end of the season. Our results show that arbuscular mycorrhiza fungi can be potential candidate enables the soybean plants to be SCN tolerant and may help offset the yield losses. However, further investigation should be conducted to know the actual mechanism how these fungi are able to increase soybean production without any change in AM colonization rate and SCN infestation in the soil.

4.2 Introduction

The soybean (*Glycine max* (L.) Merr.), is an important legume crop that supplies more than half of the word's vegetable fats, oils, and protein meal [217]. Soybean cyst nematode (SCN) *Heterodera glycines,* a sedentary endoparasite, is the most destructive pathogen to soybean. It was first identified in China in 1899 [218], in United States in 1954 in North Carolina [219-221]. This pathogen has now spread up to 90% of the soybean producing states. It causes about $1.5 billion in soybean revenue losses in the U.S and Canada [222]. SCN is distributed widely throughout north-central United States comprising of 14% to 64% field prevalence [223]. In 1995, SCN was first found in Union County in South Dakota but as of 2021 it has been increased to 34 counties [224].This plant parasitic nematode (PPN) has been difficult to control because the cyst protects the eggs which can remain viable for nearly a decade [225]. The life cycle of soybean cyst nematode has three stages i.e., egg, juvenile, and adult. Among four juvenile stages (J1 J2 J3 J4) J2 is the infecting stage that penetrates the roots of soybean and other suitable hosts via the epidermal cell then moves intercellularly to reached the vasculature and forms a syncytium which is a feeding structure for the juvenile [226, 227]. SCN can complete up to 4 life cycles in a single soybean growing season in South Dakota, depending upon the maturity group of soybeans planted and weather conditions [219].

Some SCN management strategies that are followed include, use of nematicide seed treatments, planting SCN resistant varieties [228], rotating the crops with non-host crop like corn [229, 230]. However, use of nematicides for long term is not environmentally sustainable. In some SCN resistant variety, SCN population are adapted to reproduce on them. However, the extent of reproducibility is not similar. Soybean lines PI 88788, PI

209332, or PI 548316 resulted SCN populations reproduced well but not on the soybean lines PI 548402 (Peking), PI 90763, and PI 89772 [231-233]. A huge challenge in managing SCN is that it is nearly impossible to eradicate from soil once it becomes established in a field.

Besides above-mentioned strategies, use of Arbuscular mycorrhizal fungi (AMF) is also one of the approaches for management of SCN in the field for environmental sustainability. Both root invaders, AMF and SCN utilizes host nutrients to make a specialized structure. AMF are characterized by the formation of highly branched hyphal structures for nutrient exchange between the symbionts, called arbuscules whereas SCN forms a nutrient feeding site of multinucleated giant cell called syncytium. AMF represents a well-known beneficial biological resource in relation to plant growth and production in agroecosystems, where improved host plant nutrition and health are among the most important ecosystem services provided [234]. AM fungi can improve access for plants to nutrients, and as such, it then follows those plants engaged in the AM symbiosis can be better equipped to defend themselves from biotic attackers, particularly in nutrient deficient environments. AMF have been shown to reduce development of root diseases caused by pathogens including oomycetes, fungi and nematodes [235, 236]. Interactions between AMF and plant parasitic nematodes depend on several factors including host plant, AMF strain and nematode species, but in general AMF may induce host tolerance and/or increase host resistance [237]. Main proposed modes of biocontrol traits of AMF against root pathogens include competition for space and nutrients, antagonism from mycorrhiza associated bacteria and plant defense induction [235]. In terms of plant defense, it is well

known that AMF root colonization can lead to increased levels of antioxidants and phenolic compounds [238] though often transient and weak compared to that of pathogens [239].

Multiple research projects have been done in-vitro or controlled environment to see the mechanism how arbuscular mycorrhizal fungi able to reduce the effect of PPN to the host plant. In potato, commercial AMF inocula increased biomass and the egg hatch of the *Globodera pallida* but not *G. rostochiensis*, a potato cyst nematode [240]. Similarly, study done by Pawlowski and Hartman, different types of AMF (*Claroideoglomus claroideum, Diversispora eburnean, Dentiscutata heterogama, Funneliformis mossae and Rhizophagus intradices*) tested reduced the number of cysts on soybean roots by 59 to 81% and also egg hatching by AMF exudates up to 62% done under in-vitro conditions [241]. Despite a meta-analysis which confirmed that AM fungi can suppress fungal and nematode pathogens [242], there is a wide range of open questions. We have limited information on the effectiveness of AMF on different nematodes. The research needed to evaluate in field condition to make AMF as an effective biological control agent to manage the negative impact of the SCN on soybean production. In our experiments, we not only focused on the greenhouse experiment but also evaluated the impact of commercial AMF inoculum on different location in two growing seasons in fields that are naturally infested with SCN. The population of SCN reproduction and soybean yield at the end of harvest were the most important parameters that were addressed in this study. In addition, we also determined the effect of AMF on SCN resistance soybean varieties as SCN is rapidly evolving enhanced and complex virulence against resistant crops [243, 244].

4.3 Methods and Materials

4.3.1 Greenhouse study

4.3.1.1 Soybean varieties, fungal and SCN materials

We performed two independent greenhouse experiments for SCN susceptible soybean variety i.e., Williams-82 (Experiment I) and SCN resistant soybean variety Jack (PI88788) (Experiment II). In both experiments, seeds of soybean were surface sterilized with 2% bleach for 2 min and then 70% ethanol for 90 seconds and washed three times with sterilized deionized water. These seeds were allowed to germinate on petri-plate with moist filter paper in the dark at 25 °C for 48 h. Two germinating seeds were transplanted to each pot (diameter * height = 3.8 cm * 121 cm) (Stuewe and Sons Inc., Tangent OR) containing 120 cm^3 of the potting medium. Soil used in this study was collected from Horticulture building of South Dakota State University (SDSU). The growth medium consisted of sand and soil mix at 2:1 ratio. Organic soil was sieved through 2 mm sieve and sterilized twice by autoclaving at 121 °C for 1 h over a period of 3 days, and then let it cool at room temperature for 48 hours. The soil substrate contains available Olsen phosphate (6.05 ppm), nitrate (19.7 ppm), ammonium (3.93 ppm), pH (8.08) in both experiment (AgLab Express, Sioux Falls, South Dakota, USA). Each pot was thinned to one soybean plant after 1 week of transplanting. After thinning, 0.5 g of commercial AM soil additive fungus inoculum (MycoApply- Mycorrhizal Applications, Grants Pass, Oregon, USA) was added and mixed well ~five cm below the top surface and covered with growth medium. The products contain the four different AM fungal species *Rhizophagus intraradices, Glomus mosseae, G. aggregatum, and G. etunicatum* each with 525 propagules/g. For the non-inoculated control plants (C), a twice autoclaved Mycoapply was added. For SCN inoculum, *Heterodera glycines* HG type 0 were used for the study. 2000 SCN eggs and juveniles in water suspension at 1ml per pot were added in the soybean

plants of SCN control (no AM) and AM+SCN treatments after 3 weeks of seedling emergence. SCN eggs and juvenile inoculum was obtained from plants initially grown in SCN infested soil at the SDSU greenhouse.

4.3.1.2 Experimental design and growth conditions

Both experiment I (SCN susceptible variety) and experiment II (SCN resistant variety), we conducted a greenhouse with four different treatments. This included soil inoculated with AM fungus (AM), soil without AM fungus (C), AM with soybean cyst nematode infestation (AM+SCN), and control with SCN infestation (SCN). Each treatment had 10 biological replicates. However, after plant analysis, the pots where we did not find AM colonization in AM treated plants and no SCN eggs were found in SCN infested treatment were not considered for data analysis. All the pots were kept in a 7.6-litre bucket filled with sand and was placed in a water bath in greenhouse. The temperature of water bath was maintained at 27-28^{0}C at a day length of 16 hours. we did not provide any supplemental nutrients to enhance activity of AM fungus for nutrient uptake by the soybean plants.

4.3.1.3 Plant harvest and measurement

Harvesting was done after 35 days after plantation (DAP) of SCN inoculation in the soil. The containers were taken out of the bucket, soaked in water for 30 minutes. All the plants were uprooted gently, and cyst were collected in 210 um pore sized sieve nested under 710 um pore sized sieve and sprayed with strong stream of water to dislodge the cyst from the root. Aliquots of root sections were preserved in the 50% alcohol to determine the AM colonization. To do this, the preserved roots were rinsed with tap water to remove alcohol, water bathed with 10% KOH solution at 90^{o}C for 2 hours, rinsed several times

with tap water, and stained with 5% ink at 90°C for 30 minutes [153]. We analyzed a minimum of 100 root segments to determine the percentage of AM root colonization by using the gridline intersection method [154]. Shoots and roots of soybean were separated, and fresh weight determined before drying in oven at 70^0C for 72 hours, after which dry matter weight was determined.

4.3.2 Field study

4.3.2.1 Field location, plant, and fungal materials

Field experiments were conducted in two location, South Dakota State University Research Farm in Beresford, and at growers' field near Brookings. Both locations have natural SCN infestation. For both locations, two commercial soybean varieties were used. In Brookings GH0674X (SCN susceptible soybean variety) and S06-Q9 (SCN tolerant soybean variety) while in Beresford, AG2431(SCN susceptible soybean variety) AG1935 (SCN tolerant soybean variety) were planted in 2018. We again repeated the field trial at Beresford in 2019, where S14-J7 (SCN susceptible soybean variety), AG1935 (SCN tolerant soybean variety) were used.

We collected initial soil samples for SCN eggs from each plot in early summer (just after planting) from 20-30 randomly selected spots on each plot using a soil probe. After 4 weeks of seed planting, we added commercially available mycorrhizal inoculum, Mycoapply that consists of *Rhizophagus irregularis, Glomus mossae*, *G. etunicatum* and *G. aggregatum*. The MycoApply® was mixed with water and sprayed manually at a rate of 1.9 g/m^2 (525 propagules/m^2) close to the to the roots by making two furrows in the middle two rows of the plot. Each treatment had 4 to 8 biological replicates.

4.3.2.2 AM root colonization assay

A representative sub sample of roots were washed in running tap water, cleaned by soaking in 10% KOH for 2 hours in water bath at 95^0C, acidified with 1 N HCl for 5 min, and then stained with ink-vinegar as described previously [153]. Two cm long root fragments were observed under stereomicroscope for AM colonization. Staining of soybean roots with ink -vinegar and blue color showed oval, treelike and hyphae near/within roots which revealed extensive root colonization by AMF. Estimation of percent colonization was done using the grid line-intersect method. We counted not less than 100 root segments [154].

4.3.2.3 Soil sampling and SCN eggs density

For each plot, 20-30 soil cores were randomly collected and then put in a plastic bag and labeled with field number/ plot number and collection date, then kept in the cooler with ice before transportation and stored in the cold room at 4^oC until SCN extraction was done. Before SCN extraction, each soil sample was manually mixed well and 100 cm^3 of soil was subsampled. The soil was then soaked in water for 24 hours and cysts were extracted by using mechanical elutriation [245]. Extraction of eggs and juveniles from cysts followed by previous method [246]. The eggs were then suspended in 50-mL water and the number of nematode eggs in 1 ml subsample loaded on a nematode counting slide were counted using a dissecting microscope. The total number of eggs per 100 cc soil was calculated by using formula: total number of eggs = number of eggs counted in sample volume in 1ml * total volume of egg suspension. SCN sampling was performed shortly after planting and shortly after harvesting. The soybean plots were combined at the end of the season and yield data obtained.

4.3.3 Data analysis

All the data were analyzed by using R studio [197]. The experiment was based on 4 to ten biological replicates. We used one-way analysis of variance ($P \leq 0.05$) with plant growth response, SCN egg number, mycorrhizal colonization, and seed yield as response variable. After significant p value, we used least significance difference (LSD) test or the student's t test for group comparisons. Leven's test for homogeneity of variance and the Shapiro–Wilk normality test were employed before ANOVA was done. If the data set failed these tests, we log-transformed the data prior to the analysis.

4.4 Results

4.4.1 Greenhouse study

4.4.1.1 Effect of mycorrhizal inoculum on colonization rate, plant biomass, and SCN infestation in the soil

Overall, the SCN susceptible soybean variety *Williams*-82 and SCN resistant variety showed different growth response on different colonization group. In *W-82*, the plant that had Mycoaaply treatment had higher root and shoot dry weight than non-inoculated control plants (Figure 4.1A). Plants infected with SCN also had lower shoot dry weight than Mycoapply treated plants and root dry weight followed the same trend though not statistically different at alpha=0.05. SCN resistant soybean plants did not have much variation in dry biomass in both shoot and root except the root of non-inoculated (C) plants and SCN infected plants had higher root dry weight than Mycoapply inoculated plants (AM and AM+SCN) (Figure 4.1B).. We found similar pattern in fresh biomass of shoot and root. But the level of significance was different as the fresh biomass of the plant contains water content as well. This may have caused some differences observed (Figure 4.2A, B). We

found that soybean variety had effect on the fresh and dry shoot and root weight but had no effect by different colonization group by using two-way ANOVA on variety and treatment on each of response variable (Supplementary Table 4.1).

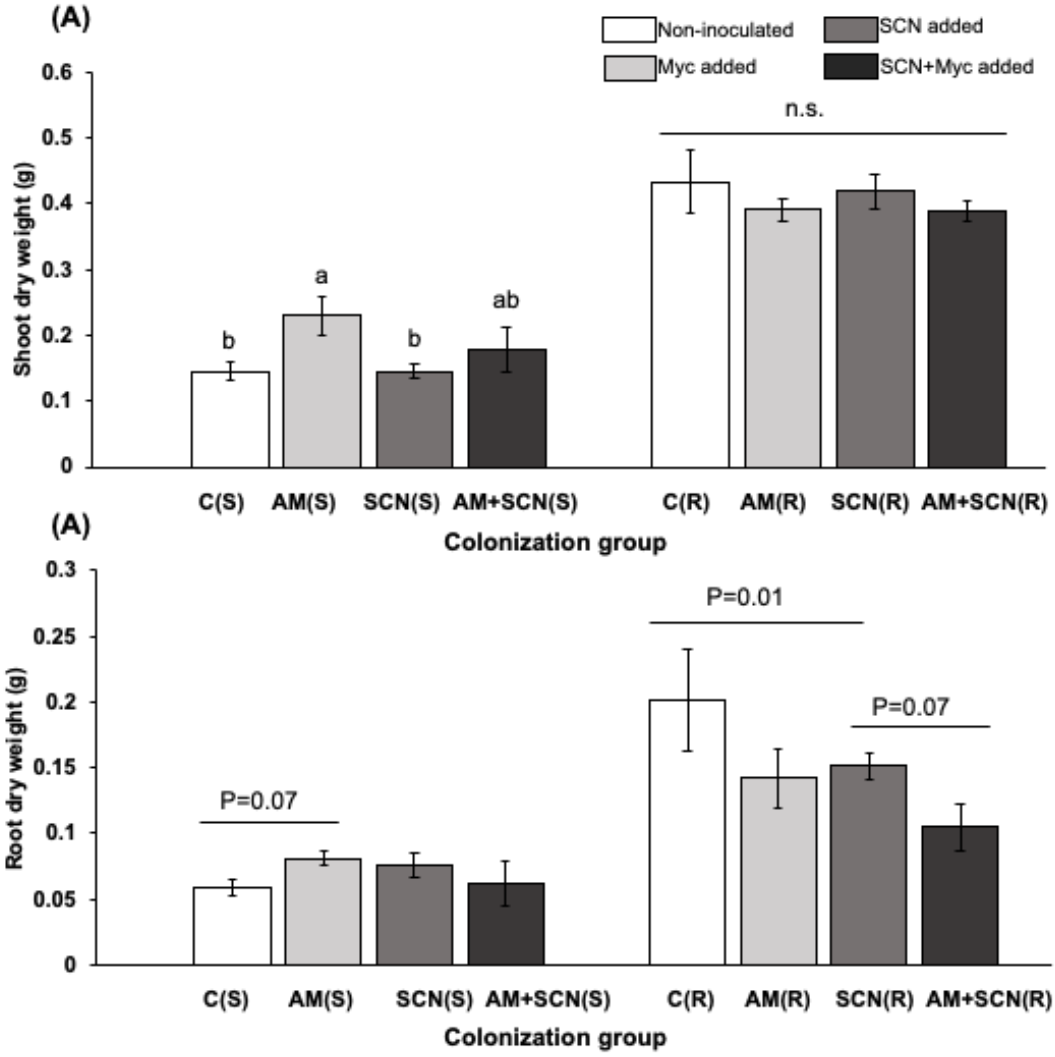

Figure 4.1: Shoot (A) and root(B) dry weight of SCN susceptible soybean (Williams-82) (left) and resistant (PI 88788) (right) variety depending on the different colonization group (white bars represent non-inoculated control plants, light grey bars represent Mycoapply® added plants/ AM inoculated, medium grey bars represent SCN infested plants and black bar represent SCN infected plants with Mycoaaply® applied plants. Root colonization abbreviations: C: controls (Non – inoculated plants); AM: addition of MycoApply® that's consists equal combination of 4 different AM, *Rhizophagus irregularis, Glomus mossae*, *G. etunicatum* and *G. aggregatum;* SCN: *Heterodera glycines* (HG type 0) infected plants; AM+SCN: MycoApply added on SCN infected plants. 'S' and 'R' in bracket of each colonization group represent SCN susceptible and resistant soybean variety respecitively. Different letters on the bars (means ± standard error of the mean) indicate statistically

significant differences within each graph according to the least significant difference test ($P \leq 0.05$)

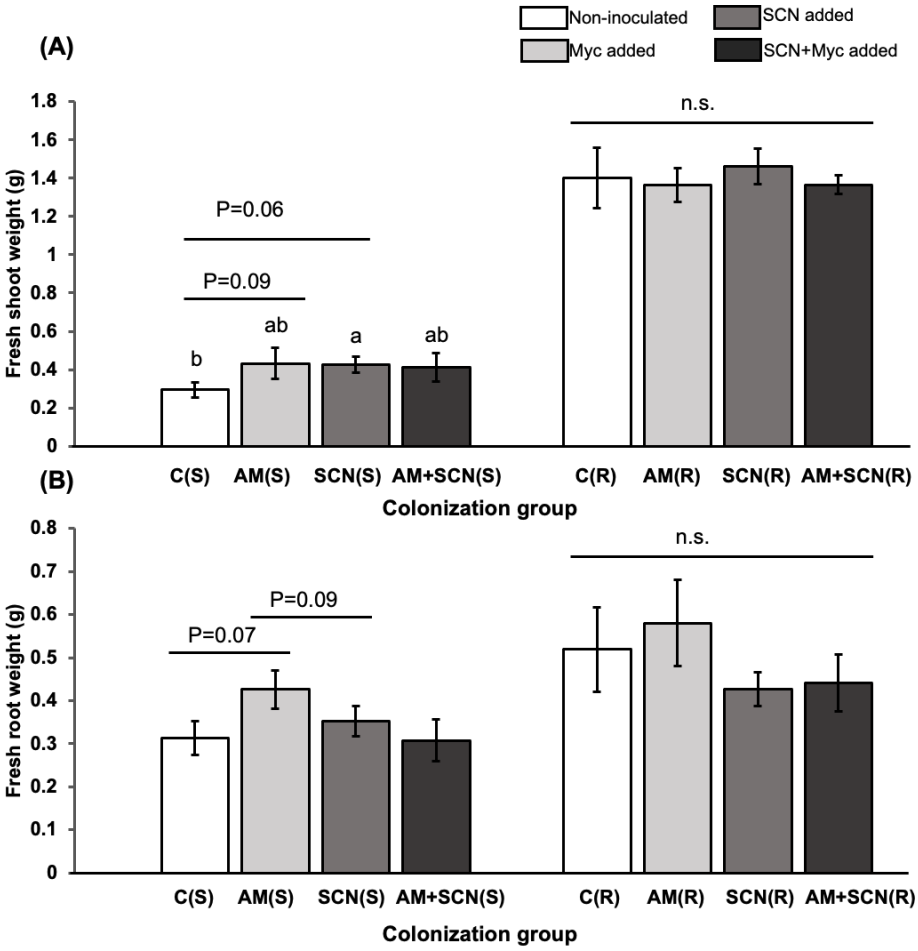

Figure 4.2: Shoot (A) and root(B) fresh weight of SCN susceptible soybean (Williams-82)(left) and resistant (PI 88788) (right) variety depending on the different colonization group (white bars represent non-inoculated control plants, light grey bars represent Mycoapply® added plants/ AM inoculated, medium grey bars represent SCN infested plants and black bar represent SCN infected plants with Mycoaaply® applied plants. Root colonization abbreviations: C: controls (Non – inoculated plants); AM: addition of MycoApply® that's consists equal combination of 4 different AM, *Rhizophagus irregularis, Glomus mossae*, *G. etunicatum* and *G. aggregatum;* SCN: *Heterodera glycines* (HG type 0) infected plants; AM+SCN: MycoApply added on SCN infected plants. 'S' and 'R' in bracket of each colonization group represent SCN susceptible and resistant soybean variety respecitively. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$)

4.4.1.2 Colonization of soybean roots by AMF and impact of fungal inoculum on SCN egg population in soil

Mycorrhizal colonization levels on soybean root (both SCN susceptible and resistant variety) ranged from 20% to 50% at 49 days after planting. When Mycoapply were added in SCN infested soil, we found a trend of increase in AM colonization rate (%) in those plants compared to only Mycoaaply added control (AM) plants (Figure 4. 3A). We did not find the differences in system with AM+ SCN and only AM inoculated plants for both W-82 and Jack (PI88788) soybean varieties respectively even after using student t test for two samples. Then we looked for the effect of soybean variety (SCN susceptible and resistant) and different colonization group (AM and AM+SCN) on the AM colonization on the plants and found that both soybean variety (p-value is 0.0045) and colonization group (p-value is 0.070) were statistically significant. These results led us to believe that the soybean variety significantly impacted the mean AM colonization rate. We also believe that when AM fungus interact with SCN, the colonization rate increased than when AM inoculated alone at p-value of 0.07.

We tested the impact of commercial Mycoapply addition in the SCN infested soil to see how arbuscular mycorrhizal fungi behave in colonization rate, soybean yield and SCN egg reproduction. The mycorrhizal treatment on SCN infected plants led to a lower but non-significant SCN eggs per plant than the check plants (SCN alone) (Figure 4.3B) after 35 days of SCN infestation in the soil. We again, analyzed the main and interaction effect on SCN egg density by two different varieties of soybean and SCN inoculation with and without Mycoapply and found no statistical differences on them (variety: p-value is 0.57; colonization group: p-value is 0.190 and interaction effect: p-value is 0.971). In addition

to this, we did not find the differences in SCN egg number in between the *w-82* and PI88788 soybean varieties indicating that SCN resistant PI88788 varieties has lost its tolerance against SCN infection.

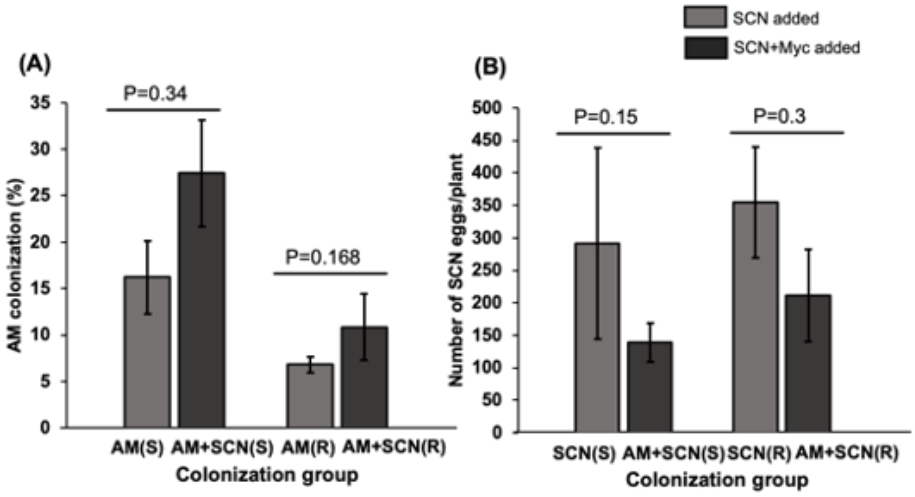

Figure 4.3: AM colonization in roots (A) and total numbers of SCN eggs (B) of SCN susceptible soybean (Williams-82) (left) and resistant (PI 88788) (right) variety depending on the different colonization group (white bars represent non-inoculated control plants, light grey bars represent Mycoapply® added plants/ AM inoculated, medium grey bars represent SCN infested plants and black bar represent SCN infected plants with Mycoaaply® applied plants. Root colonization abbreviations: C: controls (Non – inoculated plants); AM: addition of MycoApply® that's consists equal combination of 4 different AM, *Rhizophagus irregularis, Glomus mossae*, *G. etunicatum* and *G. aggregatum;* SCN: *Heterodera glycines* (HG type 0) infected plants; AM+SCN: MycoApply added on SCN infected plants. 'S' and 'R' in bracket of each colonization group represent SCN susceptible and resistant soybean variety respecitively. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$)

4.4.2 Field study

4.4.2.1 Effect mycorrhizal inoculum on colonization rate, SCN infestation in the soil, and seed yield

We examined the effects of commercial mycorrhizal addition (Mycoapply) for soybean mycorrhizal colonization rate, seed yield and SCN egg density under natural SCN field infestations. Mycorrhizal colonization between control and AM fungal inocula treated

soybean roots was not statistically different in all experiments (Figure 4.4A, Figure 4.5A) independent of different soybean variety used for both years. For both 2018 and 2019 and two locations Brookings and Beresford, the colonization ranged from 37% to 65%. The analysis was based on each location and year of the field trial by using LSD. Pairwise comparison to Mycoapply added and control plants were done by student t test. For further investigation on AM colonization rate, we also did three-way ANOVA (Table S4.2) where we calculated the main effect and interaction effect of the colonizing group, soybean variety (SCN susceptible or resistant) and different location. We found no differences on interaction effect of those three variables (p-value 0.822).

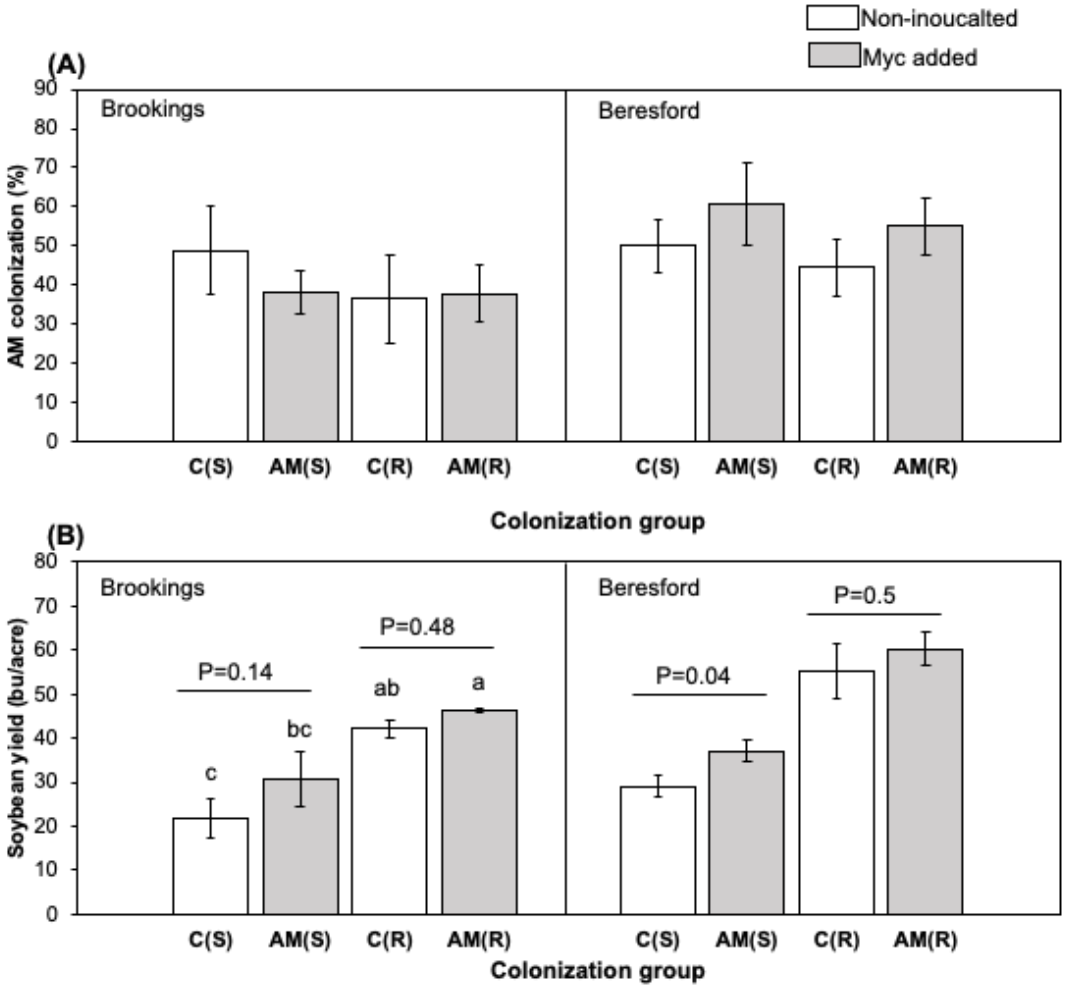

Figure 4.4: Mycorrhizal colonization in Brookings and Beresford (a) and Initial and final SCN eggs counts in Brookings and Beresford (b) in year of 2018 of SCN susceptible soybean (Williams-82) (left) and resistant (PI 88788) (right) depending on the addition of mycoapply (white bars represents plots where no Mycoapply were added, light grey bars represents Mycoapply added plots in different location and year; Black bar in right graph represent initial SCN count of whole plots. Root colonization abbreviations: C: controls (No Mycoapply added plots); AM: addition of MycoApply® that's consists equal combination of 4 different AM, *Rhizophagus irregularis, Glomus mossae*, *G. etunicatum* and *G. aggregatum.* These fields soil was already infected with Soybea Cyst nematodes. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$).

We determined the total SCN eggs count on each plot collecting from randomly selected 20-30 spots from all our plots. We collected the soil sample after sowing or before application of commercial fungal inocula and termed them as initial SCN egg counts. Before harvest or when soybean had fully grown seed, we collected final soil samples and termed as final SCN egg counts. The initial SCN egg counts were the average of all the plots in 100 cm^3 of soil sample. That is why we have only one value for initial SCN egg count for our field trial. After counting the SCN eggs we found that Brookings had higher number of SCN eggs (1046/100 cm^3 of soil) over the Beresford (247/100 cm^3 of soil in 2018) and in 2019 it was 182/100 cm^3 of soil in Beresford (Table 4.1). This also confirms that Brookings field were heavily infested with SCN compared to Beresford field. There were consistently increases in the final SCN egg counts for all field station independent of Mycoapply treatment. The increase in final SCN population were 100.6%, 163.9%, 286.9%, 88.7% at Brookings, 350.1%, 365.9%, 310.4%, 442.3% at Beresford in 2018 and 378.9%, 198.7%, 547.5%, 233.5% at Beresford in 2019 on C (S), AM(S), C (R), and AM (R) respectively based on mean value for each treatment. This indicate that mycorrhizal application did not stopped multiplication of SCN in the soil. However, increase % from initial to final SCN eggs were relatively low in Mycoapply addition in Beresford on year

of 2019 for both soybean varieties. But same trend was not found in the year of 2018 except in SCN resistant variety of Brookings field.

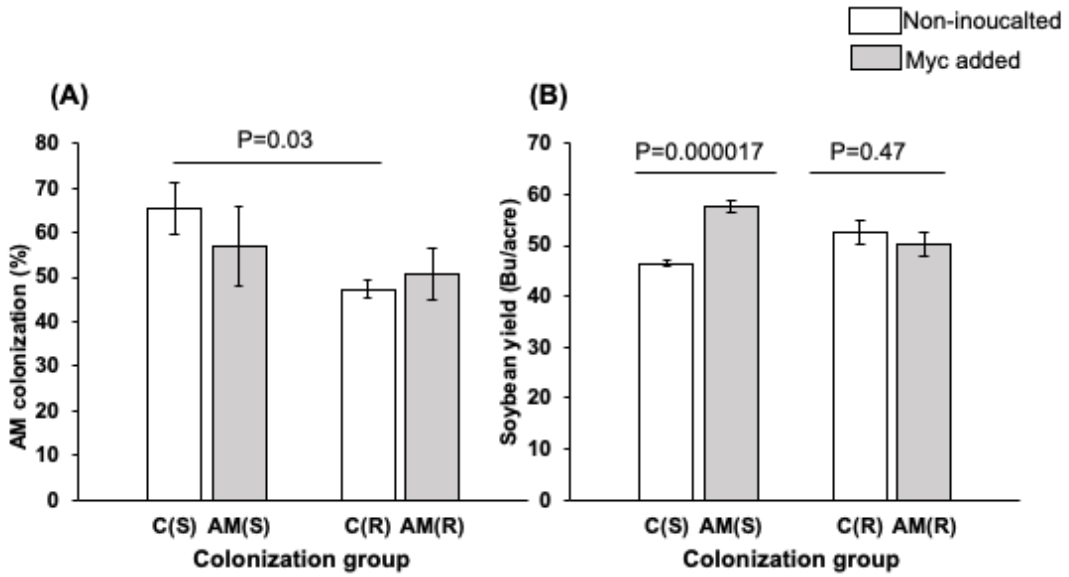

Figure 4.5: Mycorrhizal colonization in Brookings and Beresford (a) and Initial and final SCN eggs counts in Brookings and Beresford (b) in year of 2019 of SCN susceptible soybean (Williams-82) (left) and resistant (PI 88788) (right) depending on the addition of mycoapply (white bars represents plots where no Mycoapply were added, light grey bars represent Mycoapply added plots in different location and year; Black bar in right graph represent initial SCN count of whole plots. Root colonization abbreviations: C: controls (No Mycoapply added plots); AM: addition of MycoApply® that's consists equal combination of 4 different AM, *Rhizophagus irregularis, Glomus mossae*, *G. etunicatum* and *G. aggregatum.* These fields soil was already infected with Soybea Cyst nematodes. Different letters on the bars (means ± standard error of the mean) indicate statistically significant differences within each graph according to the least significant difference test ($P \leq 0.05$).

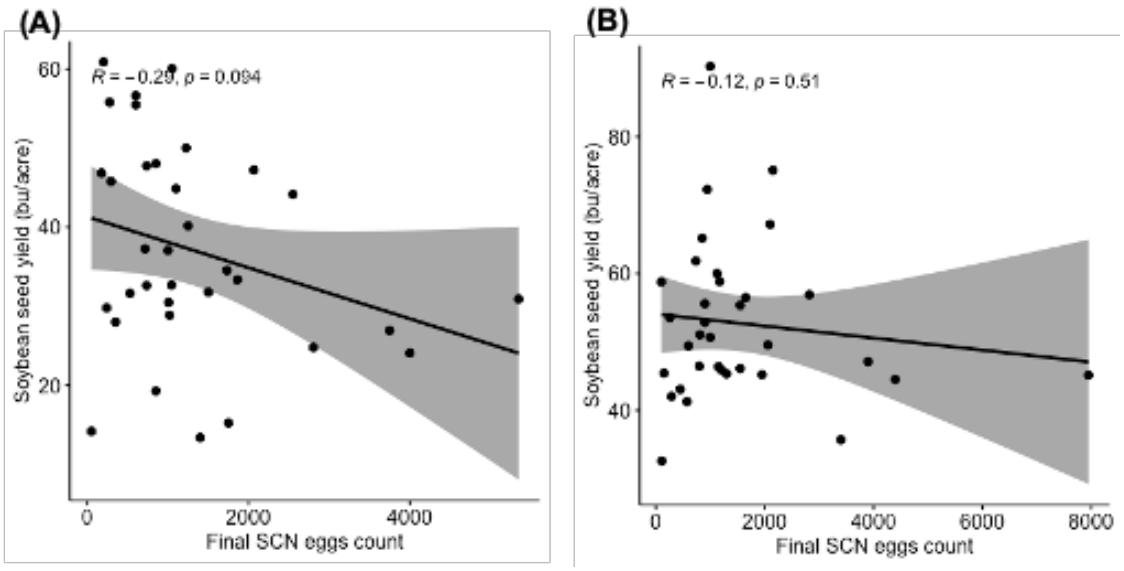

Figure 4.6: Co-relation between Final SCN egg count and soybean seed yield on different SCN susceptible varieties (A) and different SCN resistant soybean varieties (B) by using Pearson co-relation test.

	Brookings (2018)	Beresford (2018)	Beresford (2019)
Treatment	SCN eggs population	SCN eggs population	SCN eggs population
Initial SCN count	1046.875±308.945	247.21875±47.622	182.5±43.720
C (S) (Final)	2100±554.902	1112.75±284.564	874±339.839
AM (S) (Final)	2762.5±1138.04	1151.875±276.596	545.2±150.409
C(R) (Final)	4050±1546.90	1014.5±222.179	1181.6±334.43
AM(R) (Final)	1975±646.94	1340.625±328.125	608.6±152.643

Table 4. 1: The initial and final SCN eggs count in the first year (2018) and 2nd year of field experiment conducted in Brookings and Beresford field station

Mycorrhizal fungi had the greatest effect on overall soybean seed yield in all experiment that were conducted in two different years of 2018 and 2019 (Figure 4.4B and Figure 4. 5B). In 2018, addition of commercial fungal inoculant led to increase soybean seed yield by 40.03% and 27.91% bushels/acre in SCN susceptible variety (GH0674X,

AG2431) and 9.8% and 8.78% bushels/acre increase in SCN resistant soybean variety (S06-Q9, AG1935) at Brookings and Beresford respectively. Similar trend was observed in our 2nd year (2019) trial at Beresford resulting 24.26% increase in the susceptible variety (S14-J7). However, there was slight decrease (-4.67%) in the soybean seed yield on resistant variety (AG1935). Statistically, by using student t test, between the control plot and Mycoapply added plot in SCN susceptible soybean variety, we found Mycoapply treatment significantly increased the seed yield over the control plants independent of location and the year when we conducted the experiment. Except in Brookings, though seed yield increase was ~40% but not statistically different (p-value is 0.1) due to high variability in the data. In case of SCN resistant variety there was no statistical differences in soybean yield between control and Mycoapply treated plots. In addition to this, we processed all the data at same time and found significant effect on yield by variety ($p=3.50e^{-}09$), location (Brookings, Beresford (2018), Beresford (2019) (p=0.00000838) and Mycoapply application (p=0.011). But there was not any significant interaction effect of these three variables on soybean seed yield.

4.5 Discussion

Previous study showed that several other fungi, including *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., produce exudates that inhibit SCN juvenile motility [247]. Only few studies have evaluated the direct effect of AMF on SCN egg population in vivo, other studies done in-vitro to see the juvenile activity in soybean by mycorrhizal spores and egg hatching rate on potato [240], soybean [241]. In this report we can find furthermore impact of AMF on SCN infested soil in terms of growth response colonization rate, egg numbers and seed yield in natural conditions.

Throughout our experiments, addition of commercial AMF inocula in the greenhouse had a beneficial effect on plant growth specially shoot weight (Figure 4.2A). Plants infested with SCN, and AMF inoculum had slightly higher shoot dry weight than only SCN and non-inoculated control plants and SCN infestation in the soil resulted in decreased growth of *W-82* in greenhouse experiment. Growth increment by AMF inoculation was reported in other studies as well [163, 190, 191, 209]. Higher biomass in shoot were observed in W-82 soybean varieties but not in Jack (PI88788). A field experiment conducted in Illinois, Iowa, and Missouri from 1997 to 1999, biomass of soybean and yield both were negatively impacted by SCN infestation but had no any aboveground symptoms of infection on both resistant and susceptible cultivars over the entire growing season [248]. In our study, growth of *W-82* soybeans was suppressed when SCN parasitized the plants, regardless of mycorrhizal colonization status. We did not quantified all AM structures like arbuscules, vesicles, intraradical or extraradical hyphae within root but we reported total AM fungal structures inside and on the surface of roots after washing, cleaning and staining roots by ink-vinegar [153, 154]. But we found differences in the colonization rate on variety of two different soybean in greenhouse (Figure 4.3A ANOVA p value = 0.00448). This result is also supported by other reports as AM colonization rate also depend on different soybean variety [249, 250]. It is known that different species of AM fungi differ in the type of benefits they confer on the growth and development of plants [251]. The flowering and fruiting appeared increased in mycorrhizal treated plant compared to non-mycorrhizal plants when *G. mossae* were used [252]. In our experiments we have used same commercial AMF inoculum (Mycoapply®) in greenhouse and field and results of controlled and field conditions were mostly similar.

The suppression of yield in control plots emphasizes the high potential for economic loss when soybeans are grown in SCN infested fields. Application of commercial AM inoculum in our studies under field conditions had positive effects (either statistically significant or not) on soybean yield not only on SCN susceptible soybean varieties but also in first year of SCN resistant varieties (Figure 4.4B, Figure 4.5B). This is consistent with other studies for soybean yield [253, 254], corn yield [255, 256], and alfalfa [257, 258]. This is attributed to the potential ability of AMF to enhance uptake of essential nutrients and other benefits to the crops particularly phosphorus [39]. But these yield reductions were not accompanied by visually detectable symptoms related to SCN infestation. Since we used different commercial varieties in the field which may also resulted some of the differences in the results. We found decrease in the seed yield of SCN resistant varieties (AG1935) at Beresford in year of 2019. But the negative growth by AMF treatment were also found in meta-analysis done by Kaschuk et al (2010) where yield ranged from -4% to + 24%. However, there is no information whether or not the field were infested with SCN in their study [259]. As expected, we found SCN susceptible cultivars produced lower yields than resistant cultivars except the Mycoapply® added susceptible variety which resulted higher seed yield than resistant variety grown on same field (Figure 4.5B). The degree of AM benefits depends on host plant type, cultivar, and environmental condition.

Lack of differences in the final SCN egg population between the SCN susceptible and resistant soybean varieties also supports the previous report that stated 70% of SCN populations have adapted to PI 88788 at some level, reducing the effectiveness of using SCN-resistant cultivars as a crop management tool [244]. Similarly, we found no reduction in the number of eggs to the final population in both greenhouse (Figure 4.3B) and field

experiment (Table 4.1) but at the same time it has been known that AMF can colonize SCN cysts and sporulate within the cysts and infect SCN eggs [225]. AMF, including *C. claroideum, D. heterogama*, and *F. mosseae*, harbors endobacteria in cytoplasm that have not been characterized but might produce antimicrobial product which have biological functions [260]. These endobacteria possibly play role in protecting plants against soil borne pathogens like PPN. In our study we did not consider the egg hatching activity in the greenhouse and field experiment. Which also have important role in infection as only hatched egg have chances to consist infective J2 juveniles that can later create negative impact in soybean growth and production. AMF spores are also shown to harbor other types of bacteria including eight different species of actinomycetes, which were found within *F. mosseae* spores associated with guava and were shown to have antifungal properties and produced chitinolytic enzymes [261]. Since chitin is in the outer layer of SCN eggshells, it may be likely these bacteria would suppress SCN as well by degrading the outer layer of the egg. The lack of long-term suppression of SCN eggs in field by AM fungus might be the result of differences in the carrying capacities of mycorrhizal and nonmycorrhizal soybeans later in the experiment. Carrying capacity determines the maximum population density or saturation level of an organism that a system can support (21). The lack of consistent suppression of SCN by commercial AM fungi in the greenhouse and in field studies may have been caused by environmental conditions. It is also crucial to monitor J2 juveniles in roots of infected soybean to exactly track how the presence of AMF impacted in space competition with nematode. The suppression of nematode by AMF should occur consistently under field conditions specially in low P and N containing field. The level of P, NH_4 and NO_3 were enough in our field experiment also

indicate that plants were not in nutrient stressed condition where AMF plays significant role. However, we found negative co relation between the soybean seed yield and final SCN egg counts in first year of experiment in both Brookings (r=-0.29 and p-value=0.09) and Beresford (r=-0.12 and p-value=0.51). In other words, SCN infestation in the soil did not alter the performance of AM fungi in the field leading to increase in the soybean production. Furthermore, time course experiments that track the SCN egg population after application of AM fungi in each stage of growing period can be documented in natural soils, cultural practices such as...could be employed to promote the increase of these common soil-inhabiting fungi resulting in increased soybean yields and decreased levels of SCN parasitism and accompanying yield suppression.

4.6 Conclusion

It could be concluded from this study that the use of commercially available Mycoapply increased growth, seed yield of soybean, with varied response in the cultivars under the given soil conditions. Independent of mycorrhizal colonization rate and SCN egg population status, SCN susceptible soybean variety in general, performed better with mycorrhizal inoculation increased shoot growth in greenhouse and producing higher yield in field condition where soil has been infested with soybean cyst nematodes. Thus, Mycoapply has the potential to increase soybean yield even in SCN infested soil under favorable conditions.

4.7 Supplementary Materials

Factor	Shapiro-Wilk	Levene's test	ANOVA	DF
shoot dry weight	0.7453	0.7698	0.03257 *	3,23
Shoot fresh weight	0.1689	0.8935	0.201	3,23
Root fresh weight	0.3454	0.8671	0.2612	3,23
root dry weight	0.555	0.4549	0.2101	3,23
Shoot fresh weight	0.01072	0.2716	0.9322	3,23
root fresh weight	0.7075	0.3918	0.4935	3,23
Shoot dry weight	0.1329	0.1274	0.7456	3,23
Root dry weight	0.2923	0.3448	0.09148	3,23
Field (2018)				
Brookings				
AM colonization	0.5921	0.4451	0.766	3,12
Yield	0.3124	0.07342	0.004028 **	3,12
Beresford				
AM colonization	0.01146	0.5543	0.5373	3,28
Yield	0.1528	0.1814	1.646e-05 ***	3,28
Field (2019)				
AM colonization	0.6014	0.1845	0.2198	

Table S4. 1: ANOVA, Shapiro wilk, Leven’s test list on greenhouse and field experiments

Effect	DFn	DFd	F	p p<.05	ges
var	1	40	53.496	6.88e-09 *	5.72E-01
loc	1	40	9.926	3.00e-03 *	1.99E-01
Trt	1	40	4.435	4.20e-02 *	1.00E-01
var: loc	1	40	1.07	3.07E-01	2.60E-02
var: Trt	1	40	0.363	5.50E-01	9.00E-03
loc: Trt	1	40	0.0000574	9.94E-01	1.44E-06
var: loc: Trt	1	40	0.011	9.17E-01	2.75E-04

Table S4.2: Three-way ANOVA Table (Type II tests)

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