# Unravelling the importance of mycorrhiza for plant nitrogen nutrition and transfer into the networks

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Dedico

Aos meus avós, que são e sempre serão meus anjos da guarda.

Ofereço

# ABSTRACT

Most terrestrial plants establish symbiotic associations with fungi called mycorrhiza, like ectomycorrhizas (EM) and arbuscular mycorrhizas (AM), for accessing limiting plant nutrients. For improving N nutrition, some plant species may establish EM-AM dual-mycorrhizal associations, either within the same root system or at different plant's ontogenetic stages. Furthermore, EM and AM associations may interconnect plants via a common mycorrhiza network(CMN) for N exchange. However, available studies fail to provide univocal evidence on the advantage to a host plant of exploiting a dual association, compared to a single one for Nacquisition, as well as to demonstrate the potential effects of the CMN networks mediating resourcepartitioning between connected plants, since transfer can occur via several routes simultaneously. In addition, quantification of the amount of possible nutrients transferred has also shown to be challenging, leading to doubts regarding the importance of the CMN in inter-plant partitioning. With this in mind, I have developed two novel experiments to prove and distinguish the contribution of the CMN for N transfer between connected plants as well as to evaluate nutritional advantages of dual vs single mycorrhizal plants. The first experiment aimed to respond two main objectives: (A) to evaluate N nutrition benefits in plants associated with single EM or AM *versus* dual associations and (B) to evaluate the potential of a dually associated plant as N donor via a CMN with receiver plants bearing single EM or AM associations. For this purpose, I have designed a novel multi-chamber mesocosm where a central split-rooted donor, able to associate with both AM and EM simultaneously, shares an AM or EM network with one neighbour plant simultaneously. Since only dually mycorrhized donor plants had access to both fungi type, it is possible to access different N nutrition of single colonized neighbours compared to central dual mycorrhizal plant. In addition, by applying $^{15}N$ labeled solution to central dual mycorrhizal plant, I could track the preferential N allocation via AM *vs* EM network. At my knowledge, such evaluation was never made before. I hypothesized that (1) host plants establishing dual mycorrhiza associations will exhibit an enhanced N nutrition, compared to those depending on single associations. I further hypothesized (2) that dual mycorrhizal plants will preferentially share more N to plants bearing an EM association, due to its larger mycelium proliferation. Lastly, I hypothesized that (3) such mycelium proliferation might act as a sink for C, requiring higher C allocation from host plant. The data obtained demonstrate a nutritional advantage regarding N uptake for host plants holding dual mycorrhizal association, compared to single colonized plants.

However, no transfer of N occurred between donor and receiver plants. Therefore, I concluded that CMN functioning for N transfer might occur only under specific situations, such as for particular plant–fungus combination, the characteristics of connected plants or abiotic conditions.
For the second experiment, I aimed to quantify the direct transfer of N via the mycelial network in comparison to indirect pathways. I hypothesized that: (1) N transfer between connected plants occurs genuinely through hyphal connection rather than indirect pathways; (2) the proportion of N allocated from donor to receiver plants through mycorrhiza hyphae connections is significant and may improve neighboring plant nutrition and (3) by shading donor plant, N transferred to receiver plants is increased, once it might be able to produce more C to be exchanged by transported N. Thedata demonstrated a higher $^{15}N$ transfer to *ram1-1* receiver plants. The highest $^{15}N$ found in the *ram1-1* plant summed with the highest root biomass observed in this plant which increasing itsarea of nutrients absorption, highlighting the importance of indirect pathways for resources allocations in our system. Also in opposite to what was previously hypothesized, shading treatment did not increased $^{15}N$ transfer. With this, it is possible to conclude that CMN are important, but most likely by other means than discussed in the literature.

*Keywords*: Mycorrhiza network, dual-mycorrhizal plants, nitrogen, plant nutrition, arbuscular mycorrhiza, ectomycorrhizal, transfer, direct pathway, indirect pathway, isotopes.

# ZUSAMMENFASSUNG

Die meisten Landpflanzen gehen symbiotische Verbindungen mit Pilzen ein, die Mykorrhiza genannt werden, wie Ektomykorrhiza (EM) und arbuskuläre Mykorrhiza (AM), um Zugang zu limitierenden Pflanzennährstoffen zu erhalten. Um die N-Ernährung zu verbessern, können einige Pflanzenarten EM-AM-Doppelmykorrhiza-Assoziationen bilden, entweder innerhalb desselben Wurzelsystems oder in verschiedenen ontogenetischen Stadien der Pflanze. Darüber hinaus können EM- und AM-Assoziationen die Pflanzen über ein gemeinsames Mykorrhiza-Netzwerk (CMN) für den N-Austausch miteinander verbinden. Die vorliegenden Studien liefern jedoch keineeindeutigen Beweise für den Vorteil, den eine Wirtspflanze durch die Nutzung einer dualenAssoziation im Vergleich zu einer einzelnen für den N-Erwerb hat, sowie für die potenziellen Auswirkungen der CMN-Netzwerke, die die Ressourcenaufteilung zwischen verbundenenPflanzen vermitteln, da der Transfer über mehrere Wege gleichzeitig erfolgen kann. Darüber hinaus hat sich die Quantifizierung der Menge an möglichen Nährstofftransfers als schwierig erwiesen, was zu Zweifeln an der Bedeutung der CMN bei der Partitionierung zwischen Pflanzen führt. Vor diesem Hintergrund habe ich zwei neuartige Experimente entwickelt, um den Beitrag der CMN fürden N-Transfer zwischen verbundenen Pflanzen nachzuweisen und zu unterscheiden sowie um die ernährungsphysiologischen Vorteile von Pflanzen mit dualer versus einfacher Mykorrhiza zu bewerten. Das erste Experiment verfolgte zwei Hauptziele: (A) die Bewertung der N-Ernährungsvorteile von Pflanzen, die mit einzelnen EM- oder AM-Assoziationen assoziiert sind, im Vergleich zu dualen Assoziationen und (B) die Bewertung des Potenzials einer doppelt assoziierten Pflanze als N-Spender über eine CMN mit Empfängerpflanzen, die einzelne EM- oder AM-Assoziationen tragen. Zu diesem Zweck habe ich einen neuartigen Mehrkammer- Mesokosmos entworfen, in dem ein zentraler Spender mit geteilter Wurzel, der sowohl mit AM alsauch EM gleichzeitig assoziieren kann, ein AM- oder EM-Netzwerk mit einer Nachbarpflanze teilt. Da nur die duale Mykorrhizapflanze des Spenders Zugang zu beiden Pilzarten hat, ist es möglich, die N-Ernährung der einzelnen kolonisierten Nachbarpflanzen im Vergleich zur zentralen dualen Mykorrhizapflanze zu untersuchen. Darüber hinaus konnte ich durch die Anwendung von $^{15}N$- markierter Lösung auf die zentrale duale Mykorrhizapflanze die bevorzugte N-Allokation über das AM vs. EM-Netzwerk verfolgen. Meines Wissens nach wurde eine solche Auswertung noch nie gemacht. Ich stelle die Hypothese auf, dass (1) Wirtspflanzen, die duale Mykorrhiza-Assoziationen bilden, eine verbesserte N-Ernährung aufweisen, verglichen mit solchen, die von

Einzelassoziationen abhängig sind. Weiterhin stelle ich die Hypothese auf, dass (2) duale Mykorrhizapflanzen aufgrund ihrer größeren Myzelvermehrung mehr N als Pflanzen mit einer EM-Assoziation teilen. Die gewonnenen Daten zeigen einen Nährstoffvorteil hinsichtlich der N-Aufnahme für Wirtspflanzen mit dualer Mykorrhiza-Assoziation im Vergleich zu einfach kolonisierten Pflanzen. Es fand jedoch kein N-Transfer zwischen Spender- und Empfängerpflanzen statt. Daraus schlussfolgerte ich, dass die CMN-Funktion für den N-Transfer nur unter bestimmten Situationen auftreten könnte, wie z. B. bei bestimmten Pflanzen-Pilz-Kombinationen, den Eigenschaften der verbundenen Pflanzen oder abiotischen Bedingungen.

Im zweiten Experiment wollte ich den direkten N-Transfer über das Myzelnetzwerk im Vergleich zu indirekten Wegen quantifizieren. Ich stelle die Hypothese auf, dass: (1) der N-Transfer zwischen verbundenen Pflanzen wirklich durch Hyphenverbindungen erfolgt, wobei der Pilz als Transportschlauch fungiert, und nicht über indirekte Wege; (2) der Anteil des N, der durch Mykorrhiza-Hyphenverbindungen von Spender- zu Empfängerpflanzen übertragen wird, signifikant ist und die Ernährung der Nachbarpflanzen verbessern kann und (3) durch Beschattung der Spenderpflanze der N-Transfer zu den Empfängerpflanzen erhöht wird, sobald diese in der Lage sein könnte, mehr C zu produzieren, das durch den transportierten N ausgetauscht werden kann. Die Daten zeigten einen höheren $^{15}$N-Transfer zu *ram1-1* Empfängerpflanzen. Der höchste $^{15}$N-Wert, der in der *ram1-1*-Pflanze gefunden wurde, summierte sich mit der höchsten Wurzelbiomasse, die in dieser Pflanze beobachtet wurde, was die Fläche der Nährstoffaufnahme erhöhte, was die Bedeutung der indirekten Wege für die Ressourcenzuteilung in unserem System hervorhebt. Auch im Gegensatz zu dem, was zuvor angenommen wurde, erhöhte die Schattierungsbehandlung nicht den $^{15}$N-Transfer. Daraus kann man schließen, dass CMN wichtig sind, aber höchstwahrscheinlich auf andere Weise als in der Literatur diskutiert.

*Schlüsselwörter*: Mykorrhiza-Netzwerk, Dual-Mykorrhiza-Pflanzen, Stickstoff, Pflanzenernährung, arbuskuläre Mykorrhiza, Ektomykorrhiza, Transfer, direkter Weg, indirekter Weg, Isotope.

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## TABLE OF CONTENT

# ABBREVIATION LIST

| Abbreviation | Explanation |
|---|---|
| AM | Arbuscular mycorrhiza |
| EM | Ectomycorrhiza |
| CMN | Common mycorrhiza network |
| C | Carbon |
| N | Nitrogen |
| Ca | Calcium |
| Fe | Iron |
| P | Phosphorus |
| K | Potassium |
| Mg | Magnesium |
| $NH_4^+$ | Ammonium |
| $NO_3^-$ | Nitrate |
| GC | Gas chromatography |
| ICP-OES | Inductively coupled plasma-optical emission spectrometry |
| IRMS | Isotope ratio mass spectrometry |
| LA | Long-Ashton solution |
| LN | Low nutrient solution |
| Ram | Reduced arbuscular mycorrhiza |

The following publications contributed to this thesis:

1. Figueiredo, A. F., Boy, J., Guggenberger, G. (2021). Common Mycorrhiza Network: Theories and mechanisms behind underground interactions.

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2. Figueiredo, A. F., de la Fuente, A. A., Sauheitl, L., Guggenberger, G., J., Boy (2021). Higher N uptake in dual-mycorrhizal plants (AM and EM) in comparison to single-type mycorrhizal plants (AM or EM) and possible nutrient sharing via common mycorrhizal networks.

   Submitted to *Plant and Soil.*

3. Figueiredo, A. F., de la Fuente, A. A., Boy, J., Sauheitl, L., Guggenberger, G. (2021). Pathways of nitrogen transfer between plants connected through a mycorrhizal network.

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# 1. General Introduction

## 1.1. Nitrogen in Plant - Soil systems

Nitrogen (N) is one of the most important nutrients for plant growth and biomass production, being required in significant quantities. It represents 1-5% of plant dry weigh and plays a key role in metabolic processes since it is a major component of amino acids, which are required to synthesize protein and other related compounds (Poulton et al., 2012). Nitrogen is also the major component of chlorophyll, an important pigment used by the plant to convert sunlight energy, carbon dioxide and water into sugars during photosynthesis (Epstein & Bloom, 2005; Leghari et al., 2016).

Nitrogen in the soil may be found in a variety of forms, which differs in its availability for plant roots uptake. Plants are known to take N mainly up in inorganic forms, such as ammonium ($NH_4^+$) and nitrate ($NO_3^-$) (Carlisle et al., 2012; Song et al., 2015). Among them, $NH_4^+$ is the most efficient for plant uptake once it can be immediately incorporated into amino acids. However, it is not always available for uptake due to its strong adsorption to soil exchange sites and poor mobility in soil solution. Nitrate, although readily available due to its high solubility in soil water, must be reduced before incorporation, which requires higher use of energy from the plant (Persson et al., 2006; Scott & Rothstein, 2011). Recent studies have demonstrated the ability of plants to take up N in organic forms as well (Näsholm et al., 2009; Andersen, Mayor & Turner, 2017). Low molecular weight organic N compounds, primarily free amino acids, had been shown to be assimilated at rates that often competes with those of $NH_4^+$and $NO_3^-$, and may represent a major N source for plant due to their unaltered incorporation into plant protein (Näsholm et al., 2009; Liu et al., 2017). These findings are especially important for temperate and boreal forests where N mineralization rates are low and therefore considered as low-fertility habitats. In these ecosystems, there is a strong evidence that amino acid pools are comparably more abundant and therefore must be considered as a potentially important N pool for plants. This has largely been overlooked in previous studies relating soil N cycling with plant productivity (Rothstein, 2010; Enggrob et al., 2019).

In addition to naturally N-limited biomes, such as the mentioned boreal and temperate forests, N is usually heterogeneously distributed in the soil or might be frequently present in a form that can not be used by the plant, limiting therefore plant growth and development (Courty et al., 2015). To overcome such limitations, plants have developed specifics uptake mechanisms and structural features in order to improve its N uptake. One of the most common adaptation is the change in the roots structure, such as the inhibition of primary roots growth (frequently

associated with P deficiency), increase of hair roots density and growth (frequently associated with P and Fe deficiency) as well as increase in lateral roots growth and density (frequently associated with N, P, Fe, and S deficiency) (Morgan & Connolly, 2013). Those changes may increase the elongation and the overall surface area of the root system, allowing plants to have access to new nutrient sources, and resulting in higher root to shoot ratios in nutrient-limited plants (Lopez-Bucio et al., 2003).

Another important mechanism to improve access to limiting nutrients in the soil is the development of mutually beneficial symbiotic relationships with soil-borne microorganisms. In these relationships, both the host plant and the microorganism symbiont exchange valuable resources that they need for their own productivity and survival. Mycorrhizal fungi are widely recognized to improve plant nutrition by being able to access soil spaces and nutrient sources inaccessible for roots (Smith & Read 2010, Andrino et al., 2021), while plants have been shown to allocate up to 30% of recently-fixed C to their symbionts (Soudzilovskaia et al., 2015; Thirkell et al., 2020). Most terrestrial plants require an association with at least one type of mycorrhiza to adequately grow and complete their life cycle in natural ecosystems. The most common, ancient and widespread is the arbuscular mycorrhizal (AM) (Smith & Read, 2010; Schüßler & Walker, 2011). The second most common mycorrhiza type in nature is the ectomycorrhizal (EM) fungi. Although fewer plant species have been found to form symbioses with EM, in comparison to AM, the hosts of EM tend to be widely dispersed, abundant and dominant members of their groups (Brundrett 2009; Teste et al., 2019). AM and EM associations are assumed to differ in their structure and plant response, such as growth, photosynthesis rate, nutrition, survival and others (Gorzelak et al.,2015). Benefits of each fungi type for N nutrition is discussed in the following section.

## 1.2. Mycorrhiza symbiosis and N nutrition

The AM is the most common type of mycorrhizal association and is established by roughly 80% of all land plant species (Schüßler & Walker, 2011). The symbiosis is initiated by the exchange of signaling molecules between the two symbionts, followed by the germination of AM spore and growth of fungal hyphae towards the root. Then, the hyphae penetrates the epidermal cell layer until the cortex of the root to finally form the highly branched structures called arbuscules, composed by an extensive surface for nutrient exchange (Figure 1) (Luginbuehl & Oldroyd, 2017). The exchanges between plant and fungi partner is mediated by a unique transport protein composition present in the periarbuscular membrane, which is a plant-derived membrane surrounding the arbuscules (Krajinski et al., 2014; Bravo et al., 2017).

Externally, hyphae extend into the soil beyond root depletion zone, enabling the plant to acquire significantly more nutrients through its symbiotic partner than it could on its own (Karandashov & Bucher, 2005)

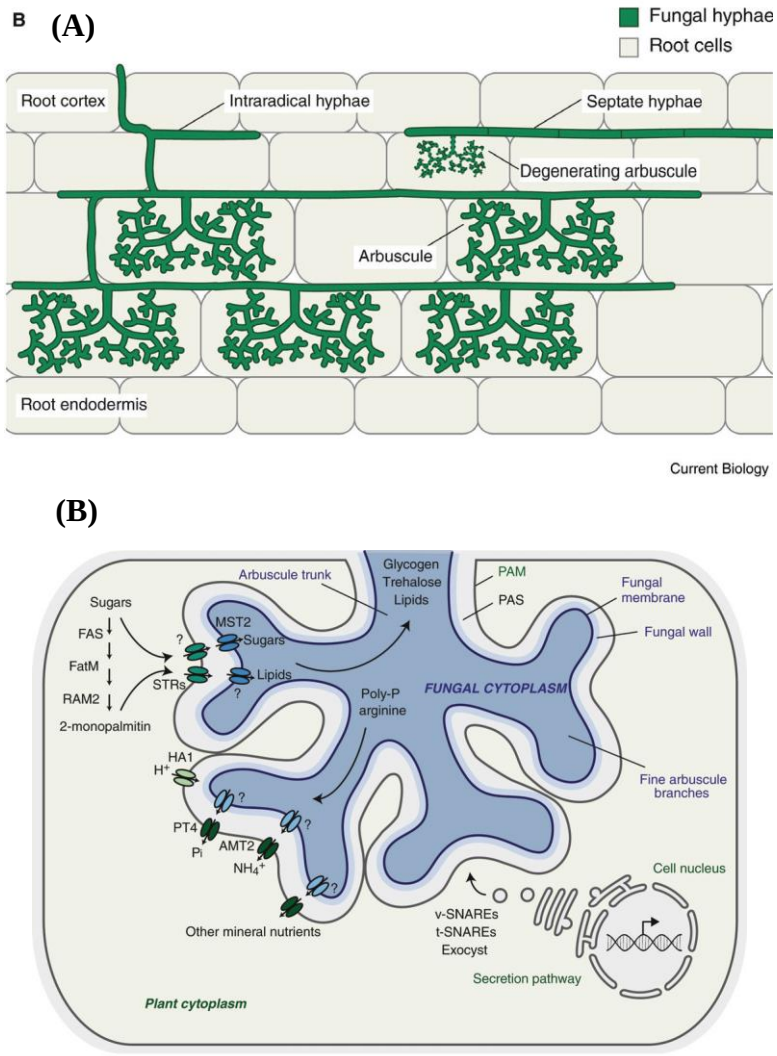


Figure 1: (A) Scheme of AM fungi colonization showing the intercellular fungal hyphae growing through the root cortex with intracellular arbuscules formation; (B) Intracellular arbuscules composed by transports protein present in the periarbuscular membrane. Figures taken from Luginbuehl & Oldroyd, (2017).

The EM fungi has a different pattern of associations with plant host. In EM, hyphae from soil propagule or from older ectomycorrhizal rootlets, attach onto epidermal cells of emerging lateral roots, proliferate and differentiate into a series of hyphal layers, to form what is known as the sheathing mantle. Inside the roots, hyphae develops around epidermal root cells in angiosperms and around both epidermal and cortical root cells in gymnosperms, forming a network of hyphae known as Hartig net. The Hartig net, with its complex labyrinthine hyphal branching and large surface area, is the main site of bi-directional exchange between fungi and

host plant (Martin et al., 2016).

These two types of mycorrhiza differ in structure and function (Phillips et al., 2013; Fisher et al. 2016). The AM fungi is a obligate symbiotic fungi and usually characterized as 'scavenging' type of fungi, since it is based on the physical exploration of the soil and uptake of mineral nutrients without changing their chemical form (Lambers et al., 2008). Although some recent studies have shown AM to be capable of acquiring N from decomposing organic sources (Hodge and Fitter, 2010; Herman et al 2012; Barrett et al., 2014; Thirkell et al., 2016) and even from organic N directly as amino acids (Whiteside et al., 2012; Zhou et al., 2020) or dipeptides (Belmondo et al., 2014), the vast majority of N acquired by AMF is thought to be as $NO_3^-$ or $NH_4^+$ (Govindarajulu et al., 2005; Bucking & Kafle, 2015). In contrast, EM fungi are generally considered a 'mining' type of fungi, meaning that they have the ability of releasing otherwise unavailable nutrients by excreting enzymes or low molecular weight organic acids (LMWOAs; Plassard & Dell, 2010). This is because EM fungi have still sapotrophic abilities, therefore exhibiting two contrasting ways of life: saprotrophic in soil (for nutrient acquisition) and biotrophic within plant living tissues.

Although AM have been more frequently reported to be important to improve plant P acquisition, both fungi types are known to improve plant N nutrition (Van der Heidjen et al., 2015; Jansa et al., 2019). However, it is believe that AM and EM may represent distinct benefits and costs for host plants. Makarov (2019) reported that, at a given plant, EM can contribute with up to 80% of plants demand for N, while AM contribute to only 20%. Goodale (2017) estimated that N uptake of EM trees may exceed that of AM trees by 50% in temperate forest. Nevertheless, benefits can vary considerably depending on biotic and abiotic factors such as the species of mycorrhiza involved in the association, the physiological state of symbionts and soil conditions (including N availability) (Johnson et al., 2015; Martin, 2016; Makarov, 2019).

Most plant types are able to associate either with AM or EM fungi. Trees forming EM association usually dominate temperate and boreal forests, while AM is more abundant in temperate grasslands, tropical forests and agricultural systems (Brundrett, 2009). Despite of the difference in AM and EM abundance in different biomes, both mycorrhizal fungi naturally co-exists in most vegetated biomes. Some special plants are able to establish dual associations with AM and EM simultaneously, either within the same root system or at different life stages (e.g. *Eucalyptus, Alnus, Populus, Salix, Quercus, Pseudotsuga, Melaleuca, Casuarina, Uapaca, Abies, Tsuga*) (Smith & Read, 2010; Teste et al., 2019).

Nutritional benefits have been frequently reported on plants hosting single mycorrhizal type, but much less is known about the benefits of hosting both. Teste et al. (2019) reviewed

the possible benefits for a plant to host dual associations. Those benefits can be nutritional, where plants bearing dual associations access a broader pool of nutrient sources, or non-nutritional, such as better ability to cope with drought events. The few studies dealing with dual inoculations *vs* single ones range from negative, neutral to positive responses (Tapwal et al., 2015; Kariman et al., 2012; Teste et al., 2019), depending on the plant and fungi species involved in the association together with abiotic effects such as climate conditions and soil nutrient availability. Thus, a better understanding of the forces driving such interactions is required, since it has profound implications for plant community composition, and it may represent an important tool for the development of management techniques for regeneration and recovery of disturbed environments (Beiler et al., 2010; Simard et al., 2015).

## 1.3. Common mycorrhizal networks (CMN) and N transfer

Despite of the significance of mycorrhiza for individual host plants, most mycorrhizal fungi can colonize simultaneously a large number of plants (van der Heijden & Horton, 2009), leading these plants to become interconnected by the so-called common mycorrhizal network (CMN) (Heaton et al., 2012; Wipf et al., 2019). The potential formation of such networks have been demonstrated mainly by *in vitro* systems (Kiers et al., 2011; van't Padje et al., 2021) in which connections can be easier visualized. In natural ecosystems, however, observation and proof of such interconnections are not an easy task since they cannot be visualized without disturbance. Some authors have been estimated the potential of plants to become interconnected by evaluating the similarity between the mycorrhizal community composition, assuming a greater similarity when plants are connected through a CMN (Beiler et al., 2010; Diédhiou et al., 2010). Other authors, like Beiler et al. (2010), considered the presence of a single genet on roots of two different trees as a proof of the network link. Yet, sharing the same genet does not necessarily indicate a direct connection among the host plants since connections can be disturbed by grazing of other soil organisms and hyphae turn over (Wu et al., 2005). This way, plants would still share the same genet besides of being physically isolated. The possibility of connections between different plants is of great importance and may represent a number of complex interactions involving multiple individuals.

Among the reported effects of such connectivity is the possibility of nutrient exchanges, which is suggested to play an important role for interplant nutrition (Bücking et al., 2016; He et al., 2019; Fang et al., 2021). The premise of possible nutrient transfers through this physical connections established by the CMN might be of great importance since such nutrients would be free of potential disruption, such as uptake for soil microorganisms, absorption in soil

particles or losses via leaching. Such transfers have been frequently reported in field and laboratory experiments using labeling compounds to trace the fate of nutrients in plants connect by a CMN, trying to demonstrate that belowground resource transfer between plants of same and different species is facilitated by mycelial connections (Teste et al., 2009, Deslippe & Simard, 2011; He et al., 2019; Fernandez et al., 2020). Some studies have shown N transfer via CMN varying from 0 to 72% under field conditions in grassland ecosystems (Farnham & George 1993). In pot experiments made in agroforestry ecosystems, N transfer rate varied from −0.1% to 12.2% (Chu et al., 2004; Meng et al., 2015), while in field conditions rates varied from 1.9% to 16% (Chapagain & Riseman, 2014; Thilakarathna et al., 2016; Zhang et al., 2020). The high variation of the amounts of N exchanged via the network has raised questions regarding its importance for plant fitness.

Although the role of the CMN for interplant N transfer has been shown for both AM (Li et al., 2009; Meng et al., 2015; Teste et al., 2015) and EM (He et al., 2004), the majority of the studies made concerned AM networks. Much less is known about the potential of EM networks to translocate N. In addition, the potential of AM and EM in transferring N over networks which involve dual-mycorrhizal plants was never evaluated. Dual-mycorrhizal networks could provide a great potential to determine costs and benefits inherent from each fungi partner without confounding host species effects. There are only few studies aiming to evaluate transfer differences between plants involved in AM and EM network, and most of them are made either by comparing single vs dual-mycorrhizal plants or by evaluating AM and EM in the same plant species but not at the same time (Teste et al., 2015).

Despite of the fungi involved in the network, several previous studies have suffered from inadequacies in clearly demonstrating the existence of a functional CMN (Leake et al., 2004; Simard & Durall, 2004); as the potential movement of isotopes through other pathways was not successfully excluded. The technical problems in unequivocally demonstrating that plant-to-plant transfer occurs genuinely through hyphal interconnections is challenging (He et al. 2004; Wilson et al., 2006). Some authors have proposed that loss of nutrients from roots or hyphae into the soil pool, followed by immediate uptake by mycorrhizal hyphae or roots of neighbouring plant, appears to be the main path for plant-to-plant transfer. Distinction and relative importance of the different pathways will determine the strength, direction and outcome of interactions among plants, requiring new technologies and ideas to address such issues.

## 1.4. Motivation and hypothesis

Although both AM and EM fungi are generally known to increase the uptake of nutrients

of host plants, their functions and benefits may not be equivalent. Several studies have been developed to evaluate single mycorrhizal plants, their nutrition and the effects of mycorrhizal network for connected plants. However, evaluation of such parameters in dual mycorrhizal plants are lacking. Available studies fail to provide unequivocal evidence on the advantage to a host plant of exploiting a dual association compared to an individual one for N acquisition. In addition, it remain unexplored whether a plant holding a dual association displays preferential N sharing with EM or AM over a CMN. Likewise, studies evaluating the role of the CMN for interplant resources exchanges has failed to quantify transfer over direct *versus* indirect pathways, and how transferred resources can affect receiver plant nutrition. I provide here a review of the previous raised theories and the news findings on the topic.

In addition, I developed two experiments to help us to cover such gaps. The first was a mesocosm system to simultaneously investigate the performance of single and dual-mycorrhizal plants with regard to N nutrition and sharing via CMNs using stable isotope labelling techniques ($^{15}N$). I hypothesized that (1) host plants establishing dual-mycorrhizal association will exhibit an enhanced N nutrition, compared to those depending on single association. I further hypothesized that (2) dual mycorrhizal plants will preferentially share more N to plants over EM network, due to its larger mycelium proliferation compared to AM. Lastlly, I hypothesized that (3) such mycelium proliferation might act as a sink for C, requiring higher C allocation from host plant. The second experiment aimed to evaluate the importance of CMN for N transfer between connected plants, by quantifying N transfer via direct hyphae connections *versus* indirect pathways. For this, I propose an experimental setup using two different types of *Medicago truncatula*: a reduced arbuscular mycorrhizal (*ram1-1*) mutant and a wild type (Wt), coupled to isotopic labelling techniques with enriched $^{15}N$, in order to overcome the limitations of previous studies to distinguish and quantify nitrogen (N) translocation over the different possible pathways. I hypothesized that (1) N transfer between connected plants occurs genuinely through hyphal connection rather than by indirect pathways; (2) the proportion of N allocated from donor to receiver plants through mycorrhiza hyphae connections is significant and may improve neighboring plant N nutrition, and (3) by shading donor plant, N transferred to receiver plants is increased, once unshaded receiver might be able to produce more C to be exchanged for transported N.

## 1.5. References


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## 2. Manuscripts

### 2.1. Common Mycorrhiza Network: Theories and mechanisms behind underground interactions

frontiers in Fungal Biology

REVIEW
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# Common Mycorrhizae Network: A Review of the Theories and Mechanisms Behind Underground Interactions

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Most terrestrial plants establish symbiotic associations with mycorrhizal fungi for accessing essential plant nutrients. Mycorrhizal fungi have been frequently reported to interconnect plants via a common mycelial network (CMN), in which nutrients and signaling compounds can be exchanged between the connected plants. Several studies have been performed to demonstrate the potential effects of the CMN mediating resource transfer and its importance for plant fitness. Due to several contrasting results, different theories have been developed to predict benefits or disadvantages for host plants involved in the network and how it might affect plant communities. However, the importance of the mycelium connections for resources translocation compared to other indirect pathways, such as leakage of fungi hyphae and subsequent uptake by neighboring plant roots, is hard to distinguish and quantify. If resources can be translocated via mycelial connections in significant amounts that could affect plant fitness, it would represent an important tactic for plants co-existence and it could shape community composition and dynamics. Here, we report and critically discuss the most recent findings on studies aiming to evaluate and quantify resources translocation between plants sharing a CMN and predict the pattern that drives the movement of such resources into the CMN. We aim to point gaps and define open questions to guide upcoming studies in the area for a prospect better understanding of possible plant-to-plant interactions via CMN and its effect in shaping plants communities. We also propose new experiment set-ups and technologies that could be used to improve previous experiments. For example, the use of mutant lines plants with manipulation of genes involved in the symbiotic associations, coupled with labeling techniques to track resources translocation between connected plants, could provide a more accurate idea about resource allocation and plant physiological responses that are truly accountable to CMN.

Keywords: resources allocation, plant fitness, mycelium connections, connected plants, direct pathway, indirect pathway

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## MYCORRHIZA NETWORK: THEORETICAL BACKGROUND

Mutualistic associations between mycorrhizal fungi andplants are well-known. Within the diverse mycorrihzatypes, the arbuscular mycorrhizae (AM), from the phylum Glomeromycota, is one of the most common, ancient and widespread, associating with around 80% of all land plant species (Schüßler and Walker, 2011). This fungi type is more predominant in warm climates and species rich ecosystems, such as tropical forests. The second most common fungi typein nature is the ectomycorrhizal (EM) fungi. Although a lower number of plant species have been found to form symbiosis with EM, in comparison to AM, the hosts of EM tend to be widely dispersed, abundant and dominant members of their groups (Brundrett, 2009; Teste et al., 2020). Different from AM, EM fungi are mainly found in colder regions and ecosystems, where less host species are present, e.g., temperate and boreal forests (Brundrett, 2009; Gorzelak et al., 2015). AM and EM networks are assumed to differ in their structure, but both affect plant responses, such as growth, photosynthesis rate, nutrition, survival, and others (Gorzelak et al., 2015). Besides, AM and EM fungi species are frequently found co-existing in thesame ecosystem. Some exceptional plants are even able to host both types of fungi in its roots, although the proportion of the association with each may differ along plant's life (Gorzelak et al.,2015).

Mycorrhizae fungi are widely recognized to improve plant nutrition by being able to access soil spaces and nutrient sources inaccessible for roots (Smith and Read, 2010, Wipf et al., 2019; Andrino et al., 2021). The great majority of mycorrhizae fungi are not host specific, being that a single mycorrhizae fungi specie is able to colonize a wide range of plant species. Once a fungi colonize the host plant, its mycelium is able to grow over large distances in the soil and may reach and colonize the roots of multiple neighboring plants, from the same or different species (Van Der Heijden and Horton, 2009). Therefore, plants sharing the same host fungi are reported to become interconnected by the so-called common mycorrhiza network (CMN) (Heaton et al., 2012; Rhodes, 2017; Wipf et al., 2019). Connectivity are therefore likely to occur between plants able to associate with the same fungi species.

As ecosystems are usually dominated by mycorrhizal plants, including most temperate and tropical grasslands as well as boreal, temperate and tropical forests (Read, 1991; Van Der Heijden, 2016), abundant and extensive mycorrhizal fungal networks are formed (Wipf et al., 2019). It is believed that plant species can interact and communicate via these CMNs (Gorzelak et al., 2015; Pickles et al., 2017; He et al., 2019). This may affect survival and behavior of connected plants as well as competitive and cooperative patterns, consequently influencing plant diversity at local and regional scales (Deslippe and Simard, 2011; Simard et al., 2012; Bücking et al., 2016). Among the reported effects of such connectivity are the improvementof seedling establishment (Bingham and Simard, 2011; Seiwaet al., 2020), impact on plant and microorganism community compositions (Meng et al., 2015; Teste et al., 2015; Kadowaki et al., 2018), induction of plant defense responses (Babikova et al., 2013; Song et al., 2014), plant communication through a variety of phytohormones such as jasmonic acid, methyl jasmonate and zeatin riboside (Song et al., 2010), and nutrient exchange, which may play a pivotal role for interplant nutrition (Bücking et al., 2016; He et al., 2019; Fang et al., 2021).

In the review made by Van Der Heijden and Horton (2009) it is stated that CMN can be compared either to "socialist" or "capitalist" systems, or even to a "superorganism." For the "socialist" behavior, individuals are able to have equal opportunities and resources are distributed more evenly providing benefits for all connected plants. For the "capitalist" network, mycorrhizal would be privately controlled for the profit of certain group of plants, increasing therefore competition between connected plants. If network behavesas a "superorganism," fungal species in the network areconsidered redundant physical extensions of the roots, which might translocate nutrients freely between plants. Therefore, the mode of interplant connection might have evolutionary consequences of CMN by substantially defining the community ecology of a site, leading to ecosystem-wide impacts (Gorzelaket al., 2015). This depends largely on which of these responses are predominant ("socialist," "capitalist," or simple physical extensions) in the moment plants are connected; together with the question whether these responses may change if plants from the same or from different species are connected.

In face of all the possible effects of CMN on plant interactions, many different theories have been raised with the intention to predict how mutual association and co-existence of species in the system is stabilized. By one hand, we have the biological market theory, for example, which is based on the assumption that fungi might recognized the best plant partner and re-allocate nutrient accordingly to its carbon (C) gain. On the other hand, we have the source-sink theory in which resource would movein a concentration gradient. This could lead resources to bedistributed more equally among partner involved in the network,which is the opposite of what is expected if the biological marketis driven resource allocations. Both theories will be more detaileddiscussed in the following sections. Nevertheless, benefits and disadvantages from the interactions between connected plants are hard to distinguish in nature, once most of the plants are colonized simultaneously by multiple fungal species, each one with its own cost–benefit. In addition, in natural ecosystems, not only mutualistic interaction between connected mycorrhizal plants takes place, but networks may also include commensalistic and even antagonistic interactions (Toju et al., 2013). Therefore, some plant species might benefit from CMN more than others, depending on the fungi and plants involved in the association. It is important to note that, even if plants would be connected mainly by a single mycorrhiza type, i.e., AM fungi, variations in the functional properties and temporal patterns of different strains can also be observed (Kiers et al., 2011). This adds further complexity to the potential mechanisms by which such network would determine plant community composition and productivity through their facilitative and antagonistic effects

on plants (Wagg et al., 2015). Therefore, predicting ecosystem dynamics of connected plants is still a huge challenge.

Due to the high complexity to discriminate effects of CMN in natural ecosystem, the majority of studies aimed to evaluate the influence of CMN for connected plants were mainly performed with few species of plants growing in pairs in microcosms and under controlled environmental conditions. Even under such controlled situation, the outcomes may still vary significantly, once benefits of connected plants may change according to host's physiological status, plants and fungal species involved, environment conditions, nutrient availability, etc. (Wagg et al., 2011). With this in mind, it is necessary to assess the mostrecent findings in literature and define still open questions, in order to guide upcoming studies in the area aimed to have a better understanding of possible plant-to-plant interactions via CMN and its effect shaping plants community. The present paper therefore evaluates results and theories of the functionality of CMN for plant-to-plant communication, especially, for resource exchange. Here, we also point the gaps of such studies in order to highlight especial points that need to be address in further studies.

## Source Sink Theory

In the source-sink model, the source is defined as the entity that can produce more of a given resource than it uses and the sink as the entity that has the potential/necessity to use more of a given resource than it produces (Heaton et al., 2012). The primary importance of plant–sink strength in governing the magnitude and direction of resource transfer through CMNs is illustrated in studies showing transfer of C to rapidly growing young EM trees with high transpiration rates, or to shaded seedlings with high respiration demands, increasing its survival and growth (Lekberg et al., 2010; Philip et al., 2010). Similarly, transfer of other resources, such as nitrogen (N), were also reported following a source-sink pattern (Montesinos-Navarro et al., 2017; Muneer et al., 2020). This mechanism has been proposed to increase the regenerative capacity of forest ecosystems (Teste et al., 2009, 2010). However, there are also reports of reduced transfer of C within a CMN to sink (shaded, defoliated, seedling) plants (Kytöviita et al., 2003; Walder et al., 2012), and even C transfer from sink (shaded) plants to source plants (Deslippe and Simard, 2011). Thus, a better understanding of the forces driving such interactions is required, since it has profound implications for our understanding of plant communities and competition. Depending on the species involved in the CMN and the possible effects for its fitness, it will drive forest community composition and dynamics (Beiler et al., 2010; Simard et al., 2015).

## Biological Market Theory

Asymmetry on resource allocation has been also demonstrated to increase competition between connected species (Merrild et al., 2013; Weremijewicz et al., 2016). Merrild et al. (2013) found that the growth suppression of small neighboring plants was diminished by clipping the shoots of large plants, which also increased the P uptake by interconnected small neighbors 6.5-fold. In order to exclude that suppression was caused by a general negative growth response, treatments including solitary vs. networked seedling was performed. In the referred study, suppression occurs only when seedlings were linked to the extraradical mycelium (ERM) of the large plant. Therefore, the authors concluded that the observed effects could solely be attributable to the CMN effect. However, such results has to be interpreted carefully, since inherent characteristics of plant species involved, such as growth rate, size, and root:shoot ratio, are likely to influence observed nutrient uptake.

Nevertheless, based on the observed results, an alternative theory has been proposed to elucidate such effects, the biological market theory. This theory is based on the assumption that both, plant and fungi, are able to detect variation in quality and amount of the resource supplied by their partner, allowing them to adjust their own resource allocation according to its gains (Kiers et al., 2011; Walder and van der Heijden, 2015; Werner and Kiers, 2015; Wang et al., 2019). Kiers et al. (2011) used molecular markers and stable isotope probing to track C flow from *Medicago truncatula* hosts into fungal RNA of roots colonized by mixed AM fungal communities with different cooperative behavior to the hostplant. The authors found greater C enrichment in the most beneficial fungal species, suggesting a preferential allocation ofC by the host, operating in a small spatial scale. The opposite flux was also observed, in which the fungi delivered more P for the host, which provided more C to fungi. Fellbaum et al. (2014)also evidenced fungal discrimination by greater N allocation to the host under elevated C allocation. If "rewards" indeed are reciprocal between mycorrhizal fungi and host plants, largerplants are supposed to obtain larger amounts of limiting nutrients by the fungal networks once they can produce and allocate much more C to the fungal partner. Increasing competition and suppressing growth of smaller individuals thus makes CMN a stronghold to avoid outcompeting its own kind.

It is important to note that the market theory proposed by some authors goes in an opposite direction to what was stated in the "source-sink" theory presented above. Neither theory should be defined as an universal framework to explain resource exchange in the mycorrhizal association nor predict plant interactions within a CMN, since the outcome of such interactions may vary with environmental conditions, functional diversity, competition for surplus resources, reciprocity and sink strength. Therefore, the effect of each variable should be tested separately and considered into the proposed models in order to define a more universal framework.

# UNDERGROUND CONNECTIVITY

Both the source-sink theory and the market theory relies on the prerequisite of an underground connectivity of plants via CMN. In general, ecologists agree on the definition of CMN as a physical linkage among plants via the mycelia of the mycorrhiza fungi and that this linkage is common in nature (Simard and Durall, 2004; Simard et al., 2012; Hoeksema, 2015). However, this premise comes from observations that species of AM fungi are often compatible with multiple host plant species. In addition, Giovannetti et al. (2001) have demonstrated the ability of genetically compatible hyphae to anastomose

(fusion), with disappearance of hyphal walls and exchange of cytoplasm and nuclei (Barreto de Novais et al., 2017). Both findings suggesting that CMNs are probably ubiquitous, although confirmation of such assumption still requires direct evidence for these linkages in the field. In this context, plants of same and different species have been reported sharing same fungi species or even same genet in several ecosystems (Simard et al., 2012; Beiler et al., 2015). Some authors have estimated the potential of plants to become interconnected by evaluating the similarity between mycorrhizal community composition, assuming a greater similarity when plants are connected through a CMN (Beiler et al., 2010; Diédhiou et al., 2010). (Beiler et al., 2010), for example, evaluated the distribution of genets of two species of ECM fungi (*Rhizopogon vesiculosus* and *R. vinicolor*) among roots of individual trees of Interior Douglas-fir (*Pseudotsuga menziesii*) as a network link. The authors proposed a model where trees of different ages were connected in a scale-free architecture and the larger trees served as hubs of nutrition, favoring understory regeneration, and functional continuity in the stand.

These achievements were of great importance to demonstrate the complexity of the CMN and the number and diversity of individuals that are potentially linked, resulting in a multitude of interactions involving multiple generations. However, sharing compatible species or even the same genets, does not necessarily indicate a direct connection among the host plants. Collembolas, for example, are known to feed on fungal hyphae. Such as AM fungi, they are widespread and abundant in the soil (Ekblad et al., 2013; Ngosong et al., 2014). By grazing the hyphae of a genet connecting two or more plants, this genet can still be identified in the roots of those plants although they would no longer be connected (Rotheray et al., 2008; Beiler et al., 2010). This is one of the examples of CMN disruption that could occur in the soil, and would be hard to identify (Wu et al., 2005; Beiler et al., 2015). Consequently, technical difficulties in proving hyphal connections between plants are the main obstacle when identifying whether any observed effect is really an intrinsic property of a CMN.

Therefore, it is also important to prove the extent and continuity of the mycelial network, together with mechanisms driving such connections and its consequences for plant fitness. In this context, there are few non-destructive methods for mycelium network observation, especially for AM fungi, mostly by the use of root observation chambers (Mikkelsen et al., 2008; Gyuricza et al., 2010) and *in vitro* dual systems (Kiers et al., 2011; Van't Padje et al., 2021). Such studies have nicely demonstrated the architecture of the extraradical mycelium of the fungi connecting two neighboring plants, but yet the relative importance of such network under realistic conditions is frequently under debate. For experiments developed in the forest, many interferences are found and the effects and mechanisms involved in the CMN cannot be excluded from other effects, such as positive and negative plant-soil feedback due to modulation of soil microbiota and biogeochemical cycles or even by production of roots exudates that might affect growth of nearby plants (Hu et al., 2018). Therefore, mycorrhizae studies still face challenges, raising questions if the data represents a natural situation, since there are no guarantees that evaluated effects are caused by mycorrhizae network.

## MECHANISMS INVOLVED FOR PLANT INTERACTION VIA CMN

Currently, the mechanisms that drive benefits and competitive interactions between plants involved into a CMN has been under debate (e.g., Fellbaum et al., 2012; Bücking et al., 2016), raising diverse theories about the mechanism in these associations. The first one is based on the assumption that established mycorrhizal plants would facilitate mycorrhization of neighboring seedlings, acting as an inoculum and C source. In this case, seedlings would be able to join a CMN, which were already stablished and supported, in terms of translocation of reduced C by the older plants. Thus, seedling would be able to get access to limiting nutrients provided by the fungi without contributing with C supply to maintain the network. The second mechanism is based on the idea that CMN will act as conduits for interplant nutrient transfer (Gilbert and Johnson, 2017; Wipf et al., 2019). In this context, depending on how resources are distributed between connected plants, plants may either benefit by a more equilibrate distribution of resources or by increasing discrepancies of resources. In the first case, plants with higher nutritional conditions may donate excess of their resources to the receiver plants by a direct transfer. In the second case, resources might be distributed unequally favoring a certain group of individuals increasing therefore competitive interactions.

### Inoculum Source and Carbon Provision

Firstly, CMN may provide an inoculum source. Association with hyphae from the CMN can be much faster in comparison to soil spore bank, by the provision of an already established fungal inoculum source by the mature tree, permitting seedlings to quickly tap into a large soil resource pool that they could not access by their own (Bingham and Simard, 2012). Thus, this faster access to mycorrhizal services in the early plant stage, where mortality is high due to drought and biotic interactions, may be of critical importance, especially under harsh environmental conditions (Simard et al., 2012; Teste et al., 2015). In the experiment developed by Varga and Kytöviita (2016), the proportion of colonized seedlings by three different AM fungi was strongly related to the fungal species as well as to the source of inoculum. Seedlings inoculate much faster from nearby mycorrhized plants than from spores, despite a high spore density. This premise is also supported by some field experiments showing a positive relationship between the survival rate of seedling and its distance from the mature tree (McGuire, 2007; Grove et al., 2019). In addition, experiments involving barriers (e.g., mesh bags) or soil disturbance to manipulated seedling contact with CMN have shown higher seedling mortality when seedling are impeded to join the network (Nara, 2006; Pec et al., 2020).

Secondly, seedling may benefit from sharing a CMN with adult established tree since adult trees might provide much more C to sustain the network while seedling invest very little C and

still obtain nutrients provided by the fungi. The maintenance of fungal symbiosis can be costly, resulting in a high C demand by the fungi for its development and activity (Smith and Read, 2010; Keymer et al., 2017; Rezáčová et al., 2017). In this context, sugars and lipids are the main C source derived from host plants transported to the fungal symbiont. Those C derived components will provide the fungi with the energy necessary for nutrient acquisition and the C skeleton for mycorrhizal growth (Bravo et al., 2017; Bezrutczyk et al., 2018). A benefit for seedlings would arise if larger trees pay the C cost required for the growth and maintenance of the CMN, so seedlings could potentially become mycorrhized and receive the benefits of this association without expending their own C for this (Diédhiouet al., 2010; Walder et al., 2012; Weremijewicz et al., 2016). In the study made by Högberg et al. (1999), for example, EM fungi connecting overstory pine trees with understory plants of different ages received 87–100% of their C from overstory trees and very little from understory trees. Walder et al. (2012) have shown a similar asymmetric pattern by using $^{13}C$ of natural abundances between $C_3$ and $C_4$ plants without disturbing the system. The authors found that the $C_4$ plant, which had the higher biomass, was invested more C to both fungal partner than the $C_3$ plant but did not have a higher nutritional benefit. In this context, nutritional benefit strongly depended on the fungus involved in the CMN, in which *Rhizophagus irregularis* allocated nutrients preferentially to the $C_3$ host plant while the CMN formed by *Glomus mosseae* were more balanced with respect to the nutrient allocation to both, $C_3$ and $C_4$, host plants. This demonstrate that C investment and nutritional benefit are not necessarily tightly linked and that some plant species can receive disproportional benefits from CMN. It is important to note that these experiments indicate that disproportional C investment by one plant does not necessarily mean a disadvantage for the other plant, especially when the cost of C is negligible for the main C donor.

## Mycorrhiza Network as Conduits for Interplant Resources Transfer

The premise of a possible nutrient transfer through a physical connection established by CMN may be of great importance in agricultural, where redistribution of symbiotic costs and benefits between individuals of the same or different plant species could increase growth of connected plants and therefore reduce amounts of chemical fertilizer input (Pena et al., 2013; Jansa et al., 2019). However, if a direct transfer of photoassimilates and nutrients between plants occurs via CMN is particularly controversially discussed (Bever et al., 2010; Courty et al., 2010). Such transfers have been frequently reported in field and laboratory experiments using labeling compounds to trace the fate of nutrients in plants connect by a CMN, trying to demonstrate belowground resource transfer between plants of same and different species is facilitated by mycorrhizal fungi (Teste et al., 2009; Deslippe and Simard, 2011; He et al., 2019; Fernandez et al., 2020).

In earlier studies, this mechanism was mainly observed in mycoheterotrophic plants, which are partly or entirely non-photosynthetic and indirectly parasitize green plants via CMN. These non-photosynthetic plants, also called epiparasites, associate with AM fungi emanating from the roots of surrounding green plants, therefore having access to C provided by those plants, together with other resources (Bidartondo et al., 2002; Girlanda et al., 2006; Selosse and Roy, 2009). In addition to mycoheterotrophic plants, some green orchids or small green perennial shrubs from the Ericaceae family have also been shown to receive considerable amounts of C from their mycorrhizal fungi (Selosse and Roy, 2009; Selosse et al., 2016). Those studies have raised the attention for the existence of a network where unrelated plants are able to transfer elemental compounds via shared fungal symbionts.

The mycorrhizal fungi which associates with mycoheterotrophic plants and green orchids usually belong to a diverse fungal taxa that also form mycorrhizae association with phototrophic tree roots (Zimmer et al., 2008; Watermanet al., 2013; Brundrett and Tedersoo, 2018). Since C transfer were observed between mycoheterotrophic and green plants and the same fungi species connecting those plants can also colonize several phototrophic trees, theories were raised regarding the possible C allocation between phototrophic trees as well. If such networks could act as a direct pathway of C and nutrients between green plants, this could play an important role for plant to plant interactions (Selosse and Roy, 2009; Smith and Read, 2010). Once C is an important resource for fungi growth, C allocation between plants would go to an opposite direction of the natural C flux commonly accepted in the symbiosis, which is from plant to fungi. In this case, one of the host plants would provide fungi with C and the fungi would not incorporate but channel this C through a neighboring plant. Some researchers believe that it might happen when networking fungus can acquire more C than it is required for its own fitness, therefore it may supply the excess to other plants in need (Gorzelak et al., 2015; Prescott et al., 2020). This has been suggested as a mechanism from the fungi to ensure survival of its host plants and therefore its access to multiple C supply, in case of a potential loss of one of the hosts (Gorzelak et al., 2015; Bücking et al., 2016). Some authors raised this theorem by using experiments involving high and low quality plants connected into a CMN (Kiers et al., 2011; Fellbaum et al., 2014; Bücking et al., 2016). In this context, the quality of a host is determined by its C investment into the mycorrhiza, in which low quality hosts have a reduced investment while high quality host can produce and allocate higher amounts of C to fungi partner. In previous studies, shading have been frequently used to reduce the plant's ability to produce C compounds to be exchanged by limiting nutrients. In such experiments, although a discrimination between plants was observed leading to higher resources (such as N and P) allocation to high quality host of the network, the fungi also transferred nutrients for the low quality host and maintained a high colonization rate in these plants (Kiers et al., 2011; Fellbaumet al., 2014; Bücking et al., 2016). Those mechanisms shows a possible strategy from the fungi in maintaining both high and low quality host into the network, to ensure that the possible loss of a high quality host is not harmful for its survival. This might be an important mechanism for fungi survival, especially under

variable environments, as suggested first by Perry et al. (1989) and Wilkinson (1998).

In this context, Simard et al. (1997a) was one of thefirst to demonstrate a bi-directional flux of C between two autotrophic plants, Douglas-fir (*P. menziesii*) and paper birch (*Betula papyrifera*) species, sharing an EM network. Here, a greatamount of C was observed to be exchanged between the plant species, with no net gain for any one of them in the end. However,in the second year of study, Simard et al. (1997b) observed a net gain of C by one of the species independently of full, partial or deep shade light intensity. However, some methodological issues regarding the experimental design of this study was unraveled later by Robinson and Fitter (1999), raising doubts regarding the ecological relevance of CMN-facilitated resource transfer. Simard et al. (1997b) used a double labeling technique ($^{14}C$ and $^{13}C$) to track C exchange between plants connected by an EM network in the field and calculate proportions of C received by each individual. However, not only EM connected plants received the applied C, but AM surrounding plants not connected to the network had access to labeled C too. That demonstrate that the movement of C between plants were not necessarily exclusively by mycorrhizal links, but could have reached neighboring plant by different pathways. This is especially likely to occur when no physical barriers are used in the experiments.

Robinson and Fitter (1999) also suggested that C transferred from neighboring photosynthetic active plant to hyphae within the roots of C-stressed plants is probably a strategy of the fungi for its own growth and survival, with minor consequences for plant communities. Teste et al. (2010) using a different experimental design also showed a low net C transfer between Douglas-fir seedlings in the field relative to total C uptake by photosynthesis. The significance of the amounts transferred have been repeatedly questioned in other works (Teste et al., 2009; Philip et al., 2010; Pickles et al., 2017), raising a center debate on whether the extent of net transfer from one plant to another is sufficiently large to affect significantly plant fitness and predict communities' dynamics. In addition, there are also reports about the accumulation of C partially or entirely in mycorrhizal roots of receiver plants, probably in fungal tissues, and not detected on shoots even under situations where root to shoot C flow is encouraged by clipping or shadding (Robinson and Fitter, 1999; Pfeffer et al., 2004; Lekberg et al., 2010). However, some authors argue that the movement of C to receiver plant, even without transfer into plant tissues, is still an important subsidy to meet the nutrient requirements of the plant, especially under stress conditions (Bever et al., 2010; Teste et al., 2015).

Mycorrhizal networks have also been frequently reported to play an important role for belowground transfer of N among plants, but as for C different studies lead to contradicting results. Patterns of N transfer have been studied using natural abundance ($\delta^{15}N$) or $^{15}N$-enriched techniques. For the $^{15}N$-enriched techniques, fertilizer is applied directly to the growth media of the N donor root or directly to the N donor plant by exposure to $^{15}N_2$ (in case of experiments using N-fixing bacteria as an additional symbiont to host plant) or foliar spray or petiole injection of labeled $^{15}N$ ($NH^+_4$, $NO^-_3$, or urea). In early studies of several intercropping systems, a substantial one-way N transfer was demonstrated via a source-sink gradient from $N_2$ fixingplants to non-$N_2$ fixing plants, within a range of 20–50% (Heet al., 2009). However, when a bi-directional flux was consideredit was possible to note a greater flux of N from non-fixing plantsto N-fixing plants, contradicting the source-sink theory initially proposed by this system (He et al., 2005, 2009; Pirhofer-Walzl et al., 2012).

Moreover, a transfer between $N_2$ non-fixing donors and receiver plants of varying amount of N has also been observed. The transfer of N usually was reported to be lower than 5 % of N added by pulse labeling, while the direction of transport was largely found to be correlated with plant size (Teste et al., 2009, 2015; He et al., 2019) or plant physiology (Meding and Zasoski, 2008; Weremijewicz et al., 2018). Teste et al. (2009) also suggested that C and N move together in form of amino acids, once the stoichiometry of the relative amounts of C and N transferred was similar of this compound, but they were never identified (Simard et al., 2015).

Interestingly, the idea of plant-to-plant transfer implies that N may flow in the "opposite" direction of what is widely known to occur. In the context of nutrient uptake, the current model suggests that P and N acquired from surrounding soil by the ERM of the fungi are transferred to the intraradical mycelium (IRM) as polyphosphate (polyP) and arginine, respectively, stored later on in vacuoles (Hijikata et al., 2010; Bücking and Kafle, 2015). Once in the IRM, polyp, and arginine are catabolized andPi and ammonium are released and transported to the plants through transporters present in the periarbuscular membrane (Breuillin-Sessoms et al., 2015; Wang et al., 2017; **Figure 1**).Therefore, for plant-to-plant transfer, N should be transferred inthe opposite direction: from plant to the IRM via transporters in the periarbuscular membrane, from IRM transferred to the ERM of the fungi and then again to the IRM of the receiverplant to be assimilated. Although many studies have been made in order to prove such transfer via connected hyphae (please check **Supplementary Table 1** for some of those studies), such fluxes were never described anywhere. In the studies presented in **Supplementary Table 1**, it is also possible to observe that amount of N transferred via CMN is quite variable, probably due to differences in the experimental design and the choice of plant and fungi combination. In addition, transfer exclusively via mycelium connection in comparison to other possible are not distinguishable, especially in those studies in which a mesh barrier is not used to prevent roots intermingle and flow ofsoil solution.

## Direct Vs. Indirect Transfer

Mechanistically, AM fungi can facilitate the transfer of N between plants by creating direct mycelial connections between donors and receivers (Høgh-Jensen, 2006; Meng et al., 2015; He et al., 2019). When it comes to resource allocation through CMN, it is easy to notice a disagreement regarding its concept within published papers, even most recent ones. On the one hand some authors report a transport of nutrients via CMN exclusivelyvia connected hyphae, thus describing hyphae as "pipelines" for resources (Klein et al., 2016; Van Der Heijden, 2016). On the other hand, other authors describe nutrient transfer to occur

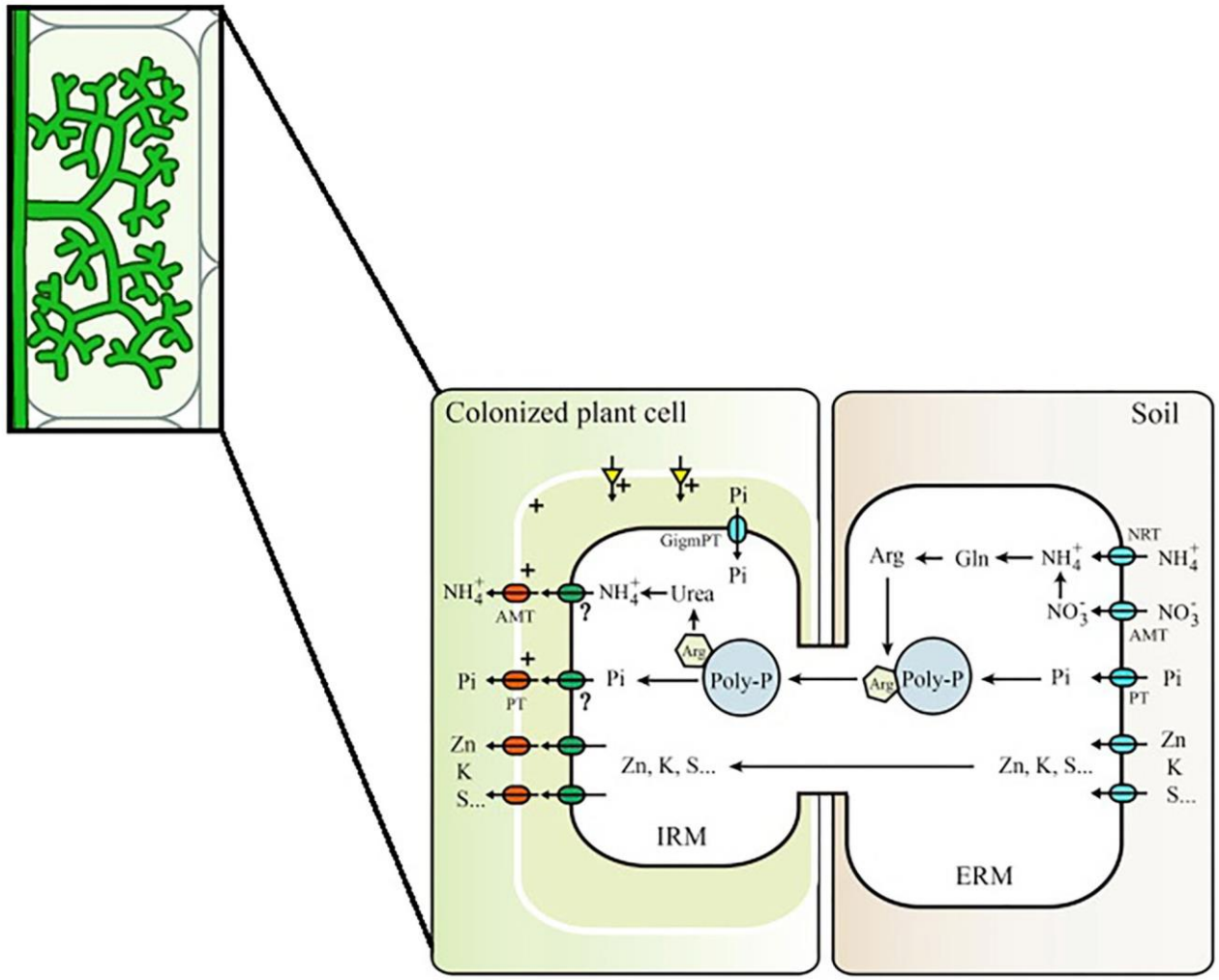


FIGURE 1 | Mycorrhizal pathways for Pi and N in AM Symbiosis. In the mycorrhizal pathway, Pi is assimilated directly via phosphate importers while ammonium ($NH_4^+$) and/or nitrate ($NO_3^-$) are assimilated into glutamine (Gln) and then into arginine (Arg). Assimilation will generate excess $H^+$ or $OH^-$ with $NH_4^+$ and $NO_3^-$, respectively. Phosphate is mainly transported in the form of polyphosphate (Poly-P) granules, which is negatively charged, making possible it association with arginine and metal ions for further transportation to the IRM. Phosphate (Pi) and $NH_4^+$ transporters from the intraradical mycelium (IRM) to the interfacial apoplast are still unknown and therefore marked with a "?," requiring further study (modified from Wang et al., 2017).

at least additionally via an indirect pathway. In such pathway, compounds are exuded or leaked into the soil pool by the roots or associated hyphae of one plant and then picked up by the rootsor associated hyphae of a neighboring plant or even by other microorganisms present in the soil (Jansa et al., 2019; Fernandez et al., 2020; Fang et al., 2021). In this context, it is frequently stated in studies that CMN simply facilitate transfers between plants without further specification of the mode of transport, although this transport may occur by several pathways simultaneously between a single pair of plants (Wang et al., 2016; Fang et al., 2021).

For these indirect pathways, resources are vulnerable for potential disruptions, such as adsorption of nutrients to soil particles, immobilization and mineralization by surrounding microorganisms, biochemical transformation, and others (Philip et al., 2010; Simard et al., 2012). Thus, a direct pathway genuinely utilizing mycorrhizal hyphae would represent a potential conduit of resource sharing, in which resources would be free of disruption by leakage and re-assimilation by other microorganisms.

In field and laboratory studies, split root designs and root restrictive screening techniques have been used to determine the different pathways in interplant transfers (Xiao et al., 2004; He et al., 2005; Meng et al., 2015; Muneer et al., 2020). These designs can effectively prevent contact between individual host plant root systems, but they do not entirely prevent bulk flow or diffusive chemical movement in the soil water. Therefore, some experimental designs rely on air gaps to avoid diffusion

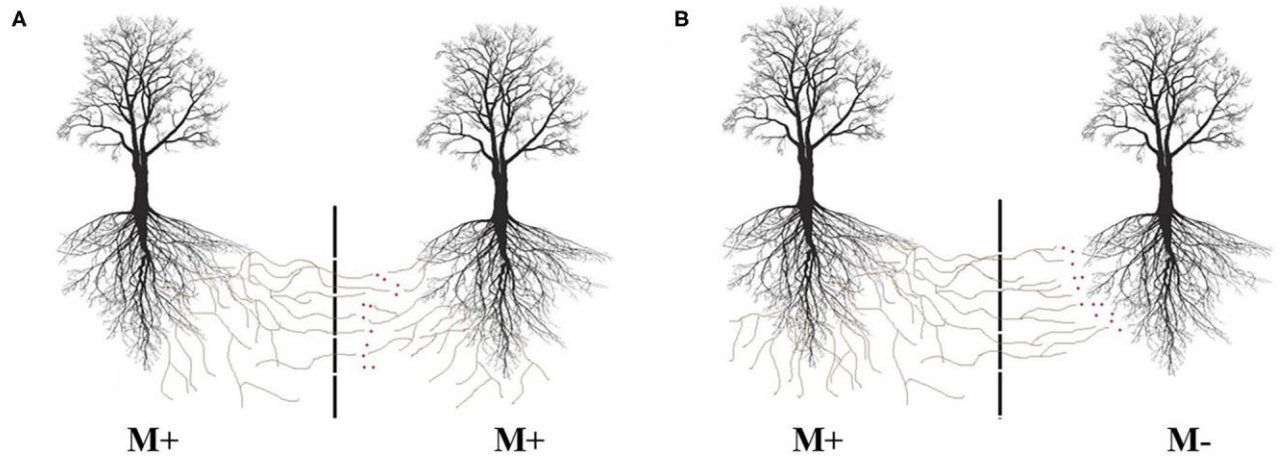


FIGURE 2 | Possible movement of resources between networked plants, even when mesh barriers are used to avoid roots intermingle and flow of soil solution between connected plants. (A) Represents disrupted hyphal connections between two mycorrhizal (M+) plants and (B) possible transfer from a mycorrhized (M+) plant to a non-mycorrhized (M−) neighbor plant.

over the soil solution flow while allowing the ingrowth of hyphae but not roots. This assures that all labeled compounds found in the receiver plant using in this system can be attributed to the mycorrhizal transport (Zhang et al., 2020; Andrino et al., 2021; Fang et al., 2021). However, these measures still do not exclude a transfer over indirect pathways, once transported resources by fungi mycelium can be released on neighboring plant compartment, leading the receiver plant to have access to resources without being connected (**Figure 2**). Moreover, connections among plants can hardly be directly visualized in soils of traditional pot experiments or even under field conditions.

Therefore, due to the technical difficulties to distinguish between transport pathways, it still remains unknown whether transfer occurs preferentially via direct hyphae connections or through indirect pathways. Creation of new experiment set-ups using new technologies to improve previous experiments should be developed for a more accurate idea about resource allocation and plant physiological responses that are truly accountable to CMN. Manipulation of the genes involved in setting up symbiotic associations between plant and fungi partner may help to differentiate the fungal effect in such networks (Merrild et al., 2013; Song et al., 2014). Mutant lines where the development of arbuscules is impaired and not functional are a promising starting point, and at least for *M. truncatula* such a mutantline is already known. Arbuscules are recognized as the main site of exchange, and comparing networks formed by wild type and mutant lines might lead to e better understanding of the effects of arbuscular network on the development of donor and receiver. Unfortunately, to our knowledge there are no such impaired mutant lines for EM fungi, therefore such studies are only possible for AM networks. In addition, some plant genera such Acacia, Alnus, Eucalyptus, Fraxinus, Populus, Salix, Shorea, and Uapaca are recognized to associate with both AM and EM fungi simultaneously (Teste et al., 2020), although frequency of each fungi type might differ along plant life. Much less research have been made in dual-mycorrhizae plants, and how AM and EM networks may affect connected plants differently. Altogether, such experiments could be helpful in order to achieve deeper understanding of mechanisms and processes behind CMN and its impact on plant community.

Nevertheless, it is important to keep in mind that, evenif resources exchanges between plants takes place mainly via indirect pathways, receiver plants can still be favored by a facilitation of its access to resource coming from neighboring plants, which may anyway play a role in plant-to-plant interaction (Høgh-Jensen, 2006; Alaux et al., 2021).

## Role of Transfer for Plant Fitness

The simple movement of elements from one plant to another does not by itself indicate a net transfer able to represent an ecological advantage on plant fitness (Kytöviita et al., 2003; Bücking et al., 2016). Quantifying the contribution of each pathway to plant fitness is likewise a matter of discussion in most studies on CMN. However, quantification of nutrient and C fluxes exclusive to the fungal hyphae is difficult. To the best of our knowledge, there are only few quantitative information on the magnitude of C fluxes between plants sharing a CMN. In general, C transfer through CMN is not frequently considered a significant pathway for mobile C transfer among plants, although some authors suggest that even small amounts may be of great importance for receiver plant survival and development (Wu et al., 2001; Deslippe and Simard, 2011; Klein et al., 2016). This can be especially true if the receiver plants are seedlings (Nara, 2006; Booth and Hoeksema, 2010; Burke et al., 2018; Liang et al., 2021). Reported amounts of C vary from 0 up to 10% in literature (Teste et al., 2010; Lin et al., 2020). Simard et al. (1997b) was the first attempting to quantify a bidirectional flux of C between plants connected via EM network, in order to evaluate its ecology significance. The authors concluded that there was no net transfer between the species. However, the

study raised debate in the literature due to its difficulty in extrapolating the data from young seedlings to mature tree and the use of relevant controls (Robinson and Fitter, 1999; Simard et al., 2012; Tedersoo et al., 2020). In addition, Simard et al. (1997b) concluded that it was not possible to distinguish whether the translocation occurred through interconnecting hyphae, soil pathways, or even both simultaneously, and, hence, did not really demonstrate the contribution of the CMN for C transfer.

A more recent approach was developed by Klein et al. (2016), attempting to evaluate C transfer between trees in a mature forest. They continuously labeled five 40-m-tall Norway spruce trees (*Picea abies*) as part of a 5-year free-air $CO_2$ enrichment experiment (FACE) with $^{13}C$-depleted $CO_2$. Despite the low difference in the $\delta^{13}C$ ratios of canopy twigs, stems, and fine roots between labeled and unlabeled control (max. 2.6‰), the isotopic signal of neighboring trees belonging to same or different taxa (*Fagus sylvatica*, *Pinus sylvestris*, and *Larix decidua*) were than measured to evaluate C allocation. The authors claimed to find evidences that reciprocal C transfer indeed occurred between trees, as $\delta^{13}C$ of fine roots of neighboring plants followed the same signal from the donor *Picea*. Most of the label was found in the fine roots, which was concluded to prove the participation of the mycorrhizae in the transfer. It was estimated that C derived from transfer represents 4% of net primary productivity.

Another point usually under discussion regarding C transfer is whether transferred C is taken up by the receiver plant for its own growth or, contrastingly, whether the C is mainly kept in the roots, probably incorporated into fungal structures, therefore not representing a meaningful advantage for the receiver plant. This was evaluated, for example, by Waters and Borowicz (1994) and Fitter et al. (1998). They assumed that by clipping the aboveground parts of living plants, additional C would be required and translocated from the roots to the re-growing clipped shoots. However, in neither of the experiments labeled C was found in the re-growing shoots of the receiver plants. Thus, the authors concluded that the transferred C remained in fungal structures. The opposite was found by Song et al. (2015) who reported labeled $^{13}C$ in the shoots of the receiver plant. Another difference in the mentioned studies is that, in the experiment developed by Song et al. (2015), C transfer from donor to receiver plant increased by increasing defoliation of donor plant. This has been suggested as an effect of the sink-source strength of the connected plants. The authors concluded authors that defoliation could have stimulated interior Douglas-fir donor to rapidly export labile C from enriched roots to the CMN, while the rapid growth rate of ponderosa pine would created a large sink. Nevertheless, even if it is assumed that mycorrhizae might be able to transfer C from one plant to its neighbors, it remains unclear if the amounts of the transferred elements are of any significance to the receiver plant. If this amount is viable for the receiver plant, a process understanding of the switch between fungal storage and delivery to the plant is still required.

Equally contradictory is the magnitude of N transfer reported in the literature. In grassland ecosystems, N transfer was reported to vary from 0 to 72% under field conditions, while it is less variable in agroforestry ecosystems, ranging from 0 up to 16%, depending of the conditions under which the experiments were performed (e.g., in pots, field, etc.; Marty et al., 2009; Chapagain and Riseman, 2014; Meng et al., 2015; Zhang et al., 2020). In general, the high variability in the literature may reflect many different factors that might interfere in plant-to-plant interaction, such as differences in environmental conditions, in the different experimental setups, or plant and fungi combinations, soil nutrient supply, additional stress conditions added (e.g., nutrients deficiency, drought, shading, etc.), and the general experimental design (e.g., field, pot or microcosmos experiments). In addition, like for C, quantification of N transfer via interconnecting hyphae is not distinguishable from other pathways (Montesinos-Navarro et al., 2016; Fang et al., 2021). The distinction and relative importance of the different pathways determines the strength, direction, and outcome of interactions among plants and soil organisms, requiring new technologies and ideas to address such issues. Nevertheless, the many researches made on this topic so far developed different hypotheses that could give us some hints on how CMN would affect plant-plant interaction.

## CONCLUSIONS

Despite of the great progress in understanding the effect of mycorrhiza network for plant-to-plant interactions, and how this might affect mycorrhizal communities, there are still important questions to be answered in future researches. Resource allocation between connected plants thereby drew the largest attention of the scientific community. Many possible effects of such transfers of resources have been described, but contrasting results were frequently found. Labeled experiments using C and N isotopes have revealed that under certain conditions a movement of such resources between donor and receiver plants seem to happen, but none of them could demonstrate unequivocally that the transfer occurred preferentially through the direct mycorrhizal pathway and not over the soil solution or simply over exudates. Moreover, quantification of this transfer demonstrated to be an even bigger challenge. Therefore, the real effect of the CMN in shaping plant communities is still not clear. Further research involving new experiment set-ups and new technologies to improve previous experiments should be developed for a more accurate idea about resource allocation and plant physiological responses that are truly accountable to CMN. The use of mutant lines with manipulation of the genes involved in setting up symbiotic associations between plant and fungi partner together with labeling techniques to track resources translocation between connected plants can be used to differentiate the fungal effect in such networks. Effects exclusively to CMN for plant interactions may help us to understand plant community and ecosystem functioning.

## AUTHOR CONTRIBUTIONS

AF, JB, and GG conceived the idea about the topic reviewed in this manuscript. AF wrote the manuscript. JB and GG contributed

with corrections and comments and approved the submitted version. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

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## 2.2. Higher N uptake in dual-mycorrhizal plants (AM and EM) in comparison to single-type mycorrhizal plants (AM or EM) and possible nutrient sharing via common mycorrhizal networks

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**Abstract**

Most terrestrial plants establish symbiotic associations with mycorrhizal fungi to accesslimiting plant nutrients such as nitrogen (N). Some plant species may establish ectomycorrhiza(EM) – arbuscular mycorrhiza (AM) dual-mycorrhizal associations. Furthermore, EM and AM associations may interconnect plants via a common mycorrhizal network (CMN) for resource exchange. However, studies have not provided univocal evidence concerning the advantage of exploiting a dual association for N acquisition to date. Likewise, dual-mycorrhizal plants have never been tested to exploit possible preferential N sharing between EM and AM CMN simultaneously. We aimed to evaluate N nutrition benefits in *P. x canescens* associated with single EM or AM vs. dual associations by evaluating the potential of the dually associated plant. Here, a dual-mycorrhizal plant acted as an N donor over a CMN with receiver plants bearing single EM or AM associations. In addition, preferential C allocation from the dual-mycorrhizal plant to a particular fungal partner was assessed. These experiments were performed by developing a mesocosm system to simultaneously observe single- and dual-mycorrhizal plants regarding N nutrition and sharing of N via CMNs in the latter. Despite the $^{15}N$ absorption and translocation to leaves and stems of labeled dual-mycorrhizal plants, N was neither allocated to roots nor transferred through a CMN to neighboring plants. Similarly, the applied $^{13}C$ label remained mainly in the labeled leaf and was rarely translocated to other plant tissues. The lack of $^{13}C$ and $^{15}N$ allocation coincided with the smallest root/shoot ratio of the labeled dual-mycorrhizal plant, which might indicate a higher investment in the development of

aboveground tissues. These findings could be a consequence of the active growth of the seedling or a reflection of improved plant nutrition in dual-colonized plants. We observed higher N contents in plants bearing dual associations, pointing toward a nutritional advantage of the host plants.

*Keywords*: Mycorrhizal network, nitrogen, transfer, nutrition, dual-mycorrhizal plants, isotopes.

**Introduction**

Nitrogen (N) is an essential element for plant growth and one of the major nutrients affecting soil fertility (Courty et al., 2015). Plants are known to take up N from inorganic and organic sources, such as ammonium ($NH_4^+$), nitrate ($NO_3^-$), amino acids and oligopeptides (Ganeteg et al., 2017; Liu et al., 2017). In Northern Hemisphere forests, such as boreal and temperate forests, N availability potentially limits plant growth (Bednarek & Tkaczyk, 2008; Zhou et al., 2019). Plants living in N-poor environments have evolved the development of symbiotic relationships with soil-borne microorganisms, such as mycorrhizal fungi. Plants establishing mycorrhizal associations exchange their photoassimilates for nutrients acquired from the soil by mycorrhizal fungi (Smith & Read, 2010). Among the different existing types of mycorrhizas, the two most widespread are ectomycorrhizas (EM) and arbuscular mycorrhizas (AM), which differ in structure and function (Phillips et al. 2013, Fisher et al. 2016). Although AMs have been more frequently reported to be important for improving plant P acquisition, both fungal types are known to improve plant N nutrition (Van der Heidjen et al., 2015; Jansa et al., 2019). However, AM and EM may represent distinct benefits for host plants. At a given plant, EM may contribute up to 80% of its assimilated N, while AM contributes only 20% (Makarov, 2019). Goodale (2017) estimated that the N uptake of EM tree species may exceed that of AM tree species by 50% in temperate forests. Benefits can vary considerably depending on biotic and abiotic factors such as the species of mycorrhiza involved in the association, the physiological state of the symbionts and soil conditions (including N availability) (Martin, 2016; Makarov, 2019).

Although the majority of plants associate with single mycorrhizal types, certain plant species simultaneously establish dual associations with arbuscular and ectomycorrhizal fungi, either within the same root system or at different life stages (e.g., *Eucalyptus*, *Alnus*, *Populus*, *Salix*, *Quercus*, *Pseudotsuga*, *Melaleuca*, *Casuarina*, *Uapaca*, *Abies*, *Tsuga*) (Smith & Read 2010; Teste, 2020). Nutritional benefits have been frequently reported for plants hosting a

single mycorrhizal type, but much less is known about the benefits of hosting both types of mycorrhizal fungi. Teste et al. (2020) reviewed the possible benefits for a plant to host dual associations, proposing nutritional benefits for plants bearing dual associations due to their access to a broader pool of nutrient sources. Other advantages suggested by the authors come from nonnutritional benefits, such as a better ability to cope with drought events. The few studies dealing with dual inoculations vs. single inoculations range from negative (Kariman et al., 2012) and neutral (Tapwal et al., 2015) to positive (Chen et al., 2000) responses.

In addition to direct N uptake from soil, in both types of mycorrhizal associations, the formation of common mycelial networks (CMNs) has been identified, which is able to interconnect plants to exchange nutrient resources such as N (Teste et al., 2015; Wipf et al., 2019) or to allow interplant communication, in which resources would be free of potential disruption by other soil microorganisms (Simard et al. 2012, 2015, 2018). In CMN, both fungal types associate with several plants of the same or different species simultaneously (Heaton et al., 2012; Rhodes, 2017; Wipf et al., 2019). The functioning of the CMNs was investigated through isotopic labeling techniques using $^{14}C$, $^{13}C$ and $^{15}N$ as tracers to track the allocation of compounds between connected plants. Movements of labeled compounds from one plant to the other have been observed for both fungal types (Philip et al., 2010; Teste et al., 2015; He et al., 2019; Fang et al., 2021). Based on the wide range of results found in the literature regarding the directions of nutrients transferred between plants, CMNs have been described to behave either as “socialist” or “capitalist” systems or even as a “superorganism” (Van der Heidjen & Horton, 2009). For “socialist” behavior, individuals are able to have equal opportunities, and resources are distributed more evenly, providing benefits for all connected plants. For the “capitalist” network, mycorrhizae would be able to recognize the most suitable partners and benefit them accordingly, increasing competition between connected plants. If the network behaves as a “superorganism”, fungal species in the network are considered redundant physicalextensions of the roots, which might translocate nutrients freely between plants. The mode of interplant connection might have evolutionary consequences for CMN by substantially defining the community ecology of a site, leading to ecosystem-wide impacts (Gorzelak et al., 2015). This phenomenon depends largely on which of these responses are predominant (“socialist”, “capitalist” or simple physical extensions) at the moment plants are connected, together with the question of whether these responses may change if plants from the same or from different species are connected.

Nevertheless, the possibility of a fast and direct pathway of N transfer between plants has received attention from the scientific community. In this context, due to intrinsic differences

between the structure and functioning of each fungus, the network formed either by AM or EM might affect connected plants differently, and some plant species might benefit from mycorrhizal networks more than others, depending on the fungi and plants involved in the association. EM, for example, forms a thick mantle around root tips from which its extensive mycelium extends beyond the root zone and turns over more slowly relative to AM hyphae (Anderson & Cairney, 2007). In addition, mycelial proliferation of EM fungi is more extensive than that of AM fungi (Ekblad et al., 2013), which might lead EM fungi to transport resources through CMN more efficiently than AM fungi to benefit neighboring plants sharing the EM network (Smith & Read, 2010; Teste et al., 2015). However, those conclusions were made mainly based on studies evaluating resource transfer via CMN on AM and EM plants separately but never simultaneously.

Thus, the present work addresses three main objectives: (A) to evaluate N nutrition benefits in plants associated with single EM or AM vs. dual associations; (B) to evaluate the potential of a dually associated plant as an N donor via a CMN with receiver plants bearing single EM or AM associations; and (C) to evaluate any preferential C allocation from the dual-mycorrhizal plant to a particular fungal partner. For this purpose, we designed a novel multichamber mesocosm in which a central split-rooted donor, able to associate with both AM and EM simultaneously, shares an AM or EM network with one neighboring plant simultaneously. Since only donor dual mycorrhizal plants have access to both fungal types, we could access different N nutrition of single colonized neighbors compared with central dual mycorrhizal plants. By applying $^{15}N$-labeled solution to central dual mycorrhizal plants, we could track the preferential N allocation via the AM vs. EM network. We hypothesized that host plants establishing dual mycorrhiza associations would exhibit enhanced N nutrition compared with those depending on single associations. We further hypothesized that dual mycorrhizal plants would preferentially deliver more N to plants sharing an EM association due to their larger mycelium proliferation. Likewise, we also hypothesized that such mycelial proliferation might act as a sink for C, requiring higher C allocation from the host plant.

## Material and Methods

### Multichamber Mesocosms

A three-compartment system was designed (Figure 1), where the central compartment was subdivided by a solid wall into two subcompartments that held the split root system of the central dual-mycorrhizal plant. The two outer compartments were separated by a “sandwich” of two layers of a 20-µm pore nylon mesh membrane (Franz Eckert GmbH, Waldkirch,

Germany) and a 5-10-µm-pore-size PTFE hydrophobic membrane (Pieper Filter GmbH, Bad Zwischenahn, Germany) in between each section to avoid root and solute crossing (Andrino et al., 2019, 2021). The front part of the rhizoboxes was made of transparent acrylic glass to follow fungal and root development without disturbing the system. The whole system was sealed with a polymer bond (PROBAU Polymer Bon + Seal Plus, Bauhaus Mannheim, Germany) and tested for leakage before the start of each experiment.

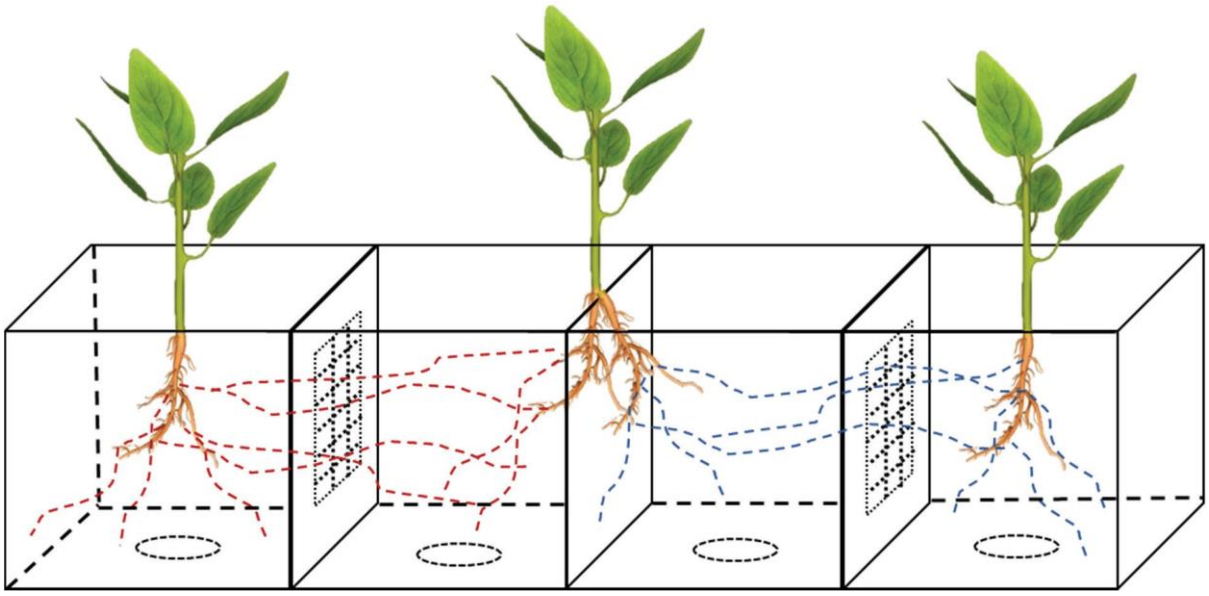

Figure 1: Scheme of the multichamber mesocosm, containing outer compartments with open walls where the "sandwich" membranes were attached and a central solid wall to avoid any roots contact or flux between the two compartments on the left and right side. Central slip roots plant is colonized by both AM and EM fungi coming from the neighbouring plants.

**Plant material and fungal inoculum**

*Populus* × *canescens* (gray poplar), a hybrid between Populus alba (white poplar) and Populus tremula (common aspen) (female clone INRA #717-1B4), recognized as a dual-mycorrhizal plant, was multiplied by micropropagation and, after rooting, transferred to pots filled with autoclaved perlite and acclimatized in a climate chamber under controlled conditions. After acclimatization, seedlings with a similar size were transplanted into the custom-made dual-compartment system. For the inoculum, one AM (*Rhizophagus irregularis* - DAOM 197198) and one EM strain (*Paxillus involutus*- Strain MAJ) were selected and grown under sterile conditions.

To inoculate *P. x canescens* with AM fungi, 2 ml of in vitro spores (total of 8000 spores) suspended in sterile distilled water (Premier Tech; Quebec, Canada) were pippeted in the vicinity of the *P. x canescens* roots, 1 ml at the time of plant transplantation and another 1 ml

one month later. The EM fungus was applied to the plants in the form of a carrier. The carrier was prepared according to Mortier et al. (1988) and Brundrett et al. (1996). In brief, 1 l Microbox containers with ventilated lids (Sac $O_2$, Deinze, Belgium) containing washed and autoclaved perlite were filled to field capacity with liquid modified Melin-Norkrans medium (MMN) (pH 5,5) containing half of the C content of the original recipe and added in the form of glucose. The inoculation of the perlite bottles containing the MMN media was performed on a clean bench by placing one agar plug taken from a pure culture of P. involutus (ATCC®; Wesel, Germany) on top of the Perlite and incubating it at 25 °C in the dark until the substrate was fully colonized. After approximately 2 months, the carrier substrate was fully colonized, and the inoculum was mixed with the substrate at a mass ratio of 1:10. For the nonmycorrhizal controls, substrate containing the inoculum was autoclaved twice to produce the mock (mycorrhiza-free inoculum).

**Experimental set-up**

The outer compartments were destined for plants that were inoculated with a pure culture of either AM (named Pop+AM) or EM (named Pop+EM) fungi. The central compartment was designated for the split root plant, in which half of its roots were placed on each side of the central solid wall. Both compartments containing the split roots were filled with the mock (autoclaved substrate). Each compartment provided a volume of 500 g of substrate. The main idea was that the hyphae of the fungi inoculated in the plants of the outer compartments grew through the mesh and colonized its neighboring portion of the split roots, forming an CMN. Therefore, one portion of the split roots of the central plant was expected to be colonized by AM fungi (PopNM(AM) Dual) prevenient of its neighboring AM plant, while the other portion was expected to be colonized by EM fungi (PopNM(EM) Dual) of its EM neighbor. This experimental setup allowed us to answer both main questions of our work regarding N nutrition and C and N allocation via CMN.

Plants were fertilized every other day with a Long Ashton (LA) low P solution (Hewitt et al., 1966). For the central plant, 50 ml was divided into two equal portions of 25 ml, which were applied to each compartment containing the half root system of the split root plant. The outer plants each received the full 50 ml portion. By dividing the amounts of fertilization in the split root chambers, AM and EM fungi colonizing roots of the central plant had relatively less N available for uptake, which allowed us to evaluate the performance of the central plant when acquiring N by dual mycorrhizal plants.

The experiment was performed for 6 months, which provided sufficient time to observe the effects of the different fungal types on N uptake and plant responses. After 6 months, dual $^{13}C$ and $^{15}N$ pulse labeling was applied to the central plant to investigate N and C allocation via CMN. The central split root plant was labeled and therefore considered the "donor" plant. The nonlabeled plants in the outer compartments were considered "receivers" plants. One week prior to labeling, the LA low P solution was replaced with a modified Low Nutrient (LN) solution (Langenfelder et al., 2007) in which nitrogen sources were excluded to avoid N dilution of the labeled N. This same solution was used for fertilization of the plants after labeling until harvest. Dual labeling of $^{15}N$ and $^{13}C$ was performed by the leaf feeding technique, adapted from Khan et al. (2002). Briefly, one young branch of each plant was immersed in a 2-ml Eppendorf™ flask containing 1.5 ml of an enriched urea solution. This solution was prepared by diluting 100 mg of $^{13}C^{15}N$-urea (99% atoms $^{13}C$ and 98% atoms $^{15}N$) (Merck, Darmstadt, Germany) in 50 ml of deionized water. The vials attached to the branches were sealed using Parafilm™ to avoid evaporation of the solution. The solution was taken up within 2-3 days by the plants. Plants were harvested 15 and 21 days after labeling. Control plants were labeled with nonlabeled urea following the same procedure and used as background for the analyses.

Plants were harvested 15 and 21 days after pulse labeling. At each harvest point, the plants were divided into leaves, stems, and roots. The roots were carefully washed to remove all adhering substrate and divided into two aliquots. A fresh subsample was stored in 70% ethanol for later staining and evaluation of the mycorrhization rate, while the second portion was rapidly frozen and stored. Prior to analysis, this frozen aliquot was freeze dried and milled. For all parts, fresh weight (FW) and dry weight (DW) were measured and used for further calculations. Labeled leaves directly immersed in the labeling solution were processed separately from the rest of the plant material. The substrate of each compartment was dried at 45 °C, ground to a fine powder and sieved < 1 mm.

**Element analyses**

The C and N contents and $\delta^{13}C$ and $\delta^{15}N$ isotopes in the plant and substrate samples were measured with an isotope cube elemental analyzer (Elementar GmbH, Hanau, Germany) coupled to an Isoprime 100 isotope ratio mass spectrometer (IRMS) (Elementar GmbH, Hanau, Germany) via a continuous flow inlet using helium (99.999% purity; Linde, Munich, Germany) as the carrier gas. The $\delta^{13}C$ values were corrected using Vienna PeeDee Belemnite (PDB) (0.011182) as an internal standard and $\delta^{15}N$ corrected using $N_2$ (0.0036764) as an internal

standard. The values were converted to atom% of $^{15}N$ and $^{13}C$ for further calculations. Transformation was performed as follows:

$$\text{atom \%} = (100 * AR * (\delta \text{ sample}/1000 + 1))/(1 + (AR * (\delta \text{ sample}/1000 + 1))) \quad (1)$$

where AR is the absolute ratio of the respective international standard (N2 or PDB), and δ sample is the value in permil (δ‰) measured by the IRMS for conversion into atom %. Atom % excess is then calculated by subtracting the background values from the samples.

To determine the nutritional composition of plant tissues, approximately 50 mg of freeze-dried and ground plant material was incinerated overnight at 480 °C for 6 h. The ashes were dissolved in 1 ml of 6 M HCl with 1.5% (w/v) hydroxylammonium chloride, and the extracted solution was diluted (1:10 v/v) with double demineralized water, filtered and measured the determine the Al, B, Ca, Cr, Co, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, Si, Sn and Zn concentrations. Measurements in all solutions were conducted by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Agilent Technologies Ireland Ltd., Cork, Ireland). The standard solutions were prepared from single element solutions of 1000 $mg \cdot L^{-1}$ (Fisher Scientific, Loughborough, UK). Here, we report only the most relevant macronutrients for plant nutrition, i.e., P, K, Ca, and Mg.

**Mycorrhization rate**

WGA-Alexa Fluor™ 488 conjugate (Molecular Probes, Eugene, OR, USA) was used according to Ramonell et al. (2005) to stain the chitin present in the fungal cell wall of AM-infected roots. Roots were incubated in 10% (w/v) KOH at 95 °C for 15 min, bleached with 0.5% $H_2O_2$ for 20 minutes, rinsed with water and incubated in 1x PBS (phosphate-buffered saline) buffer (0.14 M NaCl, 2.7 mM KCl, 1 mM $Na_2HPO_4 \times 2H_2O$, 1.8 mM $KH_2PO_4$; pH 7.3) containing 20 $\mu g.ml^{-1}$ Alexa WGA Fluor™ 488 (Thermo Fisher Scientific, Langenselbold, Germany) conjugate overnight. Visualization and photo documentation of the stained roots were performed using a Leica MZ10 F stereomicroscope (Leica Microsystems, Wetzlar, Germany) with a beam path specifically for fluorescent illumination equipped with an Olympus XC50 camera (Olympus, Hamburg, Germany) and a Zeiss Axio Observer Z1 microscope equipped with an AxioCam ICc1 (Carl Zeiss AG, Oberkochen, Germany). Fluorescence was excited with an argon laser at 488 nm and detected at wavelengths of 500–520 nm [WGA-Alexa Fluor™ 488 and green fluorescent protein (GFP)]. To calculate the mycorrhization rate of AM-colonized roots, the magnified intersection method of McGonigle et al. (1990) was used, with some modifications. In this procedure, 10-12 stained root fragments of ca. 1 cm were arranged on glass slides in parallel, covered with glass cover slips and examined under 8.0 x

magnification to account for the presence of hyphae (H), vesicles (V) and arbuscules (A). The mycorrhization rate was examined and quantified in at least three slides per plant, leading to over 300 views per sample. The frequency of occurrence of mycorrhizae was calculated according to Trouvelot et al. (1986) using the following formula:

$$F\%=100* n/N \quad (2)$$

where N is the total number of observed visual fields, and n is the number of visual fields containing mycorrhizae. The frequency of occurrence of arbuscules, vesicles and hyphae was calculated using the same formula.

For quantification of colonization in EM roots, the gridline intersection method was used (Brundrett et al., 1996). Briefly, unstained root segments were placed in petri plates, and the same Leica MZ10 F stereomicroscope (Leica Microsystems, Wetzlar, Germany) with 8.0 x magnification was used to account for the presence of mantle involving root tips. Mycorrhization was determined based on the percentage of colonized root length (RLC %), and at least 500 root tips per sample were counted.

**Mycorrhizal growth response (MGR) and root/shoot ratio**

The MGR represents the effect size of the AM and EM inoculation on dry plant biomass, and it was calculated according to (Hoeksema et al., 2010) as follows:

$$MGR = \log_e[Xi/Xn], \quad (3)$$

where Xi is the inoculated biomass, and Xn is the noninoculated biomass. For the noninoculated biomass, nonmycorrhizal controls containing the mock substrate were used.

Positive MGR values indicated that plant biomass increased in response to inoculation, whereas negative values indicated that plant biomass decreased in response to inoculation.

The root/root ratios were obtained by dividing the dry root biomass by the dry shoot biomass, with shoots being the sum of leaves and stem biomass.

**Data analysis**

All variables were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene tests, respectively. One-way ANOVA and Fisher's LSD tests were employed to test for differences in the mean values ($P < 0.05$) of measured variables between the treatment groups. Data were analyzed using XLSTAT software (ver. 2010, Addinsoft, New York, USA).

## Results

### Mycorrhization rate

All plant roots were successfully colonized by AM fungi. Comparing both harvesting points, no differences were found regarding hyphal frequency (HF%) and vesicular frequency (VF%). However, some variations could be observed for arbuscular frequency (AF%) at the second time point, in which a single mycorrhizal plant (Pop+AM) had significantly more AF% than its neighboring dual-mycorrhizal plant roots (PopNM(AM) Dual) (Table 1). Nonmycorrhizal control plants were found to be unmycorrhized.

Table 1: Hyphae (HF%), arbuscules (AF%) and vesicles (VF%) frequency of AM roots of single and dual-mycorrhizal plant roots, harvested 15 and 21 days after labeling. Comparison were made between dual and single AM plants for HF%, AF% and VF% separately for both time points. Different letters indicate statistically significance difference between values (n=4).

| | Harvest | HF% | AF% | VF% |
|---|---|---|---|---|
| PopNM(AM) Dual | 15 | 98.46 $^{a}$ | 4.90 $^{ab}$ | 0.94 $^{a}$ |
| Pop+AM | | 98.52 $^{a}$ | 5.26 $^{ab}$ | 1.03 $^{a}$ |
| PopNM(AM) Dual | 21 | 98.73 $^{a}$ | 3.01 $^{b}$ | 1.20 $^{a}$ |
| Pop+AM | | 99.18 $^{a}$ | 9.77 $^{a}$ | 2.10 $^{a}$ |

Likewise, EM roots were successfully associated with the roots of all plants in this treatment. For the EM roots, no significant differences were observed between the root length colonization (RLC%) of dual and single plants, irrespective of the time point of harvest (Table 2). The nonmycorrhizal control plants had no EM mycorrhizae in their roots, showing that they were free of any cross contamination.

Table 2: Root length colonization (RLC%) of EM roots of dual (PopNM(EM) Dual ) and single EM (Pop+EM) colonized plant roots, harvested 15 and 21 days after labeling. Comparison were made between dual and single EM plants for both time points. Different letters indicate statistically significance difference between values (n=4).

| | **Harvest** | **% RLC** |
|---|---|---|
| PopNM(EM) Dual | 15 | 51.67 [a] |
| Pop+EM | | 45.88 [a] |
| PopNM(EM) Dual | 21 | 41.76 [a] |
| Pop+EM | | 51.66 [a] |

In AM roots, arbuscules were formed, indicating an active exchange between plants and fungi (Fig. 2 a-b). Likewise, for roots colonized by EM, well-developed Hartig nets were visible between the root epidermis cells (Fig. e-f), indicating a functional association between the host plant and fungal partners.

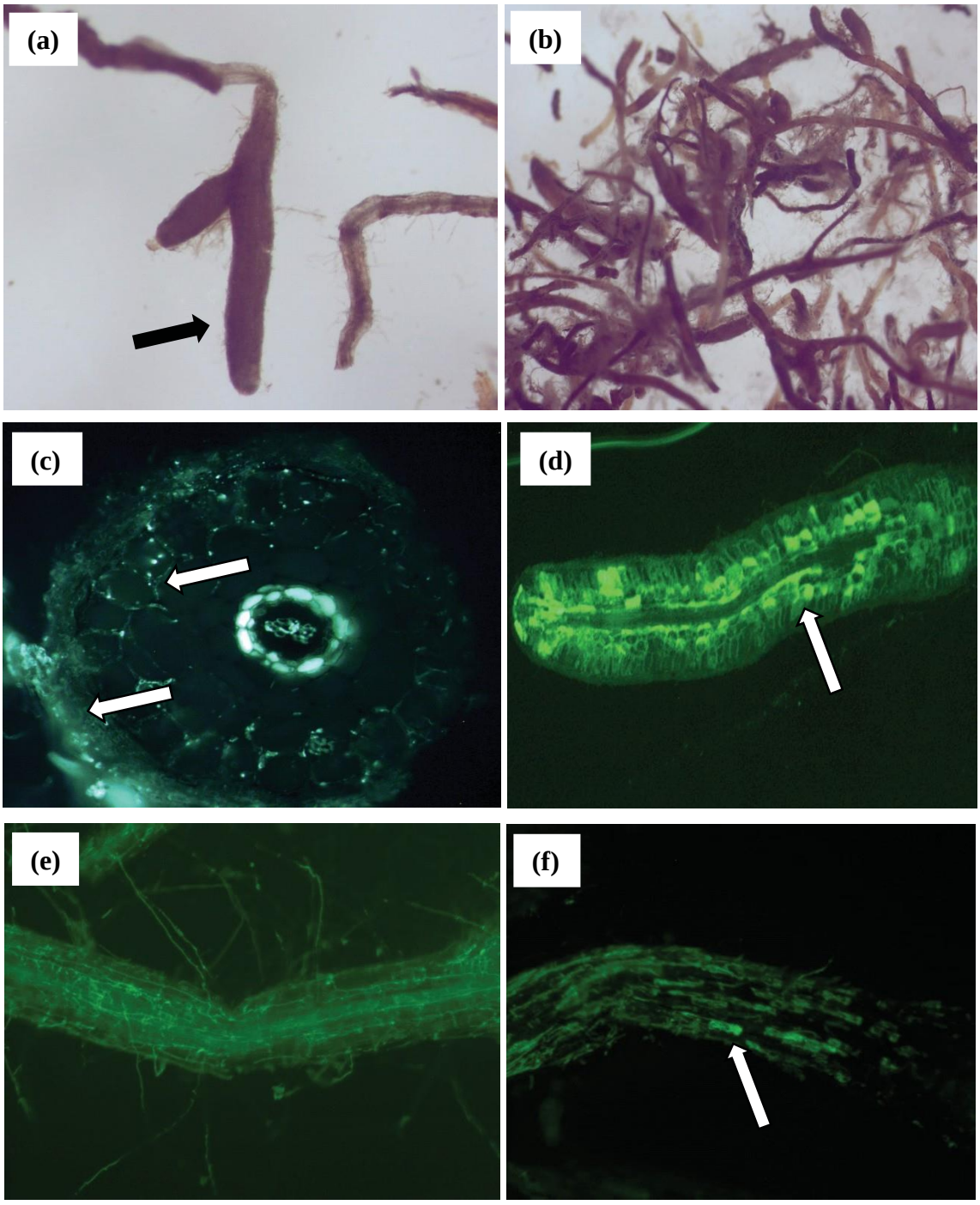


Figure 2: (a) WGA Alexa stained AM roots covered by hyphae, (b) arbuscules from stained WT *Medicago truncatula* containing fully developed arbuscules, (c) non-stained EM roots tips, (d) overview of non-stained EM roots sample, (e) vertical and (f) horizontal thin-cuts of root of 60 µm thickness, stained with WGA Alexa for the visualization of the Hartig net. Pictues (a) to (d) were taken using a Leica MZ10 F stereomicroscope (Leica Microsystems, Wetzlar, Germany) with magnification 8.0 x. Pictures (e) and (f) were made with a Zeiss Axio Observer Z1 microscope equipped with an AxioCam ICc1 (Carl Zeiss AG, Oberkochen, Germany).

**N uptake and plant response to dual vs. single colonized plants**

**Mycorrhizal Growth response (MGR) and Roots/Shoots ratio**

In general, all treatments had a positive MGR in comparison to noninoculated plants, as demonstrated by the positive values calculated. Dual mycorrhizal plants harvested 15 days after labeling showed the largest MGR effect compared with all the other treatments. The second largest MGR effect was observed in the neighboring EM plants (Table 3). Smaller values were obtained from the single colonized AM plants, especially for the second harvest made after 21 days, which had a significantly lower MGR.

Single AM-colonized plants had the largest root/shoot ratio, followed by single EM plants. The smallest values were found in dual mycorrhizal plants.

Table 3: MGR effect of central dual mycorrhizal and single AM and EM neighbours, harvested 15 and 21 days after labeling. Comparison were made for MGR and roots/shoots ratio separately, for all treatments. Different letters indicate statistically significance difference between values. (n=6).

| Treatment | Harvest | MGR | Roots/Shoots |
|---|---|---|---|
| PopNM(EM) Dual | 15 | 1.21 $^{a}$ | 0.55 $^{de}$ |
| PopNM(AM) Dual | | 0.96 $^{b}$ | 0.29 $^{e}$ |
| Pop+EM | | 1.00 $^{ab}$ | 0.93 $^{bcd}$ |
| Pop+AM | | 0.90 $^{bc}$ | 1.31 $^{b}$ |
| PopNM(EM) Dual | 21 | 0.99 $^{ab}$ | 0.58 $^{cde}$ |
| PopNM(AM) Dual | | 0.93 $^{bc}$ | 0.69 $^{bcd}$ |
| Pop+EM | | 0.89 $^{bc}$ | 1.22 $^{bc}$ |
| Pop+AM | | 0.67 $^{c}$ | 2.34 $^{a}$ |

**Nutritional composition**

No significant differences were observed in the contents of Ca, K, Mg and P in the leaves and stems of dual and single mycorrhizal plants at the first harvest date (Table 4). However, at the second harvest date, some significant differences were observed. While dual mycorrhizal plants had intermediate Ca contents, which did not differ from single colonized plants, AM plants had significantly more Ca in their leaves than EM plants. The same pattern was observed in the stem. Likewise, the K content in the leaves of dual mycorrhizal plants had intermediate values and did not differ from the single colonized AM and EM neighbors. However, EM single-colonized plants had significantly higher K contents in their leaves than AM-colonized

plants. Again, the same pattern was observed for the stem. Leaves and stems of dual mycorrhizal plants did not differ in Mg content compared with single AM plants, but both contained more Mg compared with single EM plants. In contrast, the P content in the leaves of dual mycorrhizal plants did not differ from that of single EM plants, but both had significantly more P in their leaves than single AM plants. This feature was not observed in the stem, in which no significant differences were observed between any of the plants.

In the roots, Ca did not differ between the plants in the first harvest. In the second harvest, however, larger Ca contents were found in the roots of dual mycorrhizal plants, followed by single AM, and the smallest values were found in single EM roots. AM and EM single plants did not differ. Single EM roots also had the smallest K content in the roots at both time points, while dual and single AM had the largest values. Likewise, a single EM plant had the smallest Mg content in the roots but at the second harvest point. For P, significant differences were observed only at the second harvest, at which dual mycorrhizal plants had intermediate P contents, while single AM had the largest and single EM the smallest.

Table 4: Ca, K, Mg and P in leaves, stem and roots of dual mycorrhizal plants (PopNM), single AM plants (Pop+AM) and single EM plants (Pop+EM), harvested 15 and 21 days after labeling. Comparisons between each nutrient were made for the different tissues at both time points. Different letters indicate statistically significance difference between values (n=6).

| Harvest | Sample | *Ca* | *K* | *Mg* | *P* |
|---|---|---|---|---|---|
| | | *mg.g$^{-1}$ dry weight* | | | |
| 15 | PopNM Leaves | 10.97 $^{b}$ | 6.06 $^{a}$ | 2.66 $^{bc}$ | 1.13 $^{a}$ |
| | PopNM Stem | 6.72 $^{b}$ | 3.63 $^{ab}$ | 0.81 $^{bc}$ | 0.59 $^{ab}$ |
| | PopNM(AM) Roots | 9.38 $^{ab}$ | 3.08 $^{a}$ | 0.75 $^{bc}$ | 0.55 $^{bc}$ |
| | PopNM(EM) Roots | 9.17 $^{ab}$ | 2.34 $^{bc}$ | 0.67 $^{c}$ | 0.52 $^{bc}$ |
| | Pop+AM Leaves | 12.60 $^{b}$ | 5.27 $^{ab}$ | 2.64 $^{bc}$ | 0.97 $^{ab}$ |
| | Pop+AM Stem | 6.50 $^{b}$ | 3.79 $^{ab}$ | 0.95 $^{bc}$ | 0.60 $^{ab}$ |
| | Pop+AM Roots | 9.45 $^{ab}$ | 2.22 $^{bc}$ | 0.70 $^{c}$ | 0.45 $^{c}$ |
| | Pop+EM Leaves | 10.85 $^{b}$ | 5.57 $^{ab}$ | 2.06 $^{c}$ | 1.08 $^{ab}$ |
| | Pop+EM Stem | 6.39 $^{b}$ | 3.56 $^{ab}$ | 0.60 $^{c}$ | 0.52 $^{b}$ |
| | Pop+EM Roots | 8.10 $^{b}$ | 0.96 $^{d}$ | 0.60 $^{c}$ | 0.54 $^{c}$ |
| 21 | PopNM Leaves | 18.11 $^{ab}$ | 5.29 $^{ab}$ | 4.04 $^{a}$ | 1.15 $^{a}$ |
| | PopNM Stem | 7.37 $^{ab}$ | 3.86 $^{ab}$ | 1.24 $^{ab}$ | 0.74 $^{ab}$ |
| | PopNM(AM) Roots | 11.62 $^{a}$ | 2.64 $^{ab}$ | 1.15 $^{a}$ | 0.69 $^{abc}$ |
| | PopNM(EM) Roots | 11.31 $^{a}$ | 2.92 $^{a}$ | 1.12 $^{ab}$ | 0.80 $^{ab}$ |
| | Pop+AM Leaves | 20.58 $^{a}$ | 4.41 $^{b}$ | 3.87 $^{ab}$ | 0.69 $^{b}$ |
| | Pop+AM Stem | 8.58 $^{a}$ | 2.97 $^{b}$ | 1.51 $^{a}$ | 0.84 $^{a}$ |
| | Pop+AM Roots | 9.58 $^{ab}$ | 2.04 $^{c}$ | 1.15 $^{a}$ | 1.01 $^{a}$ |
| | Pop+EM Leaves | 10.80 $^{b}$ | 5.71 $^{a}$ | 2.10 $^{c}$ | 1.30 $^{a}$ |
| | Pop+EM Stem | 7.04 $^{b}$ | 4.41 $^{a}$ | 0.67 $^{c}$ | 0.65 $^{ab}$ |
| | Pop+EM Roots | 8.44 $^{b}$ | 0.97 $^{d}$ | 0.49 $^{c}$ | 0.55 $^{bc}$ |

**C and N content**

Nitrogen contents in leaves of dual mycorrhizal plants were significantly larger than single mycorrhizal plants at both harvesting points (Table 5), while content in leaves of single AM and EM did not differ between each other. The nitrogen contents of the stem showed no significant differences within treatments.

Dual mycorrhizal plants had the largest C content in the leaves, followed by single EM plants and single AM plants. Differences between C contents in dual and single mycorrhizal

plants were even more pronounced in the second harvest, in which dual mycorrhizal plants had significantly larger C contents than both single colonized plants. Additionally, the stems of dual mycorrhizal plants had the largest C values at the first harvest, followed by the stems of single EM plants and those of the single AM plants with the lowest C contents. However, the pattern was different in the second harvest, in which EM had the highest stem C contents, followed by those in dual mycorrhizal pants. Single AM plants also had the smallest C contents at the second harvest.

Nitrogen content in roots did not follow a pattern. At the first harvest, EM roots of dual colonized plant had the largest N content while the AM plants had the smallest, and EM plants were in between. In contrast, in the second harvest, there was no difference between the N contents of EM and AM roots of the dual colonized plant, while the largest value was observed in the single AM plant. At both harvest points, single AM and EM plants had significantly larger C in their roots than dual mycorrhizal plants.

Table 5: C and N contents in leaves, stems and roots of dual mycorrhizal plants (PopNM), and single AM (Pop+AM) and EM plants (Pop+EM), harvested 15 and 21 days after labeling. Comparisons between C and N were made for the different tissues at both time points. Different letters indicate statistically significance difference between values (n=6).

| Harvest | Samples | *C* | *N* |
|---|---|---|---|
| | | *mg g$^{-1}$ dry weight* | |
| 15 | PopNM Leaves | 3359.26 $^{a}$ | 117.70 $^{a}$ |
| | PopNM Stem | 3290.49 $^{a}$ | 82.20 $^{a}$ |
| | PopNM(AM) Roots | 1696.99 $^{c}$ | 33.57 $^{c}$ |
| | PopNM(EM) Roots | 3109.48 $^{b}$ | 114.36 $^{b}$ |
| | Pop+AM Leaves | 2214.22 $^{bcd}$ | 78.75 $^{c}$ |
| | Pop+AM Stem | 1953.10 $^{bc}$ | 32.00 $^{b}$ |
| | Pop+AM Roots | 4404.98 $^{a}$ | 103.13 $^{b}$ |
| | Pop+EM Leaves | 2536.09 $^{abc}$ | 82.00 $^{bc}$ |
| | Pop+EM Stem | 2776.29 $^{ab}$ | 32.07 $^{b}$ |
| | Pop+EM Roots | 4333.38 $^{a}$ | 74.90 $^{bc}$ |
| 21 | PopNM Leaves | 2769.40 $^{ab}$ | 115.54 $^{ab}$ |
| | PopNM Stem | 2228.32 $^{bc}$ | 57.88 $^{ab}$ |
| | PopNM(AM) Roots | 2690.40 $^{bc}$ | 93.07 $^{b}$ |
| | PopNM(EM) Roots | 2409.18 $^{bc}$ | 66.10 $^{bc}$ |
| | Pop+AM Leaves | 1383.95 $^{d}$ | 48.71 $^{c}$ |
| | Pop+AM Stem | 1208.21 $^{c}$ | 33.15 $^{b}$ |
| | Pop+AM Roots | 4891.26 $^{a}$ | 173.25 $^{a}$ |
| | Pop+EM Leaves | 1646.56 $^{cd}$ | 60.81 $^{c}$ |
| | Pop+EM Stem | 2591.30 $^{ab}$ | 34.77 $^{b}$ |
| | Pop+EM Roots | 4855.88 $^{a}$ | 82.24 $^{bc}$ |

**C and N transfer via CMN**

Most of the $^{15}N$ applied to the donor dual mycorrhizal plant accumulated in the labeled leaf, reaching values up to 5.90 atom% $^{15}N$ excess (Figure 3). A significant portion of the $^{15}N$ was translocated to other leaves and the stem of the donor after 15 days, leading those tissues to become significantly enriched compared with the background. Leaves and stems reached values of 1.10 and 0.79 atom% $^{15}N$ excess, respectively. No significant enrichment was observed in the roots of donor plants, regardless of the mycorrhizal partner associated with the

roots or time of harvest. Consequently, no significant enrichment was found in any of the tissues of receiver plants, irrespective of the mycorrhizal type shared by the labeled donor plant. Additionally, the analyzed substrate of both donor and receiver plant compartments also did not show an enrichment for $^{15}N$. The applied $^{13}C$ was retained only in the labeled leaves and was not translocated to other tissues of the donor plant and likewise not to the receiver plant (data not shown).

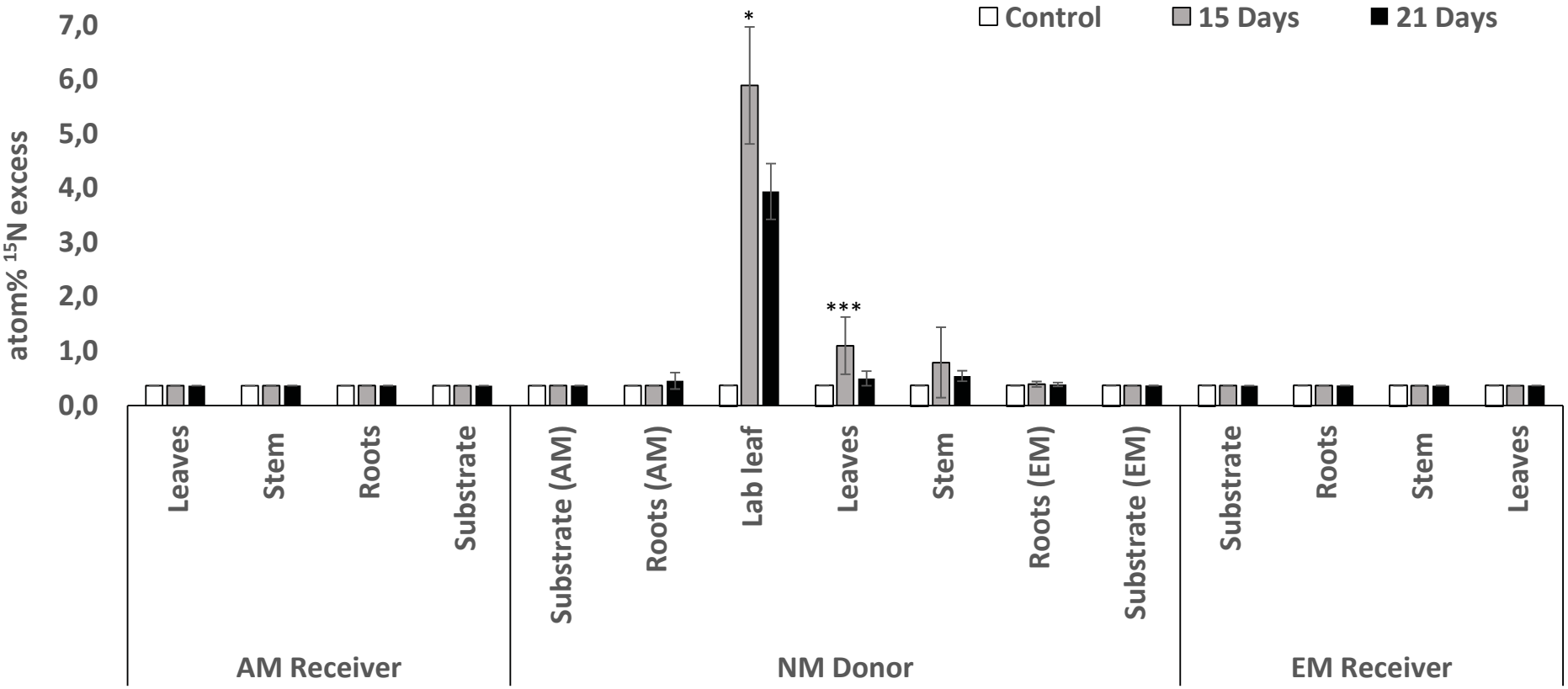


Figure 3: Atom% $^{15}N$ in the tissues of labeled donor and non-labeled receiver plants, which were sharing either an AM or an EM network with donor. Different colors represent the different harvest points. Bars with (*) had statistically different values.

## Discussion

In the current study, we aimed to evaluate both the role of establishing dual symbiotic associations for accessing a limiting soil resource such as N and the importance of AM and EM CMNs as mechanisms for plant-to-plant N exchange.

### N uptake and plant response in dual vs. single colonized plants

Both dual and single mycorrhizal plants were successfully colonized by the AM and EM strains. In our study, we observed higher colonization by EM than AM, with EM representing an average of 50% of RCL. Differential predisposition of different poplar genotypes to develop symbiotic associations with EM or AM fungi to different degrees has been demonstrated previously. Rachwal et al. (2003) evaluated the EM/AM ratio in roots of 15 Populus clones and found 8 clones dominated by AM (10–40% RLC) with low EM colonization (<5% RLC), 6 clones dominated by EM (50–90% RLC) and one exclusively ECM clone. Todeschini et al. (2007) reported 6-month-old cuttings of *Populus alba* and *Populus nigra* clones with variable levels of AM colonization (*P. alba* 7–12%, *P. nigra* 15–50%), but they had no EM. Using the same Populus species used in our experiment (*P. x canescens*), Bojarczuk et al. (2015) demonstrated that young plants have a higher predisposition for EM than AM, with plantlets cultivated for 16 weeks exclusively EM. These data are consistent with our findings.

Despite differences in colonization rates by AM and EM fungi, all treatments had a positive MGR in comparison to noninoculated plants (Table 3). Different growth responses to dual fungal colonization have been demonstrated previously, and some studies have demonstrated disadvantages associated with hosting both AM and EM simultaneously (Misbahuzzaman & Newton, 2006; Meinhardt & Gehring, 2012). Teste et al. (2020), however, showed in a review article that overall there are more frequently positive and neutral effects on dual colonization than negative ones. Our data are also in accordance with other studies using pot experiments of dual mycorrhizal plants that showed a positive MRG in dual colonized plants (Chen et al., 2000, Founoune et al., 2002, Ambriz et al., 2010, Báez-Pérez et al., 2015). Although both fungi have been shown to improve host plant growth, differences in shoot and root development in plants colonized by either AM or EM or both could be observed (Table 3). Dual-colonized plants had the smallest root/shoot ratio, which might indicate a higher investment in the development of aboveground tissues in those plants. This phenomenon could be a consequence of the seedling´s active growth and/or a reflection of the improved plant nutrition in dual colonized plants (Veresoglou et al., 2012).

In this context, higher nutrient concentrations could be expected for dual mycorrhizal plants as soon as they acquire access to complementary nutrients from both fungi. In our study, these patterns were not observed for P, Ca, K and Mg content, although AM and EM have been demonstrated to affect the concentration of these nutrients differently in the different plant tissues (Jentschke et al., 2001; Garcia & Zimmermann, 2014; Begum et al., 2019). AM fungi improved the Ca and Mg contents in aboveground tissues, while EM improved the P and K contents of the host plants. Knowledge concerning the role of the different mycorrhiza types in the uptake of nutrients other than P and N is limited and variable, especially for simultaneous colonization (Meding & Zasoski, 2008; Holste et al., 2017; Teste et al., 2020). Founoune et al. (2002), evaluating the uptake of P, Ca, Mg, Na, K, and N in dual mycorrhizal plants, observed the greatest plant growth of host colonized by EM only, followed by dual EM-AM colonization, and the lowest in single AM colonization. Our findings are partly in agreement with these results, since dual mycorrhizal plants also had intermediate values and did not differ from single colonized plants. However, comparisons made for single colonized plants by Founoune et al. (2002) have demonstrated higher concentrations of most nutrients in AM plants. Other studies have found EM to be especially important for K and Mg uptake, while AM plays an important role in Ca and P uptake (Jentschke et al., 2001; Seven & Polle, 2014; Neba et al., 2016). Variations in the data might be an effect of different plant and fungal species involved in the associations and abiotic conditions under which the experiments were developed.

In our study, differences between dual and single mycorrhizal plants regarding N nutrition were much more pronounced compared with the other evaluated nutrients. The central split root plants colonized by both fungal types had significantly higher N in the leaves and stem compared with its neighbors, which were colonized by a single fungal type. Thus, we confirmed the proposed hypothesis that central plants harboring both fungal types would have higher N acquisition due to an increased access to complementary N uptake from AM and EM. The N content of plants colonized by either AM or EM alone, however, was not different, which is in contrast to our hypothesis that AM and EM fungi would affect plant N content differently. It is well known that N concentrations in plant leaves strongly affect the photosynthetic rate and other photosynthetic parameters (Hikosaka, 2004; Luo et al., 2013; Shi et al., 2020). A higher photosynthesis rate would lead to enhanced production of C compounds that can be delivered to the fungus to obtain more access to limiting nutrients in the soil (Smith & Read, 2010). This higher production of C compounds might also compensate for the higher C sink strength of harboring both fungi simultaneously (Smith & Read, 2010; Ekblad et al., 2013), leading to a better performance of dual-colonized plants.

Single colonized plants had the highest C content in their roots, irrespective of the fungal type. This phenomenon could represent either a higher C investment from the host plant to its fungal partner when no choice is given (Fellbaum et al., 2014), or the effort of the plant to invest in root development to increase nutrient uptake (Veresoglou et al., 2012).

**C and N transfer via CMN**

With our dual $^{13}C$ and $^{15}N$ labeling, we aimed to track C and N transfer between plants, as well as the C investment of plants in different fungal partners. However, the applied $^{13}C$ to the central donor plant in the present study was retained in the labeled leaves and was not translocated to other plant tissues or even neighboring plants. Therefore, such investment could not be estimated in a precise manner, nor the C transfer between connected plants. The lack of $^{13}C$ allocation into underground tissues summed with the smallest root/shoot ratio of the labeled dual-mycorrhizal plant might indicate a higher investment in the development of aboveground tissues. This can be a consequence of the seedling´s active growth and/or a reflection of the improved plant nutrition in dual colonized plants (Veresoglou et al., 2012). Therefore, further investigations are necessary to evaluate C allocation in dual-mycorrhizal plants, probably using longer labeling times or variations in biotic and abiotic factors in the system.

For N labeling, however, a significant amount of the added $^{15}N$ was found in the leaves and stems of the donor plants harvested 15 days after labeling. Therefore, any possible translocation of N from donor to receiver plants could be evaluated. However, no $^{15}N$ allocation was observed to any neighboring plant sharing a mycorrhizal network with the labeled donor plant. This finding contradicts our hypothesis that neighboring plants sharing EM mycorrhizae would receive higher amounts of $^{15}N$ than plants sharing the AM network.

N transfer studies have been previously performed mainly from N-fixing plants to nonfixing neighbors, with data ranging from 0 to 50% transfer when using $^{15}N$ enrichment or natural abundance methods (He et al., 2003, 2009; Chalk et al., 2014). Nevertheless, other studies have involved N transfer between nonfixing plants. He et al. (2006), using woody plants, reported a very low N translocation (ranging from 0.001 to 0.01% of the added label) to nearby plants in a 4-week experimental period. The observed lack of transfer might also be an effect of plant age. Once the experiment was developed with poplar seedlings, which are considered a fast-growing wood plant (Shi et al., 2015), all additional N acquired might have been used for plant development instead of transfer.

Although N transfer has been widely recognized as an important effect of the mycorrhizal network, the high variations found in the literature and the findings of our study demonstrate

that such fluxes are difficult to quantify and that their importance might depend on parameters that are not yet known. Therefore, the lack of N and C transfer observed in the present study might not invalidate other studies showing a significant transfer but rather indicate that boundary conditions might overlay and overcompensate this transfer or even hinder it from happening at all. These might be biotic factors, such as plant type and developmental stage, nutritional status or nutrient stoichiometry, abiotic factors, such as nutrient availability and general substrate characteristics, and complex interactions of all these factors.

**Conclusions**

We demonstrated the nutritional advantage regarding N uptake for host plants holding dual mycorrhizal plants compared with single colonized plants. The observed variations in the N content of leaves and stems together with the variations in C contents of the roots of such plants indicated that higher N nutrition might be due to a decrease in C allocation to a single mycorrhizal partner in the case of dual colonization. In addition, although plants were connected through a mycorrhizal network and donor plants were significantly labeled with 15N,no transfer of N occurred between donor and receiver plants. Therefore, our results suggested that CMN functioning for N transfer might occur only under specific situations, such as for particular plant–fungus combinations, the characteristics of specific connected plants or particular abiotic conditions. Therefore, the role of CMNs in resource transfer is still a matter of discussion. The experimental setup presented in our study might represent an important prospect that can be used for further investigation (e.g., involving different plant-fungi combinations and an increase in source-sink patterns between plants) to precisely define the factors driving N transfer between AM- and EM-connected plants. Thus, we conclude that simultaneous associations of plants with AM and EM may represent a strategy of plants to improve their N nutrition and may play a role in plant species survival under favorable conditions.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author Contributions**

AF, JB and GG conceived the idea about the subject of study in this manuscript. AF, JB, GG, AA and LS contributed to the experimental design. AF conducted the experiment and analyzed the data. JB and GG supervised the research. AF wrote the paper with contributions from AA, LS, JB and GG.

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**Data Accessibility Statement**

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.5x69p8d44.

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## 2.3. Pathways of nitrogen transfer between plants connected through a common mycorrhizal network (CMN)

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**Abstract**

Most of mycorrhizal fungi are not host specific and can colonize *simultaneously* a large number of plants of the same or different species, forming a so-called common mycorrhiza network (CMN). Several studies proposed CMN as mediating resource partitioning and enhancing plant fitness. Moreover, resource transfer between plants can occur via several, hard to distinguish, pathways, blurring the picture of the contribution of CMNs in resource allocation. Additionally, quantification of the amount of transferred nutrients is challenging, leading to doubts regarding the importance of the CMN in inter-plant partitioning. In the present study, we propose a novel experimental design to prove and distinguish the contribution of the CMN for nitrogen (N) transfer between connected plants by using *ram1-1* mutant plants as receivers compared to wild type (Wt) combined with labeling techniques. With this approach, we wanted to test that most of the N transfer between donor and receiver plant occurred through fungal connection. In addition, we want to quantify N transfer via mycelial connections *vs* indirect pathways. Further, by shading of the donor plant, we expected a higher N transfer to the receiver plant, since receiver would have fully access to light and therefore able to produce more C compound to be exchanged for nutrients with the fungi partner. Our data demonstrated a larger $^{15}N$ transfer to *ram1-1* receiver plants than to Wt receiver plants. Since *ram1-1* plants had also the largest root biomass, we can conclude that N was mainly acquired via indirect pathways. Further shading did not increase the $^{15}N$ transfer plants, indicating that fungi was not in control of the transfer. With this, we conclude that CMN are important once it can still

facilitate transfer even by indirect ways, but in contrary to what is usually discussed in literature, CMN might not function as a direct pathway for resources exchanges.

**Keywords:** Common Mycorrhiza Network (CMN), nitrogen transfer, $^{15}N$, *ram1-1* mutant, stable isotope, wood wide web.

## Introduction

The symbiosis between terrestrial plants and soil microorganisms is one of the world´s most widespread forms of mutualism and is present in almost all ecosystems (Brundrett, 2009). Within soil microbiota, the arbuscular mycorrhiza (AM) fungi, are among the evolutionary oldest partners of photoautotrophs and comprise up to 50% of the total soil microbial biomass (Wang, et al., 2010; Taylor et al., 2014; Field et al., 2015; Bücking, 2016). They are obligate biotrophs able to form intimate interactions with c. 65% of all known land plant species (Wang & Qiu, 2006; Smith & Read, 2010). The main characteristic of the AM symbiosis is the penetration of its hyphae into root epidermis followed by the formation of arbuscules inside cortical cells. Arbuscules are composed of fungal hyphae ensheathed in a modified form of the cortical cell plasma membrane, the so-called periarbuscular membrane. This structure is recognized as the symbiotic interface, as it holds a unique transport system, which allows plants and fungi to exchange sugars, lipids and nutrients (Kobae & Hata, 2010; Krajinski et al., 2014; Bravo et al., 2017). On the outside, the external fungal hyphae is able to access nutrients that plants roots cannot. These nutrients are exchanged by photosynthetically fixed carbon from the plant, mainly as sugars and lipids, at the periarbuscular membrane (Smith & Read, 2010).

Associations involving only one host plant and one fungal partner are well researched and the benefits are well known. Mycorrhizal fungi may enhance plant nutrition, especially for growth limiting nutrients such as nitrogen (N) and phosphorus (P) (Smith & Read, 2010), alleviate plant abiotic stress, e.g. drought stress, salinity, and herbivory (Begum et al., 2019; Diagne et al., 2020), and protect plants against pathogens (Chen et al., 2018), among other benefits. However, these associations are often more complex under ecosystem conditions. One of the reasons for this is that plants have usually a broad host receptivity and are able to associate with a diverse array of fungal species. Likewise, fungi species are also not host specific and an individual fungus may colonize simultaneously a large number of plants of the same or different species, leading plants to become interconnected by the mycorrhizal fungi, forming the so-called common mycorrhiza network (CMN) (Simard & Durall, 2004; Heaton et al. 2012). In addition, such connections can be formed not only by an individual fungus connecting multiple

plants, but also potentially by anastomosis (or non-self hyphal fusion) of hyphae from compatible AM fungi neighbors (Giovanetti et al., 2004; 2015). Plants of same and different species have been reported sharing compatible fungi in several ecosystems (Simard et al., 2012). Some authors have estimated the potential of plants to become interconnected by evaluating the similarity between mycorrhizal community composition of plants´s roots, assuming that plants having similar mycorrhizal community composition are more likely to be connected by a CMN (Beiler et al 2010; Diédhiou et al 2010). Moreover, observations of individual fungal genets on roots of two different trees have also been used to predict whether plant roots are linked in CMN (Selosse et al., 2006; Beiler et al., 2010). However, it is important to note that in both cases the direct connection between plants are not undoubtedly proved, once it can be isolated physiologically by fragmentation such as hyphae grazing (Wu et al., 2005; Figueiredo et al., 2021).

An increasing number of research has been developed to proof the occurrence and explain the effects of such networks on, for example, plant fitness (Kytoviita et al, 2003, Bücking et al., 2016), increase of seedling survival (Weremijewicz et al., 2016, Pec et al., 2020), inoculum source (Varga et al., 2016, Grove et al., 2019) and signaling and resources transferring between connected plants (Song et al., 2014, Fang et al., 2021). A particularly interesting effect of CMN is the possibility of transferring limiting resources between connected plants. In this context, the resources provided for the plants via the fungi partner may come not only from the soil but also directly from another neighboring host plant (He et al., 2004, Meng et al., 2015; Figueiredo et al., 2021). An interesting study demonstrating that connected hyphae may work as a "pipeline" for nutrient exchanges between plants was made by Giovanetti et al., (1999), showing the ability of AM fungi of same or/and different species to become interconnected through anastomosis. They visualized cytoplasmic flow and nuclear exchange between anastomosing hyphae. Following this bidirectional flow of particles (vacuoles, mitochondria, nuclei, and fat droplets), nutrients can also be transported and reach neighboring colonized plants (Mikkelsen et al., 2008). This direct transfer compartmentalizes valuable resources away from potential disruptions, such as competition with other soil microbes, chemical adsorption of nutrients to soil particles or physical disturbances of the soil structure (Philip et al., 2010).

The premise of such a direct resource transfers has been supported by field and laboratory experiments using labeling compounds to trace the fate of nutrients in plants connect through a CMN, showing that belowground transfer between plants of same and different species is facilitated by mycorrhizal fungi (Selosse et al., 2006; Teste et al., 2009, Deslippe & Simard, 2011). Some authors have suggested that nutrients transfer may follow a source sink gradient,

where one plant that is rich in nutrients serves as a source (donor) of compounds for a neighboring plant that is poor in nutrients, which thus acts as a sink (receiver). This pattern was observed especially in experiment involving N-fixing plants as donors, acting as sources of N, and a non-fixing plants as receivers (Simard et al., 2012; Thilakarathna et al., 2016; Montesinos-Navarro et al., 2017). In this context, donor plants would donate its excess of N to the neighboring limited plant, therefore leading to positive interactions (facilitation) between connected plants, increasing the survival rate and growth of receivers. However, the opposite flux was also already observed (Li et al., 2009; He et al., 2009), in which N was transferred from non-fixing plants to N-fixing neighbors via CMN. Another theory proposes that nutrients will be preferentially allocated to the plant partner with the greater C investment (Kiers et al., 2011). Here, it is assumed that the fungi can recognize the best host plants within several options and reward it accordingly. In contrast to the previous assumption that resources would move from in a source-sink pattern, in this "reward" theory competition between largest and smallest and most shaded plants would increase. Therefore, bigger plants, or plants having more access to light, would be able to produce more C to be exchanged for larger amounts of nutrients from its fungi partner, leading the smaller or shaded partner in disadvantage. However, evidence of transfer irrespective of C inputs was also demonstrated (Walder et al., 2012). Due to discrepancies in the results obtained in the different studies performed so far, the exact mechanisms regulating nutrient exchange between plants connected to a CMN still remains obscure.

Despite contrasting evidences, resource transfer between plants can occur via several routes, such as movement and turnover of other soil organisms associated to roots and mycorrhizal hyphae or pathways through soil solution (Figueiredo et al., 2021). This can be considered as mycorrhizal–soil pathway where compounds are leaked into the soil pool by the associated hyphae of one plant and then picked up by the roots or associated hyphae of a neighboring plant (Philip et al., 2010; Simard et al., 2012). The second assumption is that resources would be transferred in a direct pathway with the hyphae connections functioning as "pipelines" for plant-to-plant exchanges (Van der Heijden, 2016; Klein et al., 2016). It is very hard to distinguish experimentally between these options. Several previous studies that aimed to demonstrate the potential effects of CMN on intra- and interspecific competition among plants have suffered from inadequacies in clearly demonstrating the existence of a functional CMN (Leake et al. 2004; Simard & Durall 2004), and the potential movement of isotopes through other pathways were not successfully excluded in the previous studies. The technical problems in demonstrating unequivocally that plant-to-plant transfer occurs genuinely through

hyphal interconnections has been shown challenging (He et al. 2004; Wilson et al., 2006), and some authors have proposed that loss of nutrients from roots or hyphae into the soil pool, followed by immediate uptake by mycorrhizal hyphae, appears to be the main path for plant-to-plant transfer (Simard & Durall 2004, Wilson et al., 2006).

Even more challenging than to prove such direct pathway of resource allocation in a CMN is the quantification of nutrient flux exclusive to the fungal hyphae over other pathways. It is important to evaluate the real contribution of the resources exchanged via direct hyphae connection, since this would represent a pathway free of disruption. Within CMN, resources could be translocated without losses by leaching or uptake by competing soil microorganisms. Some authors suggest that the amount transferred is too low to be of importance (He et al., 2009), while others say that even low amounts can be important for plants depending of the situation in which plant is found (under severe nutrients limitation sites, for example) (Montesinos-Navarro et al., 2016). Therefore, distinction and relative importance of the different pathways will determine the strength, direction and outcome of interactions among plants, requiring new technologies and ideas to address such issues.

The aim of the present work therefore was to identify the mechanisms involved in N transfer between plants connected through a CMN as well as its importance for plant fitness. For this, we propose here a novel experimental setup using two different types of *Medicago truncatula*, one with a reduced arbuscular mycorrhizal (*ram1-1*) mutant as well as wild type (Wt), coupled to isotopic labelling techniques with enriched $^{15}N$, in order to overcome the limitations of previous studies to distinguish and quantify N translocation through the direct pathway (DP) and indirect pathways (IP). The main idea by using Wt and *ram1-1* species is that, in *ram1-1* mutants, all the common symbiosis (SYM) genes are intact, and accordingly, the initial infection events, such as the formation of hyphopodia on the root surface and hyphal coils in epidermal cells, occurs normally. However, mutation in the transcription factor gene *ram1-1* impaired arbuscules branching (Park et al., 2015; Xue et al., 2015; Pimprikar et al., 2016), an important step for the establishment of the symbiotic interface and site where exchanges between plant host and fungi partner takes place. Such transport is mediated by a unique transport protein composition present in the periarbuscular membrane, formed surround arbuscules (Kobae & Hata, 2010; Krajinski et al., 2014; Bravo et al., 2017). Therefore, *ram1-1* receivers will have access to N via all indirect paths but not through arbuscules. So, the direct transfer in which nutrients will be exchanged in the arbuscules containing cells can be excluded from all other paths, being able to calculate its contribution for N transport. Using this approach, we hypothesized that (1) N transfer between connected plants occurs genuinely through hyphal

connection, with the fungus acting as a hose for transport, rather than indirect pathways; (2) the proportion of N allocated from donor to receiver plants through mycorrhiza hyphae connections may improve neighboring plant nutrition; and (3) by shading donor plant, N transferred to receiver plants is increased, once it might be able to produce more C to be exchanged by transported N.

## Material and Methods

### *Plant material and fungi inoculum*

Two different types of seeds of Medicago truncatula were used for this experiment: a wild type and a reduced arbuscular mycorrhizal (ram1-1) mutant. Seeds of both types of Medicago truncatula were scarified and germinated according to Salzer et al. (1999). Shortly, seeds were covered with concentrate sulfuric acid [95-98% v/v] for around 10 min, until small dots on the testa appeared. The, seeds were washed three times with sterile tap water followed by an incubation with 2% sodium hypochlorite for 1 min. The washing steps were repeated and the seeds soaked in water for 3 hours under the light of the phytocabinet. After this period, seeds were placed in agar-plates, covered with aluminum foil and incubated at 6°C chamber for four days and at 22°C for one day, before exposure to light. After germination, seedlings were transplanted into pots filled with autoclaved Perlite and grow for 2 more weeks for acclimation, before transplantation into custom-made dual-compartment systems. The systems were filled with either sterilized Perlite (2 h at 121°C) mixed with 10% of volume of AM inoculum, or autoclaved perlite-inoculum mixture (Mock) for the non-mycorrhizal control. For AM inoculum, the strain Rhizophagus irregularis was chosen and the inoculum consisted of a mixture of AM spores, mycelium, roots fragments and soil obtained from a trap culture where the fungi was pre-growth with Sorghum bicolor. Host plant roots from the trap culture were tested for the presence of spores before use as inoculum.

### *Experimental design*

After the acclimation of the seedling in the substrate, seedlings were transplanted into custom-made four-compartment mesocosmos (Figure 1). The mesocosmos consists in two pairs of dual-compartments, where each dual-compartment is isolated from each other by a plastic wall. Within the dual-compartments, there is an open wall, in which a “sandwich” of two layers of 20µm nylon mesh membrane (Franz Eckert GmbH, Waldkirch, Germany), that avoids roots to cross from one compartment to the other, and with a 5µm PTFE hydrophobic membrane (Pieper Filter GmbH, Bad Zwischenahn, Germany) in between, in order to avoid also fluxes of solution between compartments.

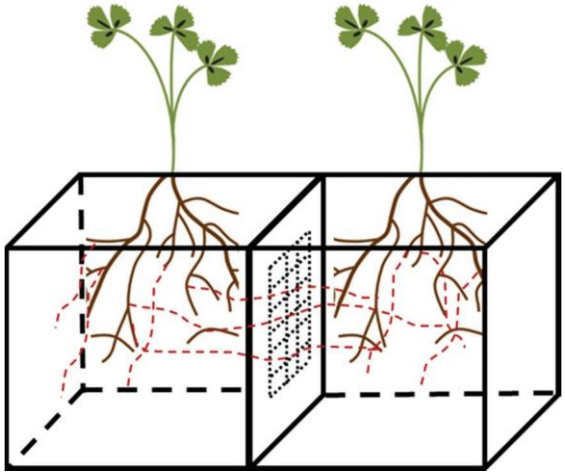

Figure 1: Scheme of the custom-made dual-compartment systems, in which plants are separated by 20µm nylon mesh membrane + 5µm PTFE hydrophobic membrane. “Donor” plant is inoculated with AM fungi and hyphae must cross the membranes and colonize neighboring plant for CMN formation.

All boxes were sealed with silicone and tested for leakage before the experiment. The plants designed as “donor” were transplanted into the compartments containing the fungi inoculum, while its neighbor “receiver” were transplanted into the mock. The fungi associate with the donor plants may grow, cross the barriers, reach the neighboring compartment and colonize the receiver plants, in a way that both plants share a CMN. The donors consists of a Wt *Medicago*, while receivers might be either another Wt, in which all pathways would occurs simultaneously, meaning that N can be translocated into the hyphae and delivery to the plant via arbuscules, but also through plant roots, in case of leakages of compounds from the hyphae into the soil and subsequent absorption into the roots of the receiver. Alternatively, the receiver is a *ram1-1* mutant, where mycorrhiza transport is absent and only indirect pathways takes place. Non-mycorrhizal plants following the same treatments were used to test for leakage. Therefore, the importance of the hyphae pathway (direct path) will be determined by the difference between the N received by the Wt compared to what was received by the *ram1-1* mutant.

$$DR = (DR + IN) - IN \quad (1)$$

Previous studies involving *M. truncatula* hosted by *R. irregularis* fungi have demonstrated maximum colonization of plants after 45 days post-inoculation (dpi) (Hartmann et al., 2019). Therefore, in order to provide time enough for the fungi to cross the compartment and fully colonize the neighboring plant, labeling and subsequent harvesting were made after three months of growth. During growth period, both plants were fertilized with equal amounts

of modified Long Ashton (LA) solution (Langenfelder Heyser et al., 2007) with reduced P content in order to induce AM mycorrhization. During the first weeks after transplantation, plants were fertilized and watered as much as needed for their survival. After its stabilization, they were fertilized every other day with 30 ml of LA solution and watered when necessary in the days where no fertilizer was applied, in order to avoid run off of solution. For the shading treatment, donor plants were entirely covered with sheath clothes with about 55% of shading effects (Fellbaum et al., 2014) on the day of labeling, meaning that plants were shaded for 2 weeks.

***$^{15}N$ and $^{13}C$ labelling***

In order to track the transfer of N and C between connected plants, the isotopic labeling techniques with enriched $^{15}N$ and $^{13}C$ was applied. One week prior labeling, the LA solution were replaced by a modified a Low Nutrient (LN) solution (Langenfelder Heyser et al., 2007), in which nitrogen sources were excluded in order to avoid N dilution of the labeled N. The same solution was used for the fertilization of the plants after labeling until harvesting. Dual- labeling of $^{15}N$ and $^{13}C$ were made by the leaf feeding technique, adapted from Khan et al., (2002). Briefly, one young branch of each plant was immersed into a 2ml Eppendorf vial containing 1.5 ml of an enriched urea solution. The solution was prepared by diluting 100mg of $^{13}C^{15}N$-urea (99% atoms $^{13}C$ and 98% atoms $^{15}N$) in 50ml deionized water. The vials attachedto the branches were sealed using parafilm to avoid evaporation of the solution. Solution was completely absorbed by the leaf within 2-3 days and plants were harvested 15 days after labeling. Control plants were labeled with non-labeled urea, following the same procedure and used as background for the analyses.

***Leachate analysis***

To provide evidence of possible transfer of nutrients via diffusion and mass flow as an indirect pathway of N transfer, we collected leachates from rhizoboxes prior to harvesting the plants. For this, each compartment of the rhizoboxes were flushed with deionized water mimicking a heavy rainfall event and the leachates were collected and frozen. The frozen leachates were than freeze dried and the remaining powder were used to determine stable N and C isotope composition.

***Harvest***

Plants were divided into leaves, shoots and roots. The roots were carefully washed to remove all the substrate adhering to it and divided into two portions: a fresh subsample was

taken and stored in 70% ethanol for later staining and evaluation of mycorrhization rate, while the second portion were rapidly frozen and posteriorly freeze dried and milled for C, N, and nutritional and isotopic composition. For all parts, fresh weigh (FW) and dry weight (DW) were accounted and used for the further calculations. Labelled leaves which were directly immersed into the labeling solution were processed separately from whole plant material, but followed the same procedure. The substrate of each compartment was dried at 45°C, ground to a fine powder, sieved and the C and N content and isotopic composition were measured.

***Mycorrhization rate***

WGA-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR, USA) was used according to Ramonell et al. (2005) to stain the chitin present in fungal cell wall in the infected roots of *M. truncatula*. Briefly, roots were incubated in 10% (w/v) KOH at 95°C for 7 min, rinsed with water and incubated in 1x PBS buffer (0.14 M NaCl, 2.7 mM KCl, 1 mM $Na_2HPO_4 \times 2H_2O$, 1.8 mM $KH_2PO_4$; pH 7.3) containing 20 μg.ml$^{-1}$ Alexa WGA Fluor™ 488 (Thermo Fisher Scientific, Langenselbold, Germany) conjugate overnight. Visualization and photo documentation of the stained roots was performed using a Leica MZ10 F stereomicroscope (Leica Microsystems, Wetzlar, Germany) with a beam path specifically for fluorescent illumination for high contrast and detailed fluorescent imaging, equipped with an Olympus XC50 camera (Olympus, Hamburg, Germany). Fluorescence was excited with an argon laser at 488 nm and detected at wave-lengths of 500–520 nm [WGA-Alexa Fluor 488 and green fluorescent protein (GFP)]. Three independent roots of each plant type (Wt and *ram1-1*) were also observed under higher magnification and used for photo documentation using Zeiss Axio Observer Z1 microscope equipped with an AxioCam ICc1 (Carl Zeiss AG, Oberkochen, Germany).

To calculate mycorrhization rate, the magnified intersection method from McGonigle et al. (1990) was used, with some modifications. For this, 10-12 stained roots fragment of c.a 1cm were arranged in parallel on glass slides and covered with glass cover slips and examined under magnification 8.0 x to account for presence of hyphae (H), vesicles (V) e arbuscules (A). The mycorrhization rate was examined and quantified in at least three slides per plant leading to over 300 views per sample. The frequency of occurrence of mycorrhiza was calculated according to Trouvelot et al. (1986) using the following formula:

$$F\% = 100 * \frac{n}{N} \quad (2)$$

where N, is the total number of seen visual fields and n is the number of visual fields containing mycorrhiza. The frequency of occurrence of the arbuscules (AF%), vesicles (VF%) and hyphae (HF%) was calculated by the same formula.

***Nutritional composition***

To determine nutritional composition of plant tissues, around 50 mg of dried andgrounded plant material was weighed into snap-jars, incinerated overnight at 480°C for 6 h. The ashes were dissolved in 1ml of 6 M HCl with 1.5%(w/v) hydroxylammonium chloride and the extracted solution diluted (1:10 v/v) with double demineralized water, filtered and measuredfor Al, B, Ca, Cr, Co, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, Si, Sn and Zn concentrations. Measurements in all solutions were carried out by ICP-OES (Agilent Technologies Ireland Ltd., Cork, Ireland). The standard solutions were prepared from single element solutions (1000 mg $L^{-1}$, Fisher Scientific, Loughborough, UK). Here we only report the macronutrients P and K, together with some other important micronutrients for plant nutrition such as Ca and Mg.

***Isotopic composition and C and N content***

The C and N content and $^{12}C/^{13}C$ and $^{14}N/^{15}N$ isotope ratios in the samples were measured with an elemental analyser Isotope cube (Elementar GmbH, Hanau, Germany) coupled to an Isoprime 100 isotope ratio mass spectrometer (IRMS) (Elementar GmbH, Hanau, Germany) via a continuous flow inlet using helium (99.999% purity; Linde, Munich, Germany) as carrier gas. The $\delta\ ^{13}C$ values were corrected using Vienna PeeDee Belemnite (PDB) (0.011182) as internal standard and the $\delta^{15}N$ corrected using $N_2$ (0,0036764) as internal standard. Isotope ratios values obtained were converted to atm% $^{15}N$ and then to atm% $^{15}N$ excess for further calculations. Transformation were made as following:

atm % = (100 * AR * (δ sample/1000 + 1)) / (1 + (AR * (δ sample /1000 + 1))) (3) where

AR is the Absolute Ratio of the standard, $N_2$ atmospheric gas (constant), and the Delta is the value in permille (δ‰) of the sample to be converted into atm %.

The atm% $^{15}N$ excess was calculated by the difference between the atm% $^{15}N$ of the sample and the atm% $^{15}N$ of background (non-labeled plants).

The same calculation was made to obtain atm% $^{13}C$ excess, using the values of the PDB for the standard values.

***N and C transfer***

The relative contribution of $^{15}N$ in the receiver derived from tracer applied to donor plant, hereafter referred as %N transfer, was calculated as described by Teste et al. (2015) according to the following equations:

$$15N\ content = \frac{\text{atm\% 15N excess donor (or receiver) x N donor (or receiver)}}{\text{atm \% 15N excess labeled N}} \quad (4)$$

$$\%N\ transfer = \frac{\text{15N content receiver x 100}}{\text{15N content receiver + 15N content donor}} \quad (5)$$

The same calculation was used to calculate C transfer.

%N transfer were calculated for the whole plant (shoots and roots in conjunction), for each pair of plants. In addition, we assume that the calculated %N transferred from donor to receiver plant represents exclusively the transfer of N compounds derived from the labeling source, and do not account for any possible non-labeled N that might also be transferred. Therefore, %N transferred here calculated might be underestimated. The same is true for %C transferred.

***Statistics***

All variables were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests, respectively. One-way ANOVA analysis of the variance and the least significant difference (LSD) test was applied to compare significant differences between plant species at $P \leq 0.05$. Data were analyzed using XLSTAT software (ver. 2010, Addinsoft, New York, USA).

**Results**

***Mycorrhization rate***

All donor and receiver plants of both treatments were fully mycorrhized at the time of harvesting, in which hyphae, arbuscules and vesicles were present in all treatments (Table 1; Figure 2). Non-mycorrhized control plants had no mycorrhiza into its roots. Shading did not significantly affect hyphae frequency, once shaded donors had the same HF% like non-shaded ones (≥ 95%) (Table 1). *Ram1-1* receivers had the lowest HF% values, although not significantly different from the Wt, except for the *ram1-1* receiver connected with the shaded donor, which had the lowest frequency. Likewise, shading also did not decrease arbuscular and vesicle frequency of donors, being that Wt shaded donors had the largest AF% and VF%. In both cases, *ram1-1* receivers had the smallest values, significantly different from the Wt. In

contrast to the well developed arbuscles in the Wt (Figure 2, a-b), the *ram1-1* mutant contained highly truncated arbuscules (Figure 2, c-d).

Table 1: Frequency of occurrence of hyphae, arbuscules and vesicle in the donors and receivers of the different treatments. Comparisons were made between all treatments. Values are mean of 4 biological replicates, and different letters indicate statistically significant differences between treatments at $p < 0,05$.

| | | **Frequency of occurence (%)** | | |
|---|---|---|---|---|
| Treatments | | *Hyphae* | *Arbuscules* | *Vesicles* |
| Wt →Wt | Donor | 97.3 $^{a}$ | 60.6 $^{ab}$ | 56.8 $^{bc}$ |
| | Receiver | 90.0 $^{ab}$ | 48.4 $^{bc}$ | 41.4 $^{c}$ |
| Wt → *ram1-1* | Donor | 95.6 $^{a}$ | 60.2 $^{ab}$ | 60.3 $^{bc}$ |
| | Receiver | 86.0 $^{ab}$ | 13.0 $^{d}$ | 5.1 $^{d}$ |
| Wt(Sh)→Wt (NSh) | Donor | 99.6 $^{a}$ | 66.7 $^{ab}$ | 74.2 $^{a}$ |
| | Receiver | 97.6 $^{a}$ | 63.4 $^{ab}$ | 66.7 $^{b}$ |
| Wt(Sh)→ *ram1-1* (NSh) | Donor | 99.7 $^{a}$ | 80.8 $^{a}$ | 94.9 $^{a}$ |
| | Receiver | 77.3 $^{b}$ | 34.7 $^{cd}$ | 18.9 $^{d}$ |

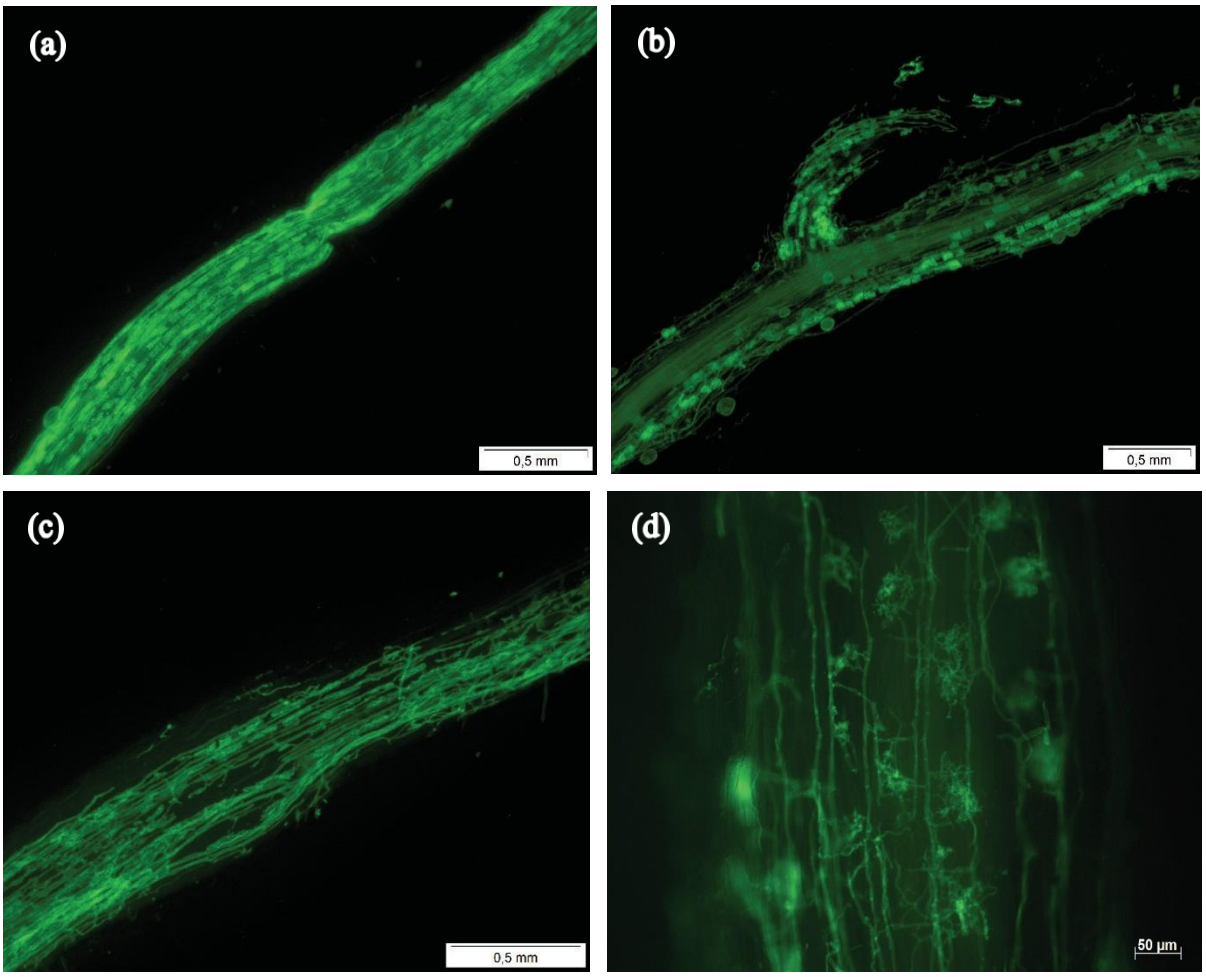


Figure 2: Alexa stained roots of Wt donor (a) and Wt receiver (b), with vesicles and fully developed arbuscules, and *ram1-1* receivers (c) and (d), containing truncated arbuscules. Hyphae are highly spread over all samples.

***Plant biomass and nutritional composition***

Shading did not decrease plant shoots biomass of donor plants, as expected. Tendency of larger shoots biomass in shaded donors could be observed (Table 2). However, shading of donor apparently affected the shoots biomass of the receiver plants in different patterns, depending if Wt donor were sharing a CMN with a Wt or *ram1-1* receiver.

In the non-shaded treatment, no differences were observed between the shoots biomass of Wt donor and Wt receiver. But, *ram1-1* receiver had significantly larger biomass when compared to the Wt donor in this treatment. Roots biomass followed the same pattern found for the shoots (Table 2), with no differences between Wt donor and Wt receiver in this treatment, but larger roots biomass on *ram1-1* receiver.

An opposite trend were found in the treatments in which Wt donor plants were shaded. The shoots biomass of Wt receiver was significantly smaller than its Wt donor plant. But, no

significant differences between roots biomass. In opposite to what was found in the non-shaded treatment, no differences between the shoots biomass of *ram1-1* receiver and shaded Wt donor were found in this case. However, roots biomass of *ram1-1* receiver were again significantly larger than Wt donors.

In general, shading seems to affected the concentrations of some nutrients within the different plant tissues. Concentrations of Ca, K, Mg and P in plants from non-shaded treatment were more equally distributed (Table 2). The largest Ca concentrations were found in the leaves of non-shaded receivers sharing CMN with shaded donor. Same pattern were observed for the Ca concentration in the stem. In the roots, however, the opposite occurred, in which Wt shaded donors had the largest Ca accumulation. Largest concentration no K were found in the roots of non-shaded receivers, while no obvious pattern was observed in the other tissues. Shaded donors had the largest P values in their leaves, while no significant difference were observed in other tissues. Concentrations of Mg did not differ in the different treatments.

Table 2: Shoots and roots dry weight (g) and nutritional composition (mg.g$^{-1}$ plant) of the donors and receivers plants from shaded and non-shaded treatments, 15 days after labelling. Values are mean of 5 or 6 biological replications. Comparisons of concentration of each nutrient and DW were made between all treatments for each plant tissues. Different letters indicate statistically significant difference between values.

| | | ***DW (g)*** | | ***Ca (mg.g )*** | | | ***K (mg.g )*** | | | ***Mg (mg.g )*** | | | ***P (mg.g )*** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | | **Shoots** | **Roots** | **Leaves** | **Stem** | **Roots** | **Leaves** | **Stem** | **Roots** | **Leaves** | **Stem** | **Roots** | **Leaves** | **Stem** | **Roots** |
| Wt →Wt | Donor | $0.72^{bcd}$ | $0.20^{c}$ | $26.84^{c}$ | $8.50^{de}$ | $12.49^{cd}$ | $14.41^{b}$ | $9.67^{bc}$ | $4.01^{bc}$ | $4.53^{a}$ | $2.91^{bc}$ | $2.87^{b}$ | $0.95^{d}$ | $0.65^{a}$ | $1.04^{ab}$ |
| | Receiver | $0.70^{bcd}$ | $0.27^{c}$ | $26.32^{c}$ | $9.32^{cd}$ | $13.18^{c}$ | $13.60^{b}$ | $9.62^{bc}$ | $4.07^{bc}$ | $4.50^{a}$ | $3.14^{ab}$ | $4.98^{a}$ | $1.05^{cd}$ | $0.70^{a}$ | $1.00^{ab}$ |
| Wt → *ram1-1* | Donor | $0.38^{d}$ | $0.16^{c}$ | $26.55^{c}$ | $8.08^{e}$ | $15.26^{bc}$ | $14.18^{b}$ | $10.09^{ab}$ | $5.30^{ab}$ | $4.53^{a}$ | $2.58^{cd}$ | $3.14^{b}$ | $1.11^{cd}$ | $0.59^{a}$ | $0.84^{ab}$ |
| | Receiver | $1.14^{a}$ | $0.64^{a}$ | $26.56^{c}$ | $11.63^{b}$ | $7.65^{e}$ | $10.44^{c}$ | $8.36^{c}$ | $6.34^{a}$ | $4.17^{a}$ | $3.58^{a}$ | $4.74^{a}$ | $0.80^{d}$ | $0.72^{a}$ | $1.08^{b}$ |
| Wt(Sh)→Wt (NSh) | Donor | $0.95^{ab}$ | $0.30^{bc}$ | $30.41^{c}$ | $9.67^{c}$ | $20.37^{b}$ | $20.96^{a}$ | $10.32^{ab}$ | $4.89^{abc}$ | $4.44^{a}$ | $2.36^{de}$ | $2.67^{bc}$ | $2.05^{ab}$ | $0.83^{a}$ | $0.98^{ab}$ |
| | Receiver | $0.46^{cd}$ | $0.20^{c}$ | $37.62^{ab}$ | $11.48^{b}$ | $8.19^{de}$ | $16.24^{b}$ | $9.43^{bc}$ | $6.99^{a}$ | $3.99^{ab}$ | $1.76^{f}$ | $2.91^{b}$ | $1.51^{bc}$ | $0.86^{a}$ | $1.09^{a}$ |
| Wt(Sh)→ *ram1-1* (NSh) | Donor | $0.65^{bcd}$ | $0.21^{c}$ | $31.46^{bc}$ | $9.84^{c}$ | $25.56^{a}$ | $19.32^{a}$ | $11.64^{a}$ | $2.80^{c}$ | $3.99^{ab}$ | $2.14^{f}$ | $1.73^{bc}$ | $2.48^{a}$ | $0.57^{a}$ | $1.23^{a}$ |
| | Receiver | $0.76^{bc}$ | $0.50^{ab}$ | $38.04^{a}$ | $12.82^{a}$ | $11.13^{cde}$ | $7.47^{d}$ | $8.43^{c}$ | $6.00^{ab}$ | $3.49^{b}$ | $1.91^{ef}$ | $1.90^{c}$ | $1.16^{cd}$ | $0.75^{a}$ | $1.21^{a}$ |

***C and N content***

In the non-shaded treatments, N were more equally distributed when two Wt plants were connected, but when *ram1-1* was the receiver plant, *ram1-1* had the largest N in its leaves compared to Wt donor (Table 3). The N content of the stem and roots had the same pattern, with no significant differences between Wt donors and receivers, but a large N content in the roots of *ram1-1* receivers. In the shading treatment, the largest N content were found in the leaves of shaded Wt donor, sharing a CMN with a Wt receiver. In the opposite to what was found in the non-shaded treatment, there were no significant differences between the N content in the leaves of shaded Wt donor and *ram1-1* receivers. Again, the same pattern were were found in the N content of stem and roots. .

C content in leaves followed the same pattern as that for N in the unshaded treatments, with *ram1-1* receiver having the largest C content. Values were significantly smaller in plant of the shading treatment, except for the Wt donor connected to a Wt receiver that had the largestC content of all other plants of the same treatment. For roots, in both treatments (shaded and unshaded) *ram1-1* receiver had the largest C content. No obvious pattern were observed for the C content in the stem.

Table 3: N and C content of leaves, stems, and roots of donors and receivers plants from shaded and non-shaded treatments, 15 days after labelling. Comparisons were made between the different tissues of all treatments. Values are mean of 5 or 6 biological replications, and different letters indicate statistically significant difference between treatments.

| | | **N (mg g$^{-1}$ )** | | | **C (mg g$^{-1}$)** | | |
|---|---|---|---|---|---|---|---|
| ***Treatment*** | | ***Leaves*** | ***Stem*** | ***Roots*** | ***Leaves*** | ***Stem*** | ***Roots*** |
| Wt →Wt | Donor | 6.50$^{b}$ | 7.68$^{b}$ | 3.15$^{cd}$ | 85.05$^{bc}$ | 215.70$^{bc}$ | 52.62$^{bc}$ |
| | Receiver | 8.19$^{ab}$ | 6.90$^{b}$ | 3.01$^{cd}$ | 108.84$^{b}$ | 194.67$^{bc}$ | 47.98$^{bc}$ |
| Wt → *ram1-1* | Donor | 2.96$^{c}$ | 3.17$^{cd}$ | 1.87$^{d}$ | 42.66$^{d}$ | 93.73$^{d}$ | 32.39$^{c}$ |
| | Receiver | 10.45$^{a}$ | 12.74$^{a}$ | 7.68$^{a}$ | 187.37$^{a}$ | 296.82$^{a}$ | 136.52$^{a}$ |
| Wt (Sh)→Wt (NSh) | Donor | 10.18$^{a}$ | 6.76$^{b}$ | 4.86$^{b}$ | 100.51$^{b}$ | 219.70$^{bc}$ | 61.97$^{b}$ |
| | Receiver | 2.11$^{c}$ | 2.87$^{d}$ | 1.93$^{d}$ | 42.61$^{d}$ | 153.91$^{ab}$ | 46.81$^{bc}$ |
| Wt (Sh)→ *ram1-1* (NSh) | Donor | 4.95$^{bc}$ | 5.16$^{bcd}$ | 3.76$^{bc}$ | 52.97$^{cd}$ | 212.57$^{bc}$ | 55.95$^{bc}$ |
| | Receiver | 2.71$^{c}$ | 5.99$^{bc}$ | 5.16$^{b}$ | 56.65$^{cd}$ | 261.21$^{cd}$ | 128.28$^{a}$ |

***C and N transfer***

As indicated by at% $^{15}$N excess and $^{15}$N content, donor plants were enriched in $^{15}$N, especially for the donors sharing a CMN with the Wt receiver (Table 4). This means that the

$^{15}$N-Urea applied to the leaf was absorbed and translocated to other plant tissues (leaves, stem and roots) and effectively made the donor enriched in $^{15}$N. The receiver plants, on the other hand, had significantly smaller $^{15}$N contents in their tissues, as compared to all donors, and the amounts did not differ between treatments.

Table 4: at% $^{15}$N excess and $^{15}$N contents of donor and receiver plants on shaded and non-shaded treatments, 15 days after labeling. Values are mean of 5 or 6 biological replications, and different letters indicate statistically significant difference between treatments.

| **Treatments** | | **at% $^{15}$N excess** | **$^{15}$N content (mg)** |
|---|---|---|---|
| Wt→Wt | Donor | 2.901 $^{a}$ | 0.492 $^{a}$ |
| Wt→ *ram1-1* | | 2.239 $^{ab}$ | 0.212 $^{c}$ |
| Wt (Sh)→Wt (NSh) | | 1.819 $^{b}$ | 0.395 $^{ab}$ |
| Wt (Sh)→ *ram1-1* (NSh) | | 1.925 $^{ab}$ | 0.311 $^{bc}$ |
| Wt→Wt | Receiver | 0.005 $^{c}$ | 0.001 $^{d}$ |
| Wt→ *ram1-1* | | 0.005 $^{c}$ | 0.002 $^{d}$ |
| Wt (Sh)→Wt (NSh) | | 0.015 $^{c}$ | 0.001 $^{d}$ |
| Wt (Sh)→ *ram1-1* (NSh) | | 0.012 $^{c}$ | 0.002 $^{d}$ |

Between 0.226 and 1.002% of the added $^{15}$N was transferred to the receiver plants (Table 5). The largest %N transfer was observed in *ram1-1* receivers, sharing a CMN with the non-shaded donor, while 0.879% N was transferred to *ram1-1* receivers sharing a CMN with shaded donor. Wt plants received significantly less $^{15}$N from donor, especially when donors were not shaded (0.226% N).

Table 5: Transfer of the $^{15}N$ added to the donor plants to the receiver plants on shaded and non-shaded treatments, 15 days after labeling. Values are mean of 5 or 6 biological replications, and different letters indicate statistically significant difference between treatments.

| **Treatment** | **%$^{15}$N transfer** |
|---|---|
| Wt→Wt | 0.226 [b] |
| Wt→ *ram1-1* | 1.002 [a] |
| Wt (Sh)→Wt (NSh) | 0.521 [ab] |
| Wt (Sh)→ *ram1-1* (NSh) | 0.879 [a] |

Differently from the $^{15}N$, $^{13}C$ was absorbed by the labeled leaf but not translocated to other leaves, stem or roots (data not shown). Therefore, transfer of C between donor and receiver plants, as well as C investment to fungi partner could not be tracked nor calculated.

***Substrate and leachate analysis***

Donor plants' substrate had the largest at% $^{15}N$ excess and $^{15}N$ contents, while very little $^{15}N$ was found in the substrate of the receiver plants (Table 6). The same pattern was observed for the collected leachate. Shaded Wt donor compartment sharing a CMN network with a non-shaded Wt neighbor had the largest $^{15}N$ contents in both substrate and leachate (0.043 and 0.030 mg of $^{15}N$, respectively).

Table 6: at% $^{15}N$ excess and $^{15}N$ contents of substrate and leachates in donor and receiver compartments, on shaded and non-shaded treatments, 15 days after labeling. Values are mean of 5 or 6 biological replications, and different letters indicate statistically significant difference between treatments.

| | | ***at%$^{15}$N excess*** | | ***$^{15}$N content (mg)*** | |
|---|---|---|---|---|---|
| **Treatment** | | **Substrate** | **Leachate** | **Substrate** | **Leachate** |
| Wt→Wt | Donor | 0.022 $^{bc}$ | 0.014 $^{b}$ | 0.012 $^{b}$ | 0.024 $^{ab}$ |
| Wt→ *ram1-1* | | 0.013 $^{c}$ | 0.010 $^{b}$ | 0.007 $^{b}$ | 0.017 $^{ab}$ |
| Wt (Sh)→Wt (NSh) | | 0.058 $^{ab}$ | 0.044 $^{a}$ | 0.043 $^{a}$ | 0.030 $^{a}$ |
| Wt (Sh)→ *ram1-1* (NSh) | | 0.068 $^{ab}$ | 0.007 $^{b}$ | 0.028 $^{ab}$ | 0.005 $^{bc}$ |
| Wt→Wt | Receiver | 0.002 $^{c}$ | 0.000 $^{b}$ | 0.001 $^{b}$ | 0.001 $^{c}$ |
| Wt→ *ram1-1* | | 0.002 $^{c}$ | 0.001 $^{b}$ | 0.001 $^{b}$ | 0.001 $^{c}$ |
| Wt (Sh)→Wt (NSh) | | 0.003 $^{c}$ | 0.003 $^{b}$ | 0.002 $^{b}$ | 0.001 $^{c}$ |
| Wt (Sh)→ *ram1-1* (NSh) | | 0.002 $^{c}$ | 0.008 $^{b}$ | 0.001 $^{b}$ | 0.004 $^{bc}$ |

**Discussion**

Donor plants, which were directly transplanted into the AM inoculum compartment, were fully colonized. Similarly, receiver plants, which were growing in the sterile substrate, were also fully colonized at the time of harvesting, meaning that AM fungi from the donor plant were able to grow, cross the membrane between plant compartments and colonize receiver plant, sharing therefore a CMN. In addition to that, the high abundance of arbuscules in both plants, which is recognized as the site of exchange during symbiosis, demonstrate that both plants were successfully connected by a common mycorrhizal network and able to exchange nutrients between partners.

Mycorrhization rate in our study remained high even when plants were shaded. In previous studies, *Medicago truncatula* has been shown to be very sensitive to shading, in which even short-term shading (for 1–2 weeks) has been shown to reduce the mycorrhizal colonization of plants (Olsson et al., 2010; Konvalinková et al., 2015), due to a decrease on C allocation to the root system. In such situations, C fluxes are particularly re-allocated to shoot meristems instead, in order to compensate for the decrease in the photosynthetic activity (Schmitt et al., 2013). This pattern was observed in previous studies when analyzing a single host plant and fungi interaction. However, when two plants share a mycorrhiza network, fungi increase their possible sources for C acquisition since its able to receive C from different connected hosts simultaneously. This might affect shading response, as observed in the present study, in which

shading did not affected mycorrhization rate. This data is in agreement with what of Fellbaum et al. (2014), where shading did not affect mycorrhization rate of the treatments in which at least one of the plants in the network were not shaded. In the cited paper, authors suggested that fungi might use part of the C provided by the non-shaded plant to sustain a high colonization rate in low-quality hosts. This was suggested as a fungi strategy in case of the loss of a high quality host. In our study, however, this could be true for the plants connected to a non-shaded Wt receiver, but not with the *ram1-1* mutant type, due to the defected arbuscules in which C could not be transported from plant to fungi partner. In this context, non-shaded *ram1-1* receivers had the smallest mycorrhization rate, especially regarding AF% and VF%, regardless the shading condition of the donor plant. This means that, even under shading, Wt donor connected to *ram1-1*, could still sustain a high mycorrhization rate in the *ram1-1* neighbor along the time of the experiment.

Likewise, shading also did not affect shoots and roots of donor plants, but affected the shoot biomass of the receiver in some cases. Root biomass was apparently driven by plant type rather than by shading treatment, with the *ram1-1* obtaining the largest root biomass. This might be due to the failure of arbuscules formation, which does not allow fungi to transfer nutrients to its host, forcing the plant to invest in its roots system in order to increase absorption of nutrients in the soil. Despite of the lack of effects of shading on plant biomass production, a reduction of C allocation to fungi partner would still be expected. Fellbaum et al. (2014) reported a reduced C allocation from shaded plants to fungi partner under short term shade (6 days), before any effect could be observed in the host plants. In the referred study, the authors also reported that fungi was able to discriminate between low and high quality donor and allocate resources accordingly during this short term shading.

In our study, we hypothesized that by shading donor plants, N transferred to receiver plants is increased, once non-shaded receivers would be able to produce more C to beexchanged by transported N. This was expected to occur if direct fungal connections are the main pathway for resource allocation. In our study, besides of a tendency of greater $\%^{15}N$ transfer to Wt receiver plants in the treatments with shaded donor plants (0.266% $^{15}N$ transfer at non-shaded treatment compared to 0.521% $^{15}N$ transfer in shaded treatment), differences found in the present study were not statistically significant. This trend, however, could be an indication that shading can potentially affect direction of N transfer, in which fungi can recognize the host plant able to allocate more C and regulate N transfer accordingly. In addition,since this pattern was observed only in the treatment in which Wt was the receiver plant, it

demonstrates that transfers to *ram 1-1* receiver occurred indeed via indirect means, in which fungi had no choice between plants involved into the network.

Another indication for a preferential indirect pathway of resource translocation between connected plants in the present study was the significantly larger %$^{15}$N transferred to *ram1-1* receivers compared toWt receivers. Therefore, transferred $^{15}$N must have occurred via leakage from the fungi hyphae into substrate and subsequent uptake by roots of receiver plant. In addition, *ram1-1* plants had larger roots biomass as compared to Wt plants (Table 2), which might have increased its access to the leaked N in the soil, explaining greater $^{15}$N found in such plants. Other authors have also stated that N transfer might occur mainly by root exudation and to a lesser extent via mycorrhizal networks (He et al. 2006; Jalonen et al. 2009).

Along with the preferential pathway for resources exchange between plants connected by a CMN, another frequent discussed topic is the importance of the transferred amounts for plant fitness. In our study, small amounts have been transferred from donor to receiver plants, with the %N transferred ranging between 0.226% and 1.002%. N transfer rate has shown a high variability in the literature. In the agroforestry ecosystem, for example, N transfer is reported to range from −0.1 to 12% in pot experiments (Chu et al. 2004; Meng et al., 2016) and from 1.9to 16% under field conditions (Chapagain and Riseman 2014; Zhang et al., 2020). He et al. (2019), for example, have reported N transfer from donor to the receiver plants between 0.09%and 0.22%. For the referred study, a microcosm experiment was performed to evaluate N transfer between three plant species (the broad-leaved evergreen tree *Ci. Camphora* as a donorand the deciduous shrub *Broussonetia papyrifera* and the annual herb *Bidens pilosa*) connectedby an AM fungi network. Donor and receivers were physically separated by mesh membrane and an air gap. Labeling were applied in a root free compartment, on the donor compartment, being accessible only by the fungi. Once labeling solution were not applied directly to donor plant, it cannot be described as a plant-to-plant transfer. The reported transfer was even smallerthan the transfer found in our experiment, which ranged from 0.02% up to 1.0%. Wang et al. (2016) by using petiole injection of $^{15}$N into soybean to detect direct N to neighboring maize plants sharing a AM network, found a net transfer of 11.4% of $^{15}$N to maize when soybean were not co-inoculated with the rhizobia, and an increase to 54% net transfer when co-inoculated with the rhizobia. In the present experiment, a double nylon mesh and an air gap were used to prevent root intermingle and nutrients diffusion through the soil. However, it is known that indirect pathways cannot be completely prevented. Despite of the wide range of results, small amounts of transfer are more frequently reported, often demonstrating less than 1% of N transfer, with just a few exceptions showing larger values (up to 50%) (He et al., 2009; Chalk

et al., 2014). Observed variations on %N transfer between connected plants might be an effect of the differences in methodologies applied, plant and fungi combinations used, inconsistency in terminologies and others. Therefore, comparison made with other publication must be made with cautions.

It is not clear how these relatively small amounts of N transfer can influence adult plants fitness. In our study, the treatment in which direct transfer could have occurred was the one with the smallest transfer, with 0.226 and 0.521% of N transferred in non-shaded and shaded treatments, respectively. In this treatment, no effect was observed in plant biomass nor nutrition, therefore rejecting our hypothesis that proportion of N allocated from donor to receiver plants through mycorrhiza hyphae connections is significant and may improve neighboring plant nutrition

**Conclusions**

Several previous studies have examined the potential effects of common mycorrhizal networks for plant-to-plant resources transfer, which might affect intra- and interspecific competition connected among plants. However, all have suffered from inadequacies in clearly demonstrating that transfer is genuinely through hyphal connections, once indirect pathways could never be completely prevented and/or quantified. In this context, the present work have successfully proposed a novel experiment to overcome the previous problem.

Our study refines the relative importance of transfer via mycorrhizal networks versus other pathways. Our data demonstrated a larger $^{15}N$ transfer to *ram1-1* receiver plants, likely caused by the larger root biomass than the Wt, thus having a larger area of nutrient absorption. This indicates a more important role of indirect pathways for resources allocations in our system, and rejects our hypothesis that N transfer between connected plants occurs genuinely through hyphal connection, with the fungus acting as a hose for transport. In addition to the small amounts of N transferred between plants, the small N content found in the Wt receiver plants, rejects also our hypothesis that proportion of N allocated from donor to receiver plants through mycorrhiza hyphae connections is significant and may improve neighboring plant nutrition.

Irrespective of the pathway for resources exchange, the importance of mycorrhizal connections between plants should not be underestimated, once it can still facilitate transfer, even by indirect ways. In this context, associated fungi hyphae from one plant can spread and cover a large area of soil, leaking nutrients in the soil and rhizosphere, which can be uptake by roots of nearby plants or even for hypha from a neighboring fungus. Thus, CMN might boost

the capacity of receivers to get resources prevenient of neighbor's plants by increasing the volume of soil they have access to. With this, we conclude that CMN are important, but most likely by other means than discussed in the literature

**Data Availability Statement**

Raw and derived data supporting the findings of this study are available from the corresponding author AF on request.

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## 3. General Discussion

Most terrestrial plants establish symbiotic associations with fungi called mycorrhiza. Ectomycorrhizae (EM) and arbuscular mycorrhizae (AM) access limiting plant nutrients, e.g nitrogen (N). Some plant species may establish EM-AM dual-mycorrhizal associations, but very few is known about the advantage to a host plant of establishing dual-type mycorrhizae. Furthermore, EM and AM may interconnect plants via a common mycelial network (CMN) for N exchange. However, available studies fail to provide univocal evidence on the potential effects of the CMN networks mediating resource partitioning between connected plants, since transfer can occur via several routes simultaneously. In addition, quantification of the amount of possible nutrients transferred has also been challenging, leading to doubts regarding the importance of the CMN in inter-plant partitioning. In the current dissertation, I developed two studies to disentangle such questions: the first one aimed to investigate the nutritional advantages of dual *vs* single mycorrhizal plants, as well as the role of the network formed by each fungi for N transfer between connected plants. The second one aimed to quantify the direct transfer of N that occurs via the mycelium network in comparison to other possible indirect pathways. I have structured the present discussion in different sub-sections to jointly examine the most important findings obtained in the three different studies.

***Mycorrhization rate and plant response***

To reach the proposed objectives I used different plants for each of the experiments. In the first study, a dual mycorrhizal plant specie *P. x canescences* was used. Despite of the hability of associating with both fungi types, there was a preference for EM colonization in thisspecies. This is in accordance to what was found by Bojarczuk et al., (2015); which demonstrated a low colonization of AM fungus on young *P. x canescences* (3% RLC) comparedto EM fungus (50-60% RLC). In the second study, two different types of *Medicago truncatula(*an AM plant specie) were used: one was a wild type (Wt) while the other had a mutation in the transcription factor gene *RAM1-1* that impaired branching of arbuscules leading to the blockade of the transfer of C and nutrients between host plant and fungi. All plants were fastlycolonized by the AM fungi *R. irregularis*, but Wt plants had significantly higher mycorrhization rate compared to *ram1-1* mutants. Shading treatment did not affected mycorrhization rate.

The biomass production of plants in both studies was evaluated in order to estimate effect of mycorrhiza on plant growth. In the first study, despite of a preference for EM association, plant total dry biomass did not differ significantly (see Table 3), irrespective whether plants were colonized by AM, EM or both, even though we could observe a tendency of higher

response in terms of biomass in the dual mycorrhizal plants. However, all colonized plants had higher biomass in comparison to non-mycorrhizal controls, demonstrating a positive response in face of colonization, in all cases. Some studies had demonstrated disadvantages associated with hosting both AM and EM simultaneously, with dual-mycorrhizal plants having a reduced plant growth compared to single colonized plants (Misbahuzzaman & Newton, 2006; Meinhardt & Gehring, 2012). This might occur because dual colonization would require a higher investment of C from the host plant to sustain both fungi partners. However, a range of positive, negative and neutral growth response have been demonstrated before. A compilation of studies made by Teste et al., 2019, has shown that, overall, there are more frequently positive and neutral effects on dual colonization than negative ones.

In the second study, AM mycorrhization had a neutral effect on shoot biomass but it clearly affected root biomass. In this context, r*am1-1* plants had the highest root biomass in comparison to Wt plants, in all treatments. This can be explained due to the failure of arbuscules formation, which does not allow fungi to transfer nutrients to its host, forcing the plant to invest in its roots system in order to increase absorption of nutrients in the soil.

Despite of effects of mycorrhiza on plant growth, host plant may benefit from association in several means, such as improvement of plant nutrition, higher resistance against drought, heavy metals, protection against pathogens (Chen et al., 2018). Therefore, a combination of parameters must be considered when evaluating mycorrhizal benefits in host plant, and some of them will be discussed below.

***Nutrition and N uptake***

Mycorrhiza may improve growth through several means. One of them is by improving mineral nutrition of the host plant. In the first experiment involving dual mycorrhizal plants, higher nutrients concentrations was expected in plants colonized by AM and EM simultaneously, due to a complementarity in nutrient acquisition provided by both fungal partners. In our study however, concentration of P, Ca, K and Mg in dual mycorrhizal plants had intermediary values and did not differ from single colonized plants. The data agrees to what was found by Founoune et al. (2002) who performed one of the few studies evaluating uptake of several nutrients in dual mycorrhizal plants. They also found intermediary nutrients levels in dual EM-AM colonization compared to single colonized one. Gange et al., (2005) also found intermediary levels of N and P in *Eucalyptus urophylla* associated with both AM and EM simultaneously. However, different from the other nutrients, in the present study N concentration were significantly higher in the leaves and stem of the dual mycorrhizal plants

compared to the singles colonized ones. This demonstrates a nutritional advantage for N acquisition .when associated with both fungi types. It is known that the plant benefits from mycorrhizal fungal colonization depends largely on the environmental conditions. In most natural environments, which are characterized by mineral nutrient deficiency and various abiotic stress conditions, mycorrhizal plants are assumed to have a selective advantage over non-mycorrhizal individuals of the same species (Chen et al., 2018). Since the N was the limitng nutrients our study, this might have lead to the more pronounced benefit in N nutrition in face of colonization, compared to the other nutrients evaluated. This is an important finding, since N concentrations in plant leafs is known to strongly affect the photosynthetic rate and other photosynthetic parameters (Hikosaka, 2004; Luo et al., 2013; Shi et al., 2020), which may also lead to a better growth and resistance of such plants compared to single colonized ones. Moreover, a higher photosynthesis rate would lead to a higher production of C compounds which can be delivered to the fungus in order to get more access to limiting nutrients in the soil (Smith & Read, 2010). This higher production of C compounds might also compensate for higher C sink strength of harbouring both fungi simultaneously (Smith & Read, 2010; Ekblad et al., 2013), therefore leading to a better performance of dual colonized plants.

Interestingly, when comparing AM and EM single colonized plants, I could observe an influence in the concentration of certain nutrients to be related to a specific fungi type. The AM fungi improved Ca and Mg content in aboveground tissues of host plants, while EM improved P and K content. Some authors have already demonstrated that AM and EM are able to affect concentration of nutrients differently in the different plant tissues (Jentschke et al., 2001; Garcia & Zimmermann, 2014; Begum et al., 2019). However, knowledge of the role of the different mycorrhiza types in the uptake of nutrients other than P and N is limited and variable, especially for simultaneous colonization (Meding et al., 2008; Holste et al., 2017; Teste et al., 2019).

In contrast, in the second study, AM mycorrhiza apparently played a minor role to determine concentration of nutrients in plant tissues, including N. Since *ram1-1* plants fail to form a functional symbioses and no exchange of nutrients occur with the association, a lower nutrition would be expected in such plants. However, *ram1-1* plants had equal or even higher nutrient content compared to the Wt plant, demonstrating that uptake must have occur via indirect pathways and not directly via fungi. Likewise, if AM would be regulating such transfers, shaded plants would be expected to have lower nutrition since its hability to produce and exchange photosynthetic derived C for nutrients uptaked by the fungi would be significantly reduced (Kiers et al., 2011; Fellbaum et al., 2014). Although I did observe some effects of the shading on the allocation of some nutrients within plant tissues, this pattern are

not likely to be controlled by the mycorrhizal association, since the highest amount of some nutrients were found in the shaded plants (such as K and P). Some authors have previously stated that mycorrhizal pathway for N uptake and transfer is quantitatively unimportant. This is based on the premise that inorganic N ($NO_3^-$ or $NH_4^+$), which is considered the main sourceof N uptake by AM fungi, is highly mobile in soil (more than other nutrients such as P) and that organic sources of N are usually unavailable to AM fungi (Smith & Smith, 2011). This high mobility is believed to increase access of roots to soil N, even in depleted soil, therefore leading to a lesser influence by the mycorrhiza on plant N uptake. This might be true for other nutrients in the soil too. In addition, variations in uptake and transfer of nutrients between mycorrhiza and host plant might be influenced by several biotic and abiotic patterns, which supports the wide rage of data observed in literature and also the difference found in our studies (Begum et al., 2019; Berger & Gutjahr, 2021).

Together with plant benefits, I also evaluated C allocation within plant tissues. In the first study, single colonized plants had the highest C content in their roots, irrespective of the fungi type. This can represent either a higher C investment from the host plant to its fungi partner when no choice is given, or the effort of the plant to invest into root development in order to increase nutrient uptake. In our second study, *ram1-1* receiver roots was the one with the highest C contant, probably as reflect of the higher root biomass in this plants type. As previously stated, higher investment in root development in *ram1-1* plants might be a results of the constraint of a functional symbiosis due to the impaired arbuscles. However, a better understanding of the C investment from host plant to associated fungi would be required for a better understanding of the exchanges patterns. I attempted to evaluate this by applying $^{13}C$ labelled solution to donor plants and tracking C allocation into fungi tissues. Results of the lableing experiment will be discussed further.

***C and N transfer via CMN***

With the dual $^{13}C$ and $^{15}N$ labeling approach, I aimed to track C and N transfer between plants, as well as the C investment of plants to fungi partner. However, the applied $^{13}C$ to the donor plant in both studies were retained in the labeled leaf and not translocated to other plant tissues or even neighbouring plants in significant amounts. Therefore, such investment could not be estimated in a more precise manner, nor the C transfer between connected plants. Further investigation would be necessary, probably using longer times or several consecutive labellings.

For the N labeling, however, a significant amount of $^{15}N$ was found in the labelled donor tissues already in the first harvesting in both experiments. Therefore, any possible translocation

of N from donor to receiver plants could be evaluated. In our first study no transfer of N was observed for any of neighboring plants sharing a mycorrhiza network with labeled donor, irrespective of the fungi type, which may indicate that no transferred occurred between plants. In our second study, however, a small transfer of $^{15}N$ was observed to both Wt and *ram1-1* receiver plants. The observed lack of transfer in our first study might be an effect of plant type and age. Once the experiment was developed with Poplar seedlings, which is considered a fast growing woody plant (Shi et al., 2015), all N acquired might have been used for plant development instead of transfer.

Nevertheless, the transfer in our second study was higher to *ram1-1* receiver plants than to Wt, which is an indication for a preferential indirect pathway of resource translocation between connected plants. Transfer of $^{15}N$ occurred likely via leakage from the fungi hyphae into substrate and subsequent uptake by roots of receiver plant. In addition, *ram1-1* had higher roots biomass compare to Wt plants, which might have increased its access to the leaked N in the soil, explaining greater $^{15}N$ found in such plants. Other authors have also stated that N transfer might occur mainly by root exudation and, to a lesser extent, via mycorrhizal networks (He et al. 2006; Jalonen et al. 2009). In this study, I also hypothesized that by shading donor plant, N transferred to receiver plants would increase, since non-shaded receivers would be able to produce more C to be exchanged by transported N. This would likely to occur if direct fungal connections were the main pathway for resource allocation. In our study, besides of greater $\%^{15}N$ transfer to Wt receiver in the treatments where donor were shaded (0,266% $^{15}N$ transfer at non-shaded treatment compared to 0,521% $^{15}N$ transfer in shaded treatment), differences found in the present study were not statistically significant. This trend, however, could be an indication that shading can potentially affect direction of N transfer, in which fungi can recognize the host plant able to allocate more C and regulate N transfer accordingly. In addition, this pattern were observed only in the treatment in which Wt was the receiver plant, demonstrating that this might likely be an effect from fungi network in this case. But, since the highest highest amounts of $^{15}N$ was transfered to *ram 1-1* receiver, I could conclude that indict pathways might play a bigger role for N transfer within the mycorrhiza network.

Along with the preferential pathway for resource exchange between plants connected by a CMN, another frequently discussed topic is the importance of the transferred amounts for plant fitness. In our study, small amounts have been transferred from donor to receiver plants, with the %N transferred ranging from 0,226% to 1,002%. N transfer rate has shown a high variability in the literature. N transfer studies have been previously performed mainly from N fixing plants to non-fixing neighbours, with data ranging from 0 to 50% of transfer when using

$^{15}N$ enrichment or natural abundance methods (He et al., 2009; Chalk et al., 2014). In the agroforestry ecosystem, N transfer is reported to range from −0.1 to 12% in pot experiments (Chu et al. 2004; Meng et al., 2016) and from 1.9 to 16% under field conditions (Chapagain and Riseman 2014; Zhang et al., 2020). He et al., (2019) have reported N transfer from donor to the receiver plant (% N transfer) between 0.09% to 0.22%. There were also studies involving N transfer between non-fixing plants, such as the one made by He et al. (2006) using woody plants, in which very low N (ranging from 0.001 to 0.01%) had been translocated to nearby plants in a 4-week experimental period, which is even lower than the transfer found in our second study. Despite of the wide range of results, small amounts of transfer are more frequently reported, often demonstrating less than 1% of N transfer, with just a few exceptions showing higher values (up to 50%) (He et al., 2009; Chalk et al., 2014).

In addition, besides all attempts of many of those experiments in preventing indirect pathways, such as the use of mesh barriers and an air gaps, indirect pathways cannot be completely prevented. Leackage from the hyphae into neighbouring compartment and subsequent uptake via roots could still occur. Therefore, the use of *ram1-1* in our second study could provide a more efficiency way to measure the real importance of fungi network for N transfer between plants. Indeed, I found a very small amount transferred via CMN (0,226 and 0,521% of N transferred) and indirect pathway (0,879 and 1,002% of N transferred) played a bigger role. It is not clear how these relatively small amounts of N transfer can influence adult plants fitness. In our study, no effect were observed in plant biomass nor nutrition, therefore it is likely that the proportion of N allocated from donor to receiver plants through mycorrhiza hyphae connections is not significant and not sufficient to improve neighboring plant nutrition

Even though N transfer has been widely recognized as an important effect of the mycorrhizal network, the high variations of transfer found in literature and also the divergence of transfer found in both of our studies, demonstrate that the importance of such fluxes might depend on parameters that are not well understood. Small or no transfer might indicate that boundary conditions might overlay and overcompensate this transfer or even hinder it from happening at all. These might be biotic factors like plant type and development stage as well as nutritional status or nutrient stoichiometry as well as abiotic factors like nutrient availability and general substrate characteristics as well as complex interactions of all these factors. Therefore, previous studies demonstrating transfer via CMN might not be invalidate. In addition, irrespective of the pathway for resources exchange, the importance of mycorrhizal connections between plants should not be underestimated, once it can still facilitated transfer, even by indirect ways. In this context, associated fungi hyphae from one plant can spread and

cover a large area of soil, leaking nutrients in the soil and rhizosphere, which can be uptake by roots of nearby plants or even for hypha from a neighboring fungus. Thus, CMN might boost the capacity of receivers to get resources prevenient of neighbor's plants by increasing the volume of soil they have access to. With this, I conclude that the CMN are important, but most likely by other means than discussed in the literature

## 4. General Conclusion

From this thesis, new knowledge on the dual mycorrhizal plant N nutrition and N translocation over network has been acquired. This is the first study that has simultaneously investigate the performance of single and dual-mycorrhizal plants with regard to N nutrition and sharing via CMNs. Also, based on the review on previous studies made on N translocation between plants connected by a CMN; I realized that evidence of connection and quantification of the direct pathway was missing. This study has shown for the first time a powerful experimental design using mutant plants with reduced arbuscular formation that can be used for a more accurate idea about resource allocation and plant physiological responses that are truly accountable to CMN over indirect pathways. Some of the hypothesis proposed at the beginning were rejected and they are summarized here in detail:

**H1:** Host plants establishing dual mycorrhiza associations will exhibit an enhanced N nutrition, compared to those depending on single associations. This hypothesis was confirmed in our experiment. I demonstrated the nutritional advantage regarding N uptake for host plants holding dual mycorrhizal plants, compared to single colonized plants. With this, I can conclude that simultaneous associations of plants with AM and EM may represent a strategy of plants to improve their N nutrition and my play a role for plant species survival, if favourable conditions are met

**H2:** Dual mycorrhizal plants will preferentially share more N to plants sharing an EM association, due to its larger mycelium proliferation compared to AM. This was rejected. Albeit plants were well connected through a mycorrhiza network and donor plants were significantly labeled with $^{15}N$, no transfer of N occurred between donor and receiver plants irrespective if neighbour were connected by an AM or EM network. Therefore, the results here presented suggest that CMN functioning for N transfer might occur only under specific situations, such as for particular plant–fungus combination, the characteristics of connected plants or abiotic conditions.

**H3:** N transfer between connected plants occurs genuinely through hyphal connection, with the fungus acting as a hose for transport, rather than indirect pathways. This hypothesis was rejected. The data demonstrated a higher $^{15}N$ transfer to *ram1-1* receiver plants. The highest

$^{15}N$ found in the *ram1-1* plant summed with the highest root biomass observed in this plant which increasing its area of nutrients absorption, may indicate a more important role of indirect pathways for resources allocations in our system. With this, I conclude that CMN are important, but most likely by other means than discussed in the literature

**H4:** The proportion of N allocated from donor to receiver plants through mycorrhiza hyphae connections is significant and may improve neighboring plant nutrition. This hypothesis was rejected. In the present study, the treatment in which direct transfer could have occured was the one with the lowest transfer, with 0,226 and 0,521% of $^{15}N$ transferred in non-shaded and shaded treatments respectively. In this treatment, no effect were observed in plant biomass nor nutrition

**H5**: By shading donor plant, N transferred to receiver plants is increased, once it might be able to produce more C to be exchanged by transported N. Our data partially agree with that. In the present study, besides of greater $\%^{15}N$ transfer to Wt receiver in the treatments where donor were shaded (0,266% $^{15}N$ transfer at non-shaded treatment compared to 0,521% $^{15}N$ transfer in shaded treatment), differences found in the present study were not statistically significant. This trend, however, could be an indication that shading can potentially affect direction of N transfer, in which fungi can recognize the host plant able to allocate more C and regulate N transfer accordingly. In addition, since this pattern were observed only in the treatment in which Wt was the receiver plant, it demonstrate that transfers to *ram 1-1* receiver occurred indeed via indict means, in which fungi had no choice between plants involved into the network.

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# 5. Appendix

## 5.1. Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der Naturwissenschaftliche Fakultät der Gottfried Wilhelm Leibniz Universität Hannover zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:
*"Unravelling the importance of mycorrhiza for plant nutrition and transfer into the networks"*am Lehrstuhl für Bodenkunde unter der Anleitung und Betreuung von Prof. Dr. Georg Guggenberger gemäß §6(1) der Promotionsordnung eigenständig verfasst habe. Ich versichere, dass ich keine anderen, außer den genannten Literaturquellen und Hilfsmitteln, verwendet habe. Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

## 5.2. Curriculum Vitae

# A L I N E  F I G U E I R E D O

Environmental Engineering and Researcher

### SHORT PROFILE

- Expertise in microbiology, soil science, plant cultivation, plant-microorganisms interactions, isotopes, ecosystem functioning, ecology.
- Work independently and self-organized
- Experience in writing scientific projects, proposal and publications
- Multicultural teamwork
- Communicative, creative and proactive

### PERSONAL INFORMATION

**CITIZENSHIP:** Brazilian

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### EDUCATION

**DOCTORATE´S DEGREE,**
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**MASTER´S DEGREE,**
Chemistry in Agriculture and Environment
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**BACHELOR´S DEGREE,**
Environmental Engineering
School of Engineering of Piracicaba – Brazil
2008-2013

### WORK EXPERIENCE

**DOCTORAL CANDIDATE**
Institute of Soil Science | Leibniz Universität Hannover
2016 — the present

- Plant and fungi cultivation
- In vitro and greenhouse experiments
- Stable isotopes
- Project management
- Writing projects reports and presentations
- Multicultural teamwork
- Supervision of Bachelor and Master students
- Publication in scientific journals

**ENTREPRENEUR AND FOUNDER OF THE COMPANY ANAUÁ AMBIENTAL Ltda**
Anauá Ambiental Ltda | 2015 — 2016

- Environmental Consultancy
- Project Management
- Supervision of projects for urban solid waste treatment
- Reports preparations for the public and private sector
- Monitoring of reforestation projects
- Monitoring projects for construction of solid waste treatment and urban water treatment plants

# A L I N E  F I G U E I R E D O

Environmental Engineering and Researcher

## S K I L L S

**|Languages**

Portuguese - native

English – fluent

German – Intermediary

Spanish – Very Good

**| Technical**

Microsoft Office

Sigma Plot

AutoCAD

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2009 — 2012

- Soil chemistry
- Soil Physics
- Soil microbiota
- Techniques for protection of native Forest
- Techniques for sustainable agriculture
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- Biogeochemistry of Tropical Ecosystems
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- Project management
- Stable Isotopes
- Physical and Chemical properties of plant litter
- Limnology

## E X T R A  C O M M I T M E N T S

- Voluntary Work with partnership with the company ArcelorMittal for Environmental Education (2009).
- 8th Internacional Symposium on Ecosystem Behavior – BIOGEOMON (Germany). Poster presentation: "C3 and C4 litterfall decomposition in streams of the Atlantic Forest under different land covers"
- Course "Stable Isotopes in Forest Ecosystem Research", Université de Lorraine, at INRA Nancy Lorraine, France (2017).
- Organization committee of the 5th Doctoral´s Research Conference - at Herrenhäuser Campus, Hannover, Germany. Talk: "Revealing pathway of N allocation between plants connected through a mycorrhiza network" (2019).

## 5.3. List of Publications

- SILVA, M. C. P.; FIGUEIREDO, A. F.; ANDREOTE, F. D.; CARDOSO, E. J. B. N. Plant growth promoting bacteria in Brachiaria brizantha. World Journal of Microbiology & Biotechnology, v. 28, p. 9, (2012).
- BINI, D.; FIGUEIREDO, A. F.; SILVA, M. C. P. da; VASCONCELLOS, R. L. F.; CARDOSO, E. J. B. N. Microbial biomass and activity in litter during the initial development of pure and mixed plantations of Eucalyptus grandis and Acacia mangium. Journal of Soil Science, v. 37, p. 76-85, (2013).
- FIGUEIREDO, A. F.; AUGUSTO, F. G.; DELLA COLETTA, L.; DUARTE-NETO, P. J.; MARTINELLI, L. A. Comparison of microbial processing of Brachiaria brizantha, a C4 invasive species and a rain forest species in tropical steams of the Atlantic Forest of South-Eastern Brazil. Marine and Freshwater Research, (2018), 69, 1397–1407.
- AUGUSTO, F. G; FIGUEIREDO, A. F.; CAMARGO, P. B.; DELLA COLETTA, L.; MAZZI, E. A.; MARTINELLI, L. A. C3 and C4 plants leaf breakdown and assimilation by aquatic macroinvertebrates in streams of the Brazilian Atlantic Forest. Marine and Freshwater Research, (2019).
- FIGUEIREDO, A. F.; BOY, J.; GUGGENBERGER, G. Common Mycorrhiza Network: Theories and mechanisms behind underground interactions. Frontiers in Fungal Biology, v. 2, p. 48, (2021).
- FIGUEIREDO, A. F.; DE LA FUENTE, A. A.; SAUHEITL, L.; BOY, J.; GUGGENBERGER, G. Higher N uptake in dual-mycorrhizal plants (AM and EM) in comparison to single-type mycorrhizal plants (AM or EM) and possible nutrient sharing via common mycorrhizal networks. – Submitted.
- FIGUEIREDO, A. F.; DE LA FUENTE, A. A.; BOY, J.; SAUHEITL, L.; GUGGENBERGER, G. Revealing pathway of nitrogen transfer between plants connected through a mycorrhiza network – To be submitted.