*Journal of Experimental Botany*
https://doi.org/10.1093/jxb/erab489 Advance Access Publication 17 November, 2021

REVIEW PAPER

# Split down the middle: studying arbuscular mycorrhizal and ectomycorrhizal symbioses using split-root assays

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Received 22 June 2021; Editorial decision 3 November 2021; Accepted 5 November 2021

Editor: Miriam Gifford, University of Warwick, UK

## Abstract

**Most land plants symbiotically interact with soil-borne fungi to ensure nutrient acquisition and tolerance to various environmental stressors. Among these symbioses, arbuscular mycorrhizal and ectomycorrhizal associations can be found in a large proportion of plants, including many crops. Split-root assays are widely used in plant research to study local and systemic signaling responses triggered by local treatments, including nutrient availability, interaction with soil microbes, or abiotic stresses. However, split-root approaches have only been occasionally used to tackle these questions with regard to mycorrhizal symbioses. This review compiles and discusses split-root assays developed to study arbuscular mycorrhizal and ectomycorrhizal symbioses, with a particular emphasis on colonization by multiple beneficial symbionts, systemic resistance induced by mycorrhizal fungi, water and nutrient transport from fungi to colonized plants, and host photosynthate allocation from the host to fungal symbionts. In addition, we highlight how the use of split-root assays could result in a better understanding of mycorrhizal symbioses, particularly for a broader range of essential nutrients, and for multipartite interactions.**

**Keywords:** Arbuscular mycorrhizal symbiosis, carbon, ectomycorrhizal symbiosis, legumes, nitrogen, nutrient transport, phosphorus, split-root, trees.

## Introduction

In the soil, roots are exposed to diverse abiotic and biotic stressors that affect the whole plant's growth, development, and physiology (Chapin *et al.*, 1987; Ramegowda and Senthil-Kumar, 2015). Distinction between root local and systemic responses to these stressors is important for understanding plant adaptation to varying environmental conditions, and often requires innovative research methods (Agapit *et al.*, 2020; Thilakarathna and Cope, 2021). Plant biologists developed a unique experimental design called a 'split-root system' in which roots are divided into two equal masses, still connected to the common shoot tissues, and transplanted into spatially separated compartments, pots, or boxes. Since root halves obtained with these techniques are physically separated yet still share common above-ground tissues, it is possible to investigate the responses triggered by any treatment applied to one root half on the other half via long-distance (root–shoot–root) signaling (Larrainzar *et al.*, 2014).

Establishing split-root assays can be challenging for some plant species, especially woody plants. In monocotyledons, the fibrous root systems can be easily divided into two equal halves,

without any destructive techniques, before transplanting into two root compartments (Bever *et al.*, 2009). In contrast, dicotyledons have one main root system dominated by a tap root. To produce two homogeneous root halves in these plants, additional steps are necessary (recently reviewed in Thilakarathna and Cope, 2021). For example, a few days after seed germination, the tap root of young seedlings is often severed to facilitate the formation of lateral roots (Gil-Quintana *et al.*, 2013; Schaarschmidt *et al.*, 2013; Kafle *et al.*, 2019). Alternatively, the main roots can be longitudinally split to produce equal root tissues on both sides (Marino *et al.*, 2007), or two independent root seedlings can be grafted to one plant (Kassaw and Frugoli, 2012; Lyu *et al.*, 2020). Subsequently, the seedlings will be grown hydroponically, aeroponically, or directly in the growth substrate (Thilakarathna and Cope, 2021).

Although quite artificial, split-root approaches have been used for decades to analyze local versus systemic regulatory mechanisms in model and crop plants (Kassaw and Frugoli, 2012; Saiz-Fernández *et al.*, 2021). For example, this method has been used in *Arabidopsis thaliana* by various groups to reveal systemic signals that result from varying availability of soil nutrients, such as nitrogen (N) (Cambui *et al.*, 2011; Guan *et al.*, 2014), phosphorus (P) (Thibaud *et al.*, 2010), or sulfur (Hubberten *et al.*, 2012). Additionally, split-root systems have been used to reveal the impact of abiotic stressors, including salt stress, flooding, or drought in multiple plant species (e.g. Jackson, 1956; Mulholland *et al.*, 2002; Hafner *et al.*, 2017), the effect of nitrate on root architecture, N assimilation, and sugar transport in rice (Wang *et al.*, 2002), or the combined effects of biochar and N application on root enzyme activity and P mobilization (Song *et al.*, 2020), to cite a few. The study of beneficial root–microbe interactions also benefited from split-root assays, in particular for the investigation of the autoregulation of nodulation pathway (Kosslak and Bohlool, 1984; Suzuki *et al.*, 2008; Kassaw *et al.*, 2015), the regulation of biological N fixation and nitrogenase activity (Larrainzar *et al.*, 2014; Thilakarathna and Cope, 2021), the composition of root exudates (Luu *et al.*, 2017; Korenblum *et al.*, 2020), or the establishment and functioning of mycorrhizal symbioses (reviewed here).

Mycorrhizal symbioses are mutualistic interactions between soil fungi and plant roots, and form a key component of most terrestrial ecosystems (Smith and Read, 2008). Multiple types of mycorrhizal associations have been described, but the two most economically and ecologically important ones are the arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) symbioses. AM fungi belong to the subphylum of Glomeromycotina and interact with >65% of land plants, including many crop species (Wang and Qiu, 2006; Spatafora *et al.*, 2016). ECM fungi, belonging to the clades of Basidiomycota and Ascomycota, interact with ~5% of land plants and are major constituents of temperate and boreal forest ecosystems (Martin *et al.*, 2001; Becquer *et al.*, 2019). In both AM and ECM associations, the extraradical hyphae of symbiotic fungi acquire hydromineral resources from the soil and actively transfer them to colonize roots via specific structures called arbuscules or Hartig nets, respectively (Peterson and Massicotte, 2004; Javot *et al.*, 2007; Becquer *et al.*, 2019). In return, the host plant transfers up to 20–25% of its photosynthetic carbon (C) to the mycorrhizal cohort (Hobbie, 2006; Konvalinková and Jansa, 2016).

In this review, we synthesize the studies that used split-root assays to investigate mycorrhizal colonization events, interactions of mycorrhizal fungi with other soil microbes via plant systemic response, hydromineral transport from fungi to host plants, and C allocation from plant to fungal partners, in both AM and ECM symbioses. Additionally, we highlight areas within each topic where additional effort would be useful, and we describe how the use of split-root systems could help continue to enrich our understanding of these symbioses in controlled environments.

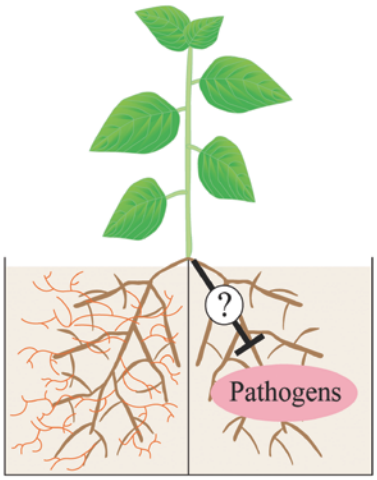
## Establishment of mycorrhizal symbioses and interactions with other soil microbes

In natural systems and agroecosystems, plant roots must cope with many types of soil microbes, beneficial, neutral, or pathogenic. In this context, split-root assays can aid in the study of successive and spatially separated colonization events with different beneficial microbes, and reveal local versus systemic regulatory mechanisms impacting microbial colonization. They can also physically separate mycorrhizal fungi and root pathogens, including fungi, bacteria, or nematodes, to investigate the systemic influence the former has on the latter (Fig. 1). In the works presented below, researchers colonized half of the roots with a mycorrhizal fungus, and the other half with another soil microbe, beneficial or not.

### *Dealing with multiple beneficial microbes*

The establishment of mycorrhizal symbioses involves a succession of steps that allows for mutual recognition between compatible plant and microbial partners (Garcia *et al.*, 2015; MacLean *et al.*, 2017; Choi *et al.*, 2018). Therefore, it is crucial for the plant to control the colonization events of compatible microbes. Experiments conducted in controlled environments using only one host plant and one fungus are reductive by definition, and do not always provide an accurate snapshot of what happens in natural ecosystems, where there are early and later fungal colonizers (Werner and Kiers, 2015).

Split-root approaches make it possible to study the interactions between early- and late-arrival AM fungi. Thus, it was observed that barley plants inoculated by different AM fungi beforehand [*Glomus mosseae*, *Rhizophagus irregularis* (formerly *Glomus intraradices*), or *Gigaspora rosea*] suppressed the subsequent colonization by *G. mosseae* in a distant root half, regardless of the external P availability (Vierheilig *et al.*, 2000*a*, *b*). Similar observations were made on subterranean clover




| | Host plant | Fungal symbiont | Pathogen | References |
|---|---|---|---|---|
| AM | *Solanum lycopersicum* | *Rhizophagus irregularis*<br>*Glomus mosseae* | *Phytophthora parasitica* | Cordier *et al.*, 1998<br>Pozo *et al.*, 2002 |
| | *Solanum lycopersicum* | *Glomus versiforme* | *Ralstonia solanacearum* | Zhu & Yao, 2004 |
| | *Hordeum vulgare* | *Glomus mosseae* | *Gaeumannomyces graminis* | Khaosaad *et al.*, 2007<br>Castellanos-Morales *et al.*, 2015 |
| | *Musa sp.* | *Rhizophagus irregularis* | *Pratylenchus coffeae*<br>*Radopholus similis* | Elsen *et al.*, 2008 |
| | *Solanum lycopersicum* | *Glomus mosseae* | *Meloidogyne incognita*<br>*Pratylenchus penetrans* | Vos *et al.*, 2012 |
| | *Vitis berlandieri* ×*V. riparia* | *Rhizophagus irregularis* | *Xiphinema index* | Hao *et al.*, 2012 |
| | *Medicago truncatula* | *Funneliformis mosseae* | *Aphanomyces euteiches* | Zhang & Franken, 2004 |
| ECM | —— | —— | —— | —— |

**Fig. 1.** Systemic resistance to root pathogens induced by arbuscular mycorrhizal and ectomycorrhizal fungi investigated in split-root assays. (A) Schematic diagram showing a split-root design to investigate the systemic impact of mycorrhizal fungi (orange lines) on pathogen infection on the host plant. (B) Reports using split-root assays to investigate systemic mycorrhiza-induced resistance in plants colonized by arbuscular mycorrhizal (AM) or ectomycorrhizal (ECM) fungi. The name of host plants, fungal symbionts, and pathogen, as well as the corresponding reference, are indicated. Missing information is represented by a horizontal line.

dually colonized by the AM fungi *Glomus* sp. and *Scutellospora calospora* (Pearson *et al.*, 1993). These experiments revealed the existence of a systemic regulatory mechanism in plants already colonized by an AM fungus that prevents the late arrival of other AM fungi, belonging to the same species or not, called 'autoregulation of mycorrhizas' (Wang *et al.*, 2018). Although still poorly understood, it has been recently reported that this autoregulation mechanism is mediated through the CLE53–SUNN genetic pathway that involves the plant CLE53 peptide whose expression is induced upon AM colonization and under high P (Müller *et al.*, 2019; Karlo *et al.*, 2020). Interestingly, hundreds of genes have been identified as possible regulated components in this pathway, indicating that many more genes involved in the autoregulation of mycorrhizas must be involved and remain to be described (Karlo *et al.*, 2020).

Legumes are also colonized by rhizobia bacteria which can fix atmospheric N in specific root structures called 'nodules'. Recent research has indicated an interdependency between AM fungi and rhizobia, and compatible combinations of these symbionts can result in synergistic effects on plant growth (Bournaud *et al.*, 2018). Controlling nodule number is crucial for the host plant, and is mediated by a mechanism called 'autoregulation of nodulation' (Ferguson *et al.*, 2019). Interestingly, components of this regulatory mechanism are also shared by the autoregulation of mycorrhiza pathway, including the shoot CLAVATA1-like receptor kinase NARK (Schaarschmidt *et al.*, 2013). Split-root approaches also revealed that previous inoculation by rhizobia was able to systemically suppress colonization by AM fungi in *Medicago sativa* (Catford *et al.*, 2003), in *M. truncatula* (Kafle *et al.* 2019), or even in barley, a non-nodulating plant (Khaosaad *et al.*, 2010). Even the application of Nod factors only, which are bacterial signaling molecules perceived by roots to trigger the symbiosis, was sufficient to systemically reduce the colonization of AM fungi (Catford *et al.*, 2003). Similarly, a *M. truncatula* mutant overproducing nodules (*nts1007*) showed distant suppression of AM symbiosis in split-root assays (Meixner *et al.*, 2005). In contrast, inoculation by *G. mosseae* or *R. irregularis* can systemically improve the number, size, and mass of nodules, as well as the nitrogenase activity, in soybean or *M. truncatula*, respectively (Ding *et al.*, 2016; Kafle *et al.*, 2019). These studies showed that multiple types of symbionts can be controlled by shared or independent regulatory mechanisms. The autoregulation of microbial symbionts is an important strategy for plants to control the allocation of fixed C towards multiple root colonizers, balancing their energy cost and optimizing the benefits provided by early- and late-arrival colonizers. Therefore, there is no doubt that split-root approaches will continue to be determinant in the identification of such regulatory mechanisms, particularly in crop species involved in multipartite beneficial associations.

In ECM symbiosis, the influence of timing of fungal introduction on colonization is crucial to maximize plant growth and benefits. Consequently, it has also been investigated using split-root approaches (Kennedy *et al.*, 2009). This system involved multiple fungal symbionts and one host to investigate whether early access to a plant gives preference to a fungus for successful colonization. Building on the role of priority in colonization, Kennedy *et al.* (2009) sought to distinguish differences between four *Rhizopogon* species colonizing the host tree *Pinus muricata*. Seedling roots were split into separated Petri dishes and each half was inoculated with spores from

one species of each *Rhizopogon*, but at different times. It was observed that a fungus introduced first was always more competitively successful in terms of root colonization, but that the second fungus to be introduced was still able to colonize the plant, to a lesser degree, if that root area was not already colonized (Kennedy *et al.*, 2009). A variation of the split-root design was also used to distinguish between effects of soil nutrient availability and host plant nutrient status on the successive colonization of early- and late-arrival ECM fungi (Lilleskov and Bruns, 2003). *Pinus muricata* was inoculated simultaneously with both the species *Rhizopogon occidentalis*, a dominant early-arrival colonizer, and *Tomentella sublilacina*, with more gradual colonization trends. To examine temporal relationships between plant status and colonization, the inoculated roots were first grown in a single compartment, then trained to grow into a separate compartment. Each compartment was supplemented, or not, with an additional nutrient source, and colonization was quantified at each phase in the initial compartment. Early colonization and decline of *R. occidentalis* were only affected by initial nutrient addition in the first phase, but *T. sublilacina* exhibited dynamic differences in colonization trends depending on nutrient addition to each compartment in both phases (Lilleskov and Bruns, 2003). This suggests that, while the colonization trends of both early- and late-arriving fungal species depend on soil nutrient availability, the successful long-term colonization of late-arrival ECM fungi may also depend on the nutrient status of the host plant. These responses in ECM plants differ from the autoregulation mechanisms described above, suggesting that strict ECM hosts might not control the colonization by their fungal partners in the same way that AM plants do. Indeed, there is no evidence so far that the presence of ectomycorrhizas on local or distant roots inhibits subsequent colonization by similar or other ECM fungal species.

Additional research is still needed to thoroughly investigate these mechanisms in ECM symbiosis, particularly on plant species, such as poplar or alder trees, that can be simultaneously colonized by AM and ECM fungi. Split-root systems can be a useful tool for further understanding the dynamics involved in these multisymbiont interactions, physically separating roots involved in different types of symbiosis, and revealing shared or distinct regulatory mechanisms.

### *Systemic resistance to pathogens induced by mycorrhizal fungi*

Significant crop loss can be attributed to plant pathogens, despite frequent and high doses of pesticide application (Fletcher *et al.*, 2006; Savary *et al.*, 2019). To reduce unintended consequences of these chemicals, bioprotectants such as mycorrhizal fungi could be an option to improve environmental health and food safety (Schouteden *et al.*, 2015; Chen *et al.*, 2018). Indeed, it has been largely documented that AM fungi can locally and/or systemically facilitate plant tolerance to root and foliar pathogens (Harrier and Watson, 2004; Campos-Soriano *et al.*, 2012; Schouteden *et al.*, 2015; Dreischhoff *et al.*, 2020). However, virus infection can also be enhanced in AM plants (Miozzi *et al.*, 2019). Only a handful of reports investigating these mycorrhiza-induced resistance/susceptibility phenomena have used a split-root system approach (Fig. 1). For example, AM fungi may play a bioprotectant role to decrease nematode infestations that can result in yield losses. Elsen *et al.* (2008) used a split-root assay to understand the possible systemic resistance in banana plants triggered by the AM fungus *R. irregularis* against two migratory nematodes, *Radopholus similis* and *Pratylenchus coffeae*. Significant reductions of both nematodes' densities were observed when the AM symbiosis was established in distant roots, indicating a systemic regulation of the pathogens. Similar observations were reported in tomato plants dually inoculated with the AM fungus *G. mosseae* and the root-knot nematode *Meloidogyne incognita* or the migratory root-lesion nematode *Pratylenchus penetrans* (Vos *et al.*, 2012), as well as in grapevine inoculated with *R. irregularis* and the ectoparasitic nematode *Xiphinema index* (Hao *et al.*, 2012). These two studies demonstrate that split-roots can be useful in understanding systemic signaling triggered by root-infesting nematodes upon mycorrhizal symbiosis.

Other studies used a similar approach to investigate bacterial and fungal pathogens. For example, it was described that tomato plants colonized by *Glomus versiforme* systemically produced more phenols in roots infected by the bacteria *Ralstonia solanacearum*, and limit bacterial proliferation (Zhu and Yao, 2004). Using a split-root assay, Cordier *et al.* (1998) and Pozo *et al.* (2002) also observed a lower disease index and lower presence of the fungal pathogen *Phytophthora parasitica* in tomato plants colonized by the AM fungi *G. mosseae* and/or *R. irregularis*. Interestingly, *G. mosseae* appeared to be more effective than *R. irregularis* in reducing *P. parasitica* infestation (Pozo *et al.*, 2002). A similar approach on four barley varieties previously colonized in split-root assays by *G. mosseae* showed contrasted biomass and symptom responses to the soil-borne fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Castellanos-Morales *et al.*, 2011). These studies indicate that the systemic resistance induced by AM fungi is dependent on both the fungal and plant species/cultivars. More recently, Zhang and Franken (2014) investigated the interaction between the AM fungus *Funneliformis mosseae* and the root fungal pathogen *Aphanomyces euteiches* using a split-root approach in the model legume *M. truncatula*. Interestingly, no systemic inhibition of the pathogen proliferation by the presence of the AM fungus was observed, and the pathogen abundance in roots was even higher in mycorrhizal plants. This observation contrasted with the results described by Hilou *et al.* (2014) where the same microbial species together colonized *M. truncatula* in a non-split-root system. Indeed, prior colonization by the AM fungus reduced pathogen proliferation and disease symptoms (Hilou *et al.*, 2014). This contradiction may be attributed to differences in plant growth conditions and/

or to direct interaction between the AM and pathogenic fungi (Zhang and Franken, 2014). This also indicates that single-pot experiments using the same microbial partners should also be considered when developing split-root assays that can be too artificial and suppress microbe–microbe interactions. Interestingly, pathogen infection and symptom severity can also be attenuated by a high degree of functional AM colonization that occurs in non-infested roots. Using a split-root approach with barley plants, Khaosaad *et al.* (2007) compared high versus low colonization by the AM fungus *G. mosseae* to understand how the degree of AM colonization systemically affects the abundance of the fungal pathogen *Gaeumannomyces graminis*. The results showed that a high degree of AM colonization was more effective at systemically suppressing the proliferation of the fungal pathogen compared with less colonized roots.

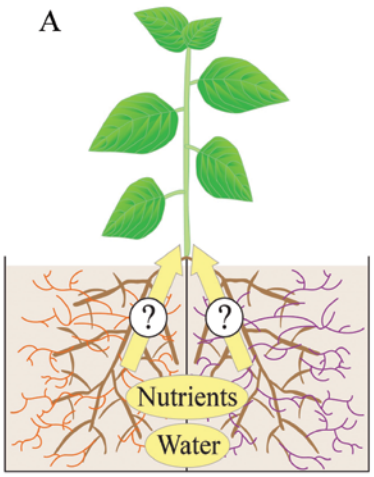
Identification of AM fungi effectively suppressing pathogens in a field setting would be an ideal application of mycorrhizal research (Chen *et al.*, 2018). However, these split-root studies revealed that the effectiveness of AM fungi to minimize the severity of pathogens is host and pathogen dependent, and that the suppression of pathogens also depends on the degree of AM root colonization. Additional work is needed to identify the regulatory mechanisms and signaling molecules translocating from AM roots to pathogen-infected roots in order to harness these mutualistic associations for pathogen mitigation in field conditions. Finally, although many studies investigated the role of ECM fungi in the resistance to some root pathogens (Smith and Read, 2008), to our knowledge, no split-root assays have been performed so far to understand the systemic resistance against pathogens induced by ECM fungi (Fig. 1).

## Resource allocation from mycorrhizal fungi to the host plant

Tracking nutrient and water transport from multiple mycorrhizal fungi to one host plant can be challenging due to the inability to differentiate the microbial origin of these resources. Using split-root assays with microbes having access to various types and/or amounts of resources can help to further understand the nutritional benefits that each symbiont provides. By separating resource access between mycorrhizal and non-mycorrhizal roots of the same plant, split-root systems can also be a powerful approach to distinguish between local and systemic signaling mediated by resource availability upon mycorrhizal symbioses (Fig. 2).

### *Arbuscular mycorrhiza-mediated nutrient transport in split-root assays*

Mycorrhizal fungi can provide their host plant with multiple resources, often including a combination of macronutrients and micronutrients in nutrient-limited soil (Courty *et al.*, 2016; Garcia *et al.*, 2016). However, when soil nutrients are not limited, plants can acquire their resources by themselves, excluding the mycorrhizal partners (Bonneau *et al.*, 2013; Nouri *et al.*, 2014; Breuillin-Sessoms *et al.*, 2015). The use of a split-root system in sorghum revealed that the root half receiving high P concentrations displayed a significantly lower colonization rate by the fungus *Glomus fasciculatus* than the half supplemented with a low P solution (Menge *et al.*, 1978). It was also described that only a colonized half of the entire root system of Carrizo citrange (*Poncirus trifoliata*×*Citrus. sinensis*) was enough to provide the host plant with necessary P amounts




| B | | Host plant | Fungal symbiont | References |
|---|---|---|---|---|
| AM | N | *Medicago truncatula* | *Rhizophagus irregularis* | Bonneau *et al.*, 2013 |
| | P | *Hordeum vulgare* | *Glomus fasciculatus* | Menge *et al.*, 1978 |
| | | *Poncirus trifoliata* x *Citrus sinensis* | *Rhizophagus irregularis* | Duke *et al.*, 1986 |
| | | *Medicago truncatula* | *Rhizophagus irregularis* | Bonneau *et al.*, 2013 |
| | | *Daucus carota* | *Rhizophagus irregularis* | Whiteside *et al.*, 2019 |
| | $H_2O$ | *Zea mays* | *Rhizophagus irregularis* | Bárzana *et al.*, 2015 |
| ECM | N | *Fagus sylvatica* | *ECM fungal community* | Mayerhofer *et al.* 2021 |
| | P | —— | —— | —— |
| | $H_2O$ | *Populus trichocarpa* x *deltoides* | *Laccaria bicolor* | Calvo-Polanco *et al.*, 2019 |

**Fig. 2.** Nutrient and water allocation from arbuscular mycorrhizal and ectomycorrhizal fungi to host plants in split-root assays. (A) Schematic diagram showing a split-root design to investigate the allocation of nutrients and water from two mycorrhizal fungi (orange and purple lines) to the host plant. Arrows and question marks represent the unknown amount of resource transported into the soil–fungus–plant continuum. (B) Reports using split-root assays to investigate nitrogen (N), phosphorus (P), other nutrients (Other), and water ($H_2O$) transport from arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi to host plants. The name of host plants and fungal symbionts, as well as the corresponding reference, are indicated. Missing information is represented by a horizontal line.

(Duke *et al.*, 1986). More recently, a split-root approach combined with quantum dot technology demonstrated that AM carrot roots were able to take up significantly more apatite (phosphate mineral) than non-colonized roots (Whiteside *et al.*, 2019).

To investigate the interaction between limited N and P supply in AM symbiosis, Bonneau *et al.* (2013) used a split-root system on *M. truncatula*. In these experiments, one half of the root system was inoculated with the AM fungus *R. irregularis* and supplied with limited N and P solution, while the other root half was kept non-colonized and watered with limited or sufficient N and P solutions, or solutions limited in N or P only. It was demonstrated that when non-colonized roots had access to P and N, a reduced colonization by the AM fungus was observed in the other root half (Bonneau *et al.*, 2013). Moreover, Balzergue *et al.* (2011) demonstrated that high external P concentrations triggered a systemic down-regulation of root excretion of strigolactones, which are a group of secondary metabolites acting as signals to attract AM fungi, suggesting that nutrient availability may also influence signaling for the symbiosis (Carbonnel and Gutjahr, 2014). This observation was confirmed at the molecular level. Indeed, the expression of *MtPT4* and *MtBCP1*, two genes coding for a P transporter and a blue copper protein, respectively, which are specifically expressed upon AM symbiosis, was strongly reduced in the AM roots when non-colonized roots were not limited in P and N (Bonneau *et al.*, 2013). This indicates that the fungal colonization was regulated by a systemic feedback repression influenced by the P and N status of non-colonized roots. Interestingly, *MtPT4* and *MtBCP1* were up-regulated in AM roots not only when the non-colonized roots were limited in P, but also when they were limited in N or in both nutrients, indicating a combined systemic effect triggered by both P and N limitations on the symbiotic functioning.

Other soil microbes can have a positive effect on plant nutrition. For example, protists colonize the rhizosphere of many plants and feed on soil bacteria, resulting in the release of a large amount of N that can then be taken up by roots (Uikman *et al.*, 1991). However, it is still unclear how a plant interacting with both types of symbionts would discriminate between these microbial N sources. Using a split-root approach, Henkes *et al.* (2018) recently demonstrated that distant inoculation by *R. irregularis* had a negative effect on N acquisition in roots colonized by protists (*Acanthamoeba castelanii*), indicating that the AM fungus dominated the N allocation in this tripartite association.

Although rather limited, the majority of studies investigating resource allocation in mycorrhizal symbioses focused on P and N in AM symbiosis. However, other nutrients are transported from the fungus to the plant, including potassium, sulfur, and micronutrients (Wipf *et al.*, 2014; Garcia *et al.*, 2017; Ruytinx *et al.*, 2020; Frank and Garcia, 2021). Therefore, efforts are still needed to investigate systemic versus local signaling induced by these other resources using split-root approaches in both AM and ECM associations (Fig. 2). Additionally, knowledge on shared and distinct transport mechanisms governing resource allocation originating from different beneficial microbes is still limited and would benefit from split-root assays.

### *Water allocation in mycorrhizal associations using a split-root approach*

The ability of AM and ECM fungi to improve water acquisition, as well as the molecular mechanisms ensuring this transport, are mainly studied in single-pot experiments, particularly under osmotic stress (Casieri *et al.*, 2013; Santander *et al.*, 2017; Xu and Zwiazek, 2020). However, to our knowledge, very few studies have used a split-root system to investigate water transport in AM association. Liu *et al.* (2007) reported a 4-fold up-regulation of a gene putatively coding for an aquaporin in *M. truncatula* roots colonized by the AM fungus *R. irregularis*, compared with non-mycorrhizal roots in a split-root assay. More recently, Bárzana *et al.* (2015) investigated the local and systemic effects of AM fungi on the expression of aquaporin-coding genes and the accumulation of osmolytes under water-limiting conditions affecting the whole plant or only one root half. Interestingly, it resulted in a both local and systemic accumulation of proline, an osmolyte involved in water potential, upon AM symbiosis, that was abolished when one of the root halves was well watered (Bárzana *et al.*, 2015).

A distinguishing feature of ECM symbiosis is the fungal mantle that forms around short root tips and acts as a physical barrier between the root and the surrounding soil. In these symbiotic interactions, the fungus plays a significant role in resource acquisition, as everything must first pass through the fungal mantle (Becquer *et al.*, 2019). Water limitation is a critical concern in plant and ecosystem health, and ECM fungi can improve drought tolerance and water uptake in host woody plants (Gehring *et al.*, 2017). To assess this role in laboratory conditions and to identify the underlying molecular mechanisms, Calvo-Polanco *et al.* (2019) developed a split-root assay on *Populus trichocarpa*×*deltoides* cuttings in which each root half was inoculated or not by the ECM fungus *Laccaria bicolor*, and exposed to well-watered or drought conditions. Expression levels of plant aquaporins and hormone production were investigated and compared between each root half. Multiple genes coding for plant aquaporins were up-regulated under drought, independently of fungal colonization. Other plant aquaporin genes were down-regulated only in the presence of *L. bicolor*, suggesting a local switch from the direct to the ectomycorrhiza-mediated water transport pathway. Additionally, in drought conditions, fungal aquaporin-coding genes were up-regulated, and ECM roots showed significantly higher levels of abscisic acid, a phytohormone integral in managing drought stress (Aroca *et al.*, 2003). To our knowledge, this is the only example of a split-root approach in a

woody plant to study the hydromineral transport in ECM symbiosis.

Altogether, these results reveal critical connections in the contribution of an ECM fungus to drought management and show promise for the use of split-root systems to develop more robust understanding of the involvement of ECM fungi in both water and nutrient allocation to host plants. They also indicate that further research using split-root approaches is needed to better understand the impact of both AM and ECM fungi in water transport towards plants (Fig. 2).

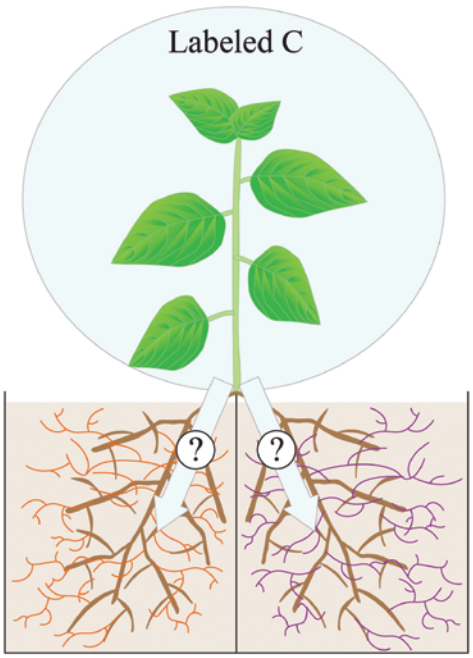
## Carbon allocation from the host plant to colonized roots

Using split-root assays can further elucidate the biochemical and molecular mechanisms of C transport from the host plant to roots colonized by mycorrhizal symbionts with access to different amounts or types of resources. Indeed, by physically separating mycorrhizal and non-mycorrhizal roots, it becomes possible to evaluate whether the host plant can preferentially allocate fixed C to the roots receiving more resources from the fungal symbiont (Fig. 3).

### *Carbon allocation in split arbuscular mycorrhizal roots*

In most terrestrial ecosystems, plants can interact with multiple species of AM fungi that provide various functional benefits, including improved mineral nutrition and stress tolerance. In return, host plants allocate photosynthetically fixed C to these symbionts in the form of sugars and lipids (Rich *et al.*, 2017). Multiple studies reported the existence of reciprocal reward mechanisms in AM symbiosis in which resource allocation is maximized in proportion to what the other partner provides (Hammer *et al.*, 2011; Kiers *et al.*, 2011; Fellbaum *et al.*, 2014; van't Padje *et al.*, 2021). Plants invest a considerable amount of energy and resources to produce photosynthetic products, therefore it is crucial for them to interact with the most beneficial AM partners and avoid the less cooperative ones. So far, C allocation in AM associations has been mostly conducted using different mycobionts in a single pot with a single, unsplit root system. A split-root approach in which root halves are simultaneously colonized by different AM fungi with access to different resources would help to identify which fungus offers the most efficient C/nutrient exchange rates. To our knowledge, the first studies demonstrating C partitioning in mycorrhizal roots using split-root assays were performed on sour orange (*Citrus aurantium*) and Carrizo citrange (*P. trifoliata*×*C. sinensis*)

A




B

| | Host plant | Fungal symbiont | References |
|---|---|---|---|
| AM | *Citrus aurantium* | *Rhizophagus irregularis* | Koch & Johnson, 1984 |
| | *Poncirus trifoliata* x *Citrus sinensis* | *Rhizophagus irregularis* | Koch & Johnson, 1984<br>Douds *et al.*, 1988 |
| | *Acer saccharum*<br>*Hordeum vulgare* | *Glomus mosseae*<br>*Gigaspora rosea*<br>*Rhizophagus irregularis* | Lerat *et al.*, 2003 |
| | *Allium vineale* | *Glomus spp.*<br>*Gigaspora margarita*<br>*Claroideoglomus candidum* | Bever *et al.*, 2009<br>Zheng *et al.* 2015 |
| | *Plantago lanceolata*<br>*Trifolium pratense* | *Rhizophagus irregularis*<br>*Funneliformis mosseae* | Argüello *et al.*, 2015 |
| | *Medicago truncatula* | *Rhizophagus irregularis* | Kafle *et al.*, 2019 |
| ECM | *Eucalyptus grandi* | *Pisolithus albus*<br>*Pisolithus microcarpus* | Hortal *et al.*, 2017 |
| | *Larix occidentalis* | *Suillus clintonianus*<br>*Suillus grisellus*<br>*Suillus spectabilis* | Bogar *et al.*, 2019 |
| | *Fagus sylvatica* | *ECM fungal community* | Gorka *et al.*, 2019<br>Mayerhofer *et al.* 2021 |

**Fig. 3.** Carbon allocation from host plants to arbuscular mycorrhizal and ectomycorrhizal fungi in split-root assays. (A) Schematic diagram showing a split-root design to investigate the allocation of carbon (C) from the host plant to two mycorrhizal fungi (orange and purple lines). Arrows and question marks represent the unknown amount of carbon transported into the plant–fungus continuum. (B) Reports using split-root assays to investigate carbon transport from host plants to arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi. The name of host plants and fungal symbionts, as well as the corresponding reference, are indicated.

colonized by *R. irregularis* (Koch and Johnson, 1984; Douds *et al.*, 1988). In these reports, AM roots received 3–5% more C from the host than non-colonized roots. Later, Lerat *et al.* (2003) compared photosynthetic C demand of three different AM fungi colonizing barley roots, also using a split-root system. They found that roots inoculated with *G. rosea* and *R. irregularis* were high and moderate C sinks, respectively, in comparison with non-inoculated roots. In addition, no differences in C sink strength were observed in roots colonized by *G. mosseae* compared with the control roots. Similar experiments on sugar maples showed that only *G. rosea* behaved as a strong C sink, while *G. intraradices* and *G. mosseae* did not show any effect on C allocation (Lerat *et al.*, 2003). Also, C sink strength of *G. rosea* and *R. irregularis* was positively correlated with the percentage of root colonization. In another study, the roots of *Allium vineale* plants were split and each half was colonized with either *Glomus* spp. or *Gigaspora margarita* (Bever *et al.*, 2009). In this experiment, the host plant allocated more photosynthetic C to roots colonized with *Glomus* spp. than those inoculated with *G. margarita*. When plants were colonized by one fungal species, their biomass was improved only with *Glomus* spp. Altogether, these studies using split-root systems demonstrate that C allocation to mycorrhizal symbionts is greatly dependent on the host plant species and colonization rate.

Furthermore, to understand the photosynthetic C partitioning of *A. vineale*, Zheng *et al.* (2015) conducted an assay in which plant roots were split and each half was colonized with a different AM fungus; *Claroideoglomus candidum* and *G. margarita*. Plants were shaded for a long, medium, or short period of time, or kept unshaded. The AM fungus *C. candidum* facilitated the plant growth in unshaded and short shading conditions, but P transferred by this fungus significantly decreased during longer shading treatments. In the meantime, shading did not affect C transfer to *G. gigaspora*, or fungal P transfer to the plant, but this symbiont was able to provide the plant with far less P than *C. candidum* (Zheng *et al.*, 2015). This indicates that host photosynthetic ability and C allocation are critical for the host plant to receive nutritional benefits from efficient AM fungi, and that plants do not invest much C to less cooperative fungi, as demonstrated in *in vitro* studies (Kiers *et al.*, 2011). Since all of these split-root assays were conducted in systems where both plants and fungi have access to the same nutrient solutions, it is hard to distinguish between the direct and mycorrhiza-mediated acquisition of nutrients. Consequently, some recent studies upgraded the split-root approach by having separate compartments containing nutrients only accessible to the AM fungi and not the host roots, such as for *Plantago lanceolata* and *Trifolium pratense* (Argüello *et al.*, 2016). In this set of experiments, roots were colonized by the AM fungi, *R. irregularis* and/or *Funneliformis mosseae*, or not, and only the fungi had access to $^{33}P$. To quantify the C cost in exchange for P, plants were exposed to $[^{14}C]CO_2$, and $^{14}C$ was tracked in the fungi. It was shown that the host plants received more P from *F. mosseae* only in the presence of *R. irregularis*. Interestingly, in the absence of alternative fungal options, *F. mosseae* was able to obtain more C in exchange for less P to the host than *R. irregularis*, which appeared to be a more cooperative partner. Using the host *M. truncatula* and the AM fungus *R. irregularis*, Kafle *et al.* (2019) also reported that if the fungus had access to a separate compartment containing N, both root halves (colonized or not) were equally provided with C. However, if N was absent from the plant and fungal compartments, a reduction of C allocation was noted in the non-colonized roots only. This indicates that the host plant was able to allocate C to the roots having the potential to offer N through the fungal colonization. Altogether, these works indicate that plants can benefit from multiple and simultaneous AM associations, and can allocate C to the roots having the potential to offer nutrients through the fungal colonization. In addition, they demonstrate that split-root assays can provide a unique way to explore the dynamics involved in host plant C allocation depending on the ability of one or multiple symbionts to access soil nutrients.

As mentioned above, legumes simultaneously interact with AM fungi and N-fixing bacteria to maximize their potential nutritional benefit. For these multipartite interactions, little is known about how the host plants differentially allocate photosynthetic C to their symbiotic partners. Studies aimed at tracking fixed C to roots colonized by either AM fungi or N-fixing bacteria have mainly been conducted in single root systems, which may be ideal to investigate synergistic effects, but not differences between symbioses (Mortimer *et al.*, 2008; Larimer *et al.*, 2014; Afkhami and Stinchcombe, 2016). Some groups explored C partitioning to roots dually colonized by AM fungi and rhizobia, such as in *Vicia faba* (Pang and Paul, 1980; Paul and Kucey 1981; Kaschuk *et al.*, 2009), but without physically separating the microbial symbionts. Alternatively, many groups investigated tripartite interactions between host, rhizobia, and AM fungi in split-root assays, as cited above (e.g. Koch and Johnson, 1984; Ames and Bethlenfalvay, 1987; Catford et al., 2003, 2006), but a few addressed the difference in C allocation between fungal and bacterial partners. Recently, Kafle *et al.* (2019) used a split-root system to physically separate the inoculation of *M. truncatula* roots by the AM fungus *R. irregularis* and the N-fixing bacteria *Sinorhizobium meliloti*, and to track the partitioning of C in each type of colonized roots. The amount of C allocated to the roots colonized by the AM fungus, having access to N or not, or to those inoculated with *S. meliloti*, was quantified using labeled C. This demonstrated that a greater proportion of C was allocated to the roots colonized with *S. meliloti* when no N was added to the fungal compartment. However, an equivalent proportion of C was provided to the roots colonized by either symbiont when the fungus had access to an N source (Kafle *et al.*, 2019). This observation was confirmed by the differential expression of plant C transporters from the Sugars Will Eventually be Exported Transporter (SWEET) family. In plants, SWEETs mediate the transport of sucrose and hexose, and are involved in multiple processes, including phloem loading, seed filling, or root exudation (Eom *et al.*, 2015). It has

also been demonstrated that some SWEETs can be involved in symbiotic C transport towards AM fungi and N-fixing bacteria (Manck-Götzenberger and Requena 2016; Kryvoruchko *et al.*, 2016; Sugiyama *et al.*, 2017; Doidy *et al.*, 2019; An *et al.*, 2019). In split-root assays, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* were up-regulated in the roots colonized by the N-fixing bacteria if the fungus reached an N-free compartment, or in AM roots when the fungus had access to N (Kafle *et al.*, 2019). These observations demonstrate that split-root assays are an efficient approach to unravel the molecular basis of C allocation in tripartite interactions.

### *Carbon allocation in split ectomycorrhizal roots*

Little is known about C allocation from woody plants to ECM fungi and other symbiotic partners. One way in which split-root assays have been used was to look at host–partner determination to distinguish differences in C allocation from the plant and colonization of root tips, relative to the amount of N the plant received from multiple fungal partners (Hortal *et al.*, 2017). N was traced from the ECM fungi *Pisolithus microcarpus* and *Pisolithus albus* to the host tree, *Eucalyptus grandis*, and C from the host to the fungi, in a split-root Petri dish system. Although quite artificial, this set-up revealed that, rather than allocating more fixed C to the fungi that provided more N, the plants up-regulated defense reactions to exclude less valuable fungi in favor of the better mutualists. More recently, Bogar *et al.* (2019) used a similar Petri dish design with three ECM fungi in the genus *Suillus* and split-root seedlings of *Larix occidentalis*, and observed higher levels of C allocated to the fungal species that provided more N to the plant.

In natural settings, most ECM plants are colonized by many fungi that may release host C into the soil through their hyphae (Rambelli, 1973; Johansson *et al.*, 2004). To investigate C movements from host plants to soil bacteria through the mycelium of ECM fungi, Gorka *et al.* (2019) used a split-root approach on 3- to 4-year-old beech trees (*Fagus sylvatica*). Tree roots were split and planted in pots that contained a separate compartment that could be reached only by fungal hyphae, and shoots were labeled with $[^{13}C]CO_2$. In these multicompartment systems, labeled C was transferred to soil bacteria that were present in the compartment colonized by ECM hyphae only, indicating C fluxes through the root–hyphae–soil continuum. Additionally, this transfer of C to soil microbes was dependent on the external N availability in the litter (Gorka *et al.*, 2019).

More recently, Mayerhofer *et al.* (2021) conducted a split-root experiment investigating the localization of reciprocal C/N exchange between *F. sylvatica* and ECM fungi. The trees were transplanted from forest soil along with wild-colonizing ECM fungi, and were grown in a split-root chambers where the fungi on each side were given access to separate litter compartments with or without $^{15}N$. The tree canopy was exposed to $[^{13}C]CO_2$ to trace the translocated photosynthetic C in mycorrhizal roots. Interestingly, no localized allocation of $^{13}C$ to the fungi was correlated with the fungal-transported $^{15}N$ when comparing the separate root halves. However, visualization of a cross-section of a mycorrhizal root tip with nanoscale secondary ion MS revealed a localized preferential exchange of C for N between plant and fungal tissues at a microscopic level (Mayerhofer *et al.*, 2021). This may indicate a possible limitation of split-root designs for identifying localized direct plant responses to ECM fungi at smaller scales.

Although revealing different outcomes (i.e. growth inhibition or reduction of C allocation for less beneficial symbionts), these recent studies indicate the ability of the plant host to influence their fungal partners with C, which is crucial in natural ecosystems where trees can be colonized simultaneously by multiple fungal species. These conclusions are indicative of the opportunity that split-root experiments offer an opportunity to understand reciprocal nutrient fluxes in complex ECM symbioses. The complicated mechanisms that dictate ECM mutualisms are probably not determined by individual factors, but rather layered interactions that result in complex networks of symbioses. Split-root assays offer a method to compare multiple factors in a controlled setting.

## Conclusion

Split-root techniques have been used for decades in several plant species to study local versus systemic signaling triggered by external factors, such as nutrient availability, abiotic stressors, and plant–microbe interactions. Split-root assays are quite artificial, can be difficult to establish for some plants, and are time-consuming, but they offer a unique way to investigate symbiotic establishment, mycorrhiza-induced systemic resistance to pathogens, or hydromineral resource allocation in AM and ECM symbioses. For example, they have been successfully used to describe the autoregulation of mycorrhiza mechanisms in AM symbiosis, that fungal colonization was regulated by a systemic feedback repression influenced by the P and N status of non-colonized roots, and that colonized plants can differentially allocate C to various microbial partners depending on the resources they provide. Mainly focusing on P, N, and C transport, split-root assays have the potential to be used more widely in the study of mycorrhizal associations, particularly on mycorrhiza-mediated micronutrient transport, on ECM systemic-induced resistance, or on model plants that can form both AM and ECM associations such as poplar or alder trees.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Funding

This work is supported by the AFRI program (grant no. 2020-67013-31800) from the USDA National Institute of Food and Agriculture, the

North Carolina Soybean Producers Association (2019-1656), and the North Carolina Agriculture Research Service (NCARS).

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