Exploring the potential for novel Ri T-DNA transformed roots to cultivate arbuscular mycorrhizal fungi

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Abstract

Arbuscular mycorrhizal (AM) fungi are key soil symbiotic microorganisms, intensively studied for their roles in improving plant fitness and their ubiquity in terrestrial ecosystems. Research on AM fungi is difficult because their obligate biotrophic nature makes it impossible to culture them in the absence of a host. Over the last three decades, Ri T-DNA transformed roots have been the gold standard to study AM fungi under *in vitro* conditions. However, only two host plant species (*Daucus carota* and *Cichorium intybus*) have been routinely used to *in vitro* propagate less than 5% of the known AM fungal species. There is much evidence that host identity can significantly affect AM symbioses, therefore, we investigated any potential host-specific effects of two novel Ri T-DNA transformed root species, *Medicago truncatula* and *Nicotiana benthamiana*, by associating them with seven AM fungal species selected based on their contrasting behaviors when grown with Ri T-DNA transformed *D. carota* roots. To evaluate the performance of new Ri T-DNA transformed roots to host and propagate AM fungal species, a factorial set-up was used to generate nine unique pairs of hosts (*M. truncatula*, *N. benthamiana*, *D. carota)* and AM fungi (*Rhizophagus irregularis*, *R. clarus*, *Glomus* sp.). Using statistical modeling, all pairs of hosts and AM fungi were compared by their symbiosis development (SD) and sporulation patterns in the hyphal compartments (HCs) of two-compartment Petri dishes. Our results show that 1) most of the variation between host and AM fungus pairs relating to SD or HC sporulation was explained by an interaction between host and AM fungal identity, i.e., host identity alone was not sufficient to explain AM fungal behaviour, 2) AM symbioses involving different combinations of symbiont identities trigger heterogenous fungal behaviours. This work provides a robust framework to develop and evaluate new Ri T-DNA roots for the *in vitro* propagation of AM fungi, an important asset for germplasm collections and biodiversity preservation.

Résumé

Les champignons mycorhiziens arbusculaires (MA) sont des microorganismes symbiotiques importants du sol, intensivement étudiés pour les bénéfices qu'ils apportent aux plantes et leur ubiquité dans les écosystèmes terrestres. La recherche sur les champignons MA est difficile, car étant des biotrophes obligatoires, leur culture en l'absence d'hôte est impossible. Au cours des trois dernières décennies, les racines transformées par Ri T-DNA ont été la référence pour étudier les champignons MA en conditions *in vitro*. Cependant, seules deux espèces de plantes hôtes (*Daucus carota* et *Cichorium intybus*) ont été couramment utilisées pour propager *in vitro* moins de 5% des espèces connues de champignons MA. De nombreuses études montrent que l'identité de l'hôte impacte significativement les symbioses mycorhiziennes arbusculaires, par conséquent, nous avons étudié ces effets potentiels en produisant deux nouvelles espèces de racines transformées par Ri T-DNA, *Medicago truncatula* et *Nicotiana benthamiana*, et en les associant à sept espèces de champignons MA, sélectionnées pour leurs comportements contrastés en culture avec les racines transformées de *D. carota*. Pour évaluer la performance des nouvelles racines transformées à héberger et propager les espèces de champignons MA, un système factoriel a été utilisé pour générer neuf paires uniques d'hôtes (*M. truncatula, N.benthamiana, D. carota*) et de champignons MA. (*Rhizophagus irregulis, R. clarus, Glomus* sp.). Le développement de la symbiose et ainsi que des profils de sporulation dans les compartiments mycéliens en boîtes de Petri à deux compartiments ont été comparées par modélisation statistique pour chacune des paires d'hôte et de champignon MA. Nos résultats montrent que 1) la variation du développement de la symbiose et des profils de sporulation entre les paires hôte et champignons MA était principalement expliquée par une interaction entre l'identité de l'hôte et du champignon MA, l'identité seule de l'hôte n'étant pas suffisante pour expliquer le

comportement du champignon MA, 2) les symbioses mycorhiziennes arbusculaires impliquant différentes combinaisons d'identités de symbiotes produisent des comportements fongiques hétérogènes. Ces travaux fournissent une méthodologie rigoureuse pour développer et évaluer de nouvelles racines transformées destinées à la propagation *in vitro* des champignons MA, une ressource importante pour les collections de matériel génétique et la préservation de la biodiversité.

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Abbreviations

AM – arbuscular mycorrhizal

CCAMF – the Canadian collection of arbuscular mycorrhizal fungi

ER – extraradical

FA – fatty acid

GFP – green fluorescent protein

GINCO-BEL – the Glomeromycota *in vitro* collection – Belgium

HC – hyphal compartment

IR – intraradical

LMM – linear mixed model

MLR – multinomial linear regression

RC – root compartment

ROC – root organ culture

SD – symbiosis development

TAG – triacylglycerol

VIPS – viability, identity, purity, and stability

WPI – weeks post inoculation

CHAPTER ONE

Introduction

1.0 Introduction

1.1 Overview of Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal (AM) fungi are an ancient taxon within the Glomeromycota phylum, which form symbioses with over 70% of terrestrial plants (Brundrett & Tedersoo, 2018). It is essential for these fungi to locate and colonize plant roots as they are unable to obtain their own carbon and are therefore dependant on hosts to complete their life cycle (Lanfranco *et al.*, 2016; Roth & Paszkowski, 2017). The AM symbiosis is based upon an exchange of resources with plant hosts transferring photosynthates to AM fungi and in return, receiving a variety of nutrients, including phosphorus and nitrogen. This transfer of resources takes place inside root cortical cells where host plasma membrane accommodates highly branched fungal structures called arbuscules (Luginbuehl & Oldroyd, 2017). Specifically, nutrients are exchanged across the periarbuscular space, which is the region between the plant host and the fungal symbiont's cell membranes (MacLean *et al.*, 2017). The bi-directional exchange of nutrients within the AM symbiosis is not a random process as there is evidence that both symbionts are capable of imposing sanctions upon the other to prevent being cheated (Kiers *et al.*, 2011).

AM fungi have also been found to improve the tolerance of their hosts for a variety of abiotic and biotic stressors such as drought, high-salinity, heavy metal toxicity, and soil-borne pathogens. Under conditions where plants are subjected to a low soil water potential (e.g., drought or high soil salinity), non-AM plants often display a decline in plant growth due to water and nutrient deficiency (Farooq *et al.*, 2009). AM plants however, are able to weather drought and high-salinity stress via an increased uptake of water from extraradical (ER) hyphae as well as an improved uptake of nutrients (Khalvati *et al.*, 2005; Evelin *et al.*, 2009). AM fungi can also ameliorate soils contaminated by heavy metals via hyphal absorption and adsorption in order to

immobilize them in the mycorrhizosphere, which effectively sequesters these metals away from host roots and shoots (Joner *et al.*, 2000; Christie *et al.*, 2004; Bhargava *et al.*, 2012). Finally, in several different instances, hosts colonized by AM fungi have been found to be better equipped to resist soil-borne pathogens through mycorrhiza-induced priming (Pozo & Azcón-Aguilar, 2007).

1.2 Bi-directional nutrient exchange

1.2.1 Carbon

Up to 30% of the photosynthates in hosts can be transferred to the AM fungi colonizing their roots, thus the AM symbiosis can represent a strong carbon sink (Drigo *et al.*, 2010). Early research found that host-derived carbon was primarily taken up by fungi in the form of hexose sugars (Solaiman & Saito, 1997; Roth & Paszkowski, 2017). This was supported by the identification of a high-affinity monosaccharide transporter (*MST2*) in *Rhizophagus irregularis* (DAOM 197198) that is expressed in both intraradical (IR) hyphae as well as arbuscules (Helber *et al.*, 2011). However, several findings made it seem rather unlikely that AM fungi primarily rely upon sugars as a primary source of carbon. To begin with, AM fungi predominantly translocate and store carbon in the form of triacylglycerols (TAGs), therefore most, if not all, root-borne sugars would need to be converted to TAGs, which is energetically inefficient (Bago *et al.*, 2002). Secondly, no gene encoding a multidomain fatty acid (FA) synthase have been identified in the genome of any species of AM fungi (Wewer *et al.*, 2014). Therefore, AM fungi are likely incapable of *de novo* synthesis of FAs, which suggests that FAs must be supplied by a host. Recently, a lipid biosynthetic pathway was identified inside arbuscule-filled root cortical cells wherein plants synthesize and transfer C16:0 FAs across the periarbuscular membrane to

their fungal symbiont (Bravo *et al.*, 2017; Luginbuehl *et al.*, 2017). Ultimately, since AM fungi are capable of receiving carbon in the form of either monosaccharides or FAs, the form in which carbon is received is likely situation-dependant (Rich *et al.*, 2017).

1.2.2 Phosphorus

Phosphorus is an essential nutrient for all organisms, and yet it is quite often the growth-limiting nutrient for plants. This is because in many agricultural systems more than 80% of phosphorus in the soil is either immobile or is present in organic forms that are unavailable to be taken up by plant roots (Schachtman *et al.*, 1998). For both plant hosts and AM fungi, acquiring phosphorus is facilitated by high-affinity transporter proteins from the *Pht1* gene family, which specifically take up the inorganic form of phosphorus, orthophosphate (Koide and Kabir 2000; Bucher 2007). Due to soil microbial activity, the rate at which phosphorus diffuses through soil is much slower than root phosphorus uptake; resulting in depletion zones in the rhizosphere (Schachtman *et al.*, 1998). Fortunately for AM plants, fungal mycelium can access a larger pool of soil-borne phosphorus than hosts because 1) hyphae can extend further into the surrounding soil past the rhizosphere and 2) hyphae have a much smaller diameter than roots, and thus ER mycelium has a much larger surface area than a root system (Smith *et al.*, 2011). Furthermore, AM fungi can recruit phosphate solubilizing bacteria to mineralize organic phosphorus by releasing carbon-filled hyphal exudates (Zhang *et al.*, 2016).

1.2.3 Nitrogen

AM fungi have also been found to be involved in the uptake of nitrogen, another macronutrient essential to plant growth. According to the nitrogen cycle, much of the nitrogen in any given

ecosystem is unavailable to plants as it is either present in soil as complex, organic molecules or in the atmosphere in a gaseous state. However, similar to phosphorus, AM plants can compensate for low nitrogen availability by relying upon ER hyphae to access areas beyond their rhizosphere. Moreover, within the *Rhizophagus irregularis* (DAOM197198) genome, genes (*GintAM1* and *GintAM2*) encoding for functional ammonium (NH_4^+) transporters has been identified (Pérez-tienda *et al.*, 2011). Utilizing NH_4^+ transporters, AM fungi scavenge areas with decomposing organic matter to take up the nitrogen that is released (Hodge *et al.*, 2001). Specifically, ER mycelium uptake both NH_4^+ and nitrate (NO_3^-), which are then converted to arginine ($C_6H_{14}N_4O_2$), transported to the IR mycelium, and then converted to NH_4^+ prior to host root uptake (Govindarajulu *et al.*, 2005).

1.2.4 Resource exchange rate

The resource exchange rate between hosts and AM fungi is dependent upon numerous factors and is therefore not always equal. For example, the quality of partner will vary depending upon two pools of resources. The first resource pool is the amount of nutrients each symbiont is capable of producing (e.g., rate of photosynthesis or rate of soil nutrient uptake) and the second pool is the amount of nutrients transferred to its plant or fungal counterpart(s). The latter pool is the basis for a biological market between symbionts (Noë & Hammerstein, 1994). Specifically, it is hypothesized that hosts and AM fungi are capable of assessing the amount of resources supplied by their partners and then modifying their own resource allocation accordingly (Kiers *et al.*, 2011). Biological market theory in the AM symbiosis is not without criticism. For instance, if reciprocal rewards were the sole determinant of resource exchange, then all AM symbioses should be strictly mutualistic. However, this is not the case. Johnson et al. (2015) explored the

Law of the Minimum within an AM context and found that under differing soil conditions (carbon-limited, phosphorus-limited, or nitrogen-limited), the relationship between hosts and fungi can vary from mutualistic, to commensal, to even parasitic. An additional argument against biological market theory is that the identity of symbiotic partners may strongly affect the rate of resource exchange more so than a reciprocal rewards system (Walder & van der Heijden, 2015). Within common mycorrhizal networks linking multiple hosts, AM fungi have been found to favour specific hosts over others, resulting in uneven terms of trade and a clear effect of symbiont identity on resource exchange (Walder *et al.*, 2012). Furthermore, under *in vitro* conditions, a common mycorrhizal network may negatively affect AM fungal growth, possibly due to host-host conflict (Engelmoer & Kiers, 2015). Ultimately, resource exchange in the AM symbiosis is dependent on a variety of abiotic and biotic factors and is therefore highly complex.

1.3 Culturing AM fungi

While plants are not wholly dependent on AM fungi, the numerous benefits they receive from their fungal partners are critical to the functioning of ecosystems globally. As such, many efforts have been made to better understand these microorganisms, starting with learning how to culture them (Koide & Mosse, 2004). Although there are undoubtedly plenty of unique set-ups used to culture AM fungi, there are ultimately two main categories: *in vitro* and *in vivo* (**Figures 1-4**).

1.3.1 Culturing in vivo

The most rudimentary means of culturing AM fungi is to gather soil filled with fungal propagules (spores, hyphae, and/or colonized roots) from the field and then add said soil to a pot (hence “pot cultures”) containing sterilised substrate and plant host. Pot cultures are sometimes

referred to as “trap cultures” when after several months post-field collection, newly produced fungal propagules are isolated and identified (Smith & Read, 2008). Pot cultures are quite effective at culturing a wide range of AM fungal species due to their similarity to more natural conditions, e.g., autotrophic host and presence of a soil-borne community of microbiota. However, due to the presence of a soil substrate, it is difficult to obtain real-time, *in situ* observations of fungal growth and to easily assess the culture viability, identity, purity and stability (VIPS; Declerck et. al., 2005). One must first harvest soil or root samples to confirm fungal viability (germination and/or production of daughter spores) or any association between roots and fungi. Unfortunately, harvesting roots to observe IR fungal colonization requires destructive steps such as boiling the roots, effectively preventing any continued growth and observation (Vierheilig *et al.*, 1998). Screening all the fungal propagules in the soil is almost impossible and highly time consuming compared to a Petri dish.

By initiating a trap culture, the end goal is to eventually obtain a culture containing only a single species or strain of AM fungus, which is difficult to assess in a soil matrix unless the culture was initiated with a single spore and protected from external contamination. Spores isolated from pot cultures can then be characterized morphologically or molecularly or used to initiate an *in vitro* culture. Pot cultures can be sub-cultured to maintain an active symbiosis and/or to separate the multiple spore morphotypes to obtain single species subcultures. In addition, they can be stored in the fridge for biopreservation.

Finally, the maintenance required for *in vivo* cultures, relative to *in vitro* cultures, can be far more laborious and time-consuming due to the watering and fertilizing necessary for autotrophic hosts (**Figure 2**). In addition, pot cultures are significantly larger than their *in vitro*

counterparts and as such take up far more storage space in growth chambers, oftentimes requiring greenhouses for additional space (**Figure 3**).

1.3.2 Culturing in vitro

The movement towards culturing AM fungi *in vitro* began with a trio of seminal publications by Barbara Mosse. In 1956, Mosse described the first successful mycorrhization under aseptic conditions by associating strawberry seedlings with surface sterilized *Endogone* sp. (likely *Funneliformis* mosseae) spores in test tubes filled with sterilized soil substrate (Mosse, 1956). Six years later, Mosse (1962), followed up her previous work by once more reporting root colonization under sterile conditions but this time using gelled medium as opposed to a soil substrate. Finally, Mosse & Hepper (1975), detailed a series of methods on root organ cultures (ROCs), a system in which untransformed excised roots were cultivated on mineral-based medium under *in vitro* conditions. The authors reported critical information to the field of AM fungal research such as the optimal pH level for AM infection, the impracticality of AM liquid ROCs, and that AM fungi do not require shoot- or leaf-borne metabolites for growth or infection (Mosse & Hepper, 1975).

A major step towards better understanding AM fungi came with the popularization of using transgenic root hosts generated via *Agrobacterium*-mediated transformation. Specifically, a species of gram-negative bacteria, *A. rhizogenes,* is used to genetically transform plant roots by inserting a bacterial plasmid into the nuclear genome of a susceptible plant (Tepfer, 1984). Such a plasmid contains root inducing (Ri) transfer DNA (T-DNA) which elicit morphogenic effects on transformed plants. Once transformed, roots will express oncogenic *rol* genes which induce the production of numerous adventitious roots or “hairy roots” that are capable of limitless

regeneration so long as they are routinely sub-cultured onto fresh, nutrient-rich medium (Nilsson & Olsson, 1997). The specific strain of *A. rhizogenes* used for root transformation is important as the level of strain virulence can determine whether hairy root structure is abnormal (high strain virulence) or morphologically similar to that of wild-type roots (low strain virulence; Chabaud et al. 2006).

The practice of using hairy roots as a means to cultivate AM fungi *in vitro* began with pioneering work by Mugnier and Mosse (1987) who associated *Funneliformis mosseae* and *Gigaspora margarita* with transformed *Convolvulus sepium* L. (bindweed) and found that the symbiosis progressed to the development of arbuscules. The following year, Bécard and Fortin (1988) co-cultured transformed *Carota Daucus* (carrot) roots with *Gigaspora margarita* and demonstrated for the first time ROCs potential as a reliable means of obtaining large amounts of viable, aseptic fungal spores.

The implication of cultivating AM fungi *in vitro* meant that virtually every drawback associated with pot cultures could be overcome. This was best described by Declerck, Strullu, and Fortin (2005) where the authors outlined the VIPS guidelines for the long-term preservation of *in vitro* AM fungi culture collections. The viability of host roots and fungi within a ROC can easily be visually confirmed under a stereomicroscope due to the transparency of both the media on which symbionts are grown as well as the Petri dish in which all components are housed. Furthermore, researchers can track the fungal life cycle undisturbed and in real time (**Figure 4**).

Another aspect of ROCs which makes them such a powerful tool is the ability to culture the AM symbiosis aseptically (**Figure 2**). Under monoxenic conditions, pure cultures (i.e., of a single fungal species or strain) can be obtained by inoculating ROCs with a single spore such that over time all subsequently produced daughter spores can be traced back to a single fungal

genotype. The significance of having access to pure cultures of AM fungi is two-fold. First, the results of any subsequent analyses (morphological, genetic, biochemical, etc.) can be interpreted in a context where there is only one host and AM fungus present, and no confounding effect associated with the presence of other microorganisms or abiotic stressors. Secondly, pure cultures provide researchers with contaminant-free, fungal DNA, which can be used for any number of molecular methods such as identification via molecular markers or genome sequencing (Croll *et al.*, 2008; Tisserant *et al.*, 2013). In meeting the first three guidelines, viability, identity, and purity, a germplasm collection can then meet the last guideline, stability. Opting to culture AM fungi in ROCs as opposed to pot cultures makes it possible for researchers to continually sub-culture from properly identified, pure cultures over multiple generations, which is referred to as "continuous culture" (Declerck *et al.*, 2005).

One of the more common *in vitro* set-ups involves the use of a two-compartment Petri dish that contains a plastic barrier dividing a designated root compartment (RC) from a hyphal compartment (HC; **Figure 2**). Both symbionts are initially associated within the RC. However, as symbioses develop and ER fungal growth becomes more extensive, hyphae will grow over the barrier and into the HC. Hairy root growth is trimmed to stay restricted to the sucrose-filled media in the RC (**Figure 3**). Creating an environment for fungi to grow isolated from host roots is significant as there is evidence that AM fungi can sporulate significantly more in the HC compared to the RC (St-Arnaud *et al.*, 1996). It is thought that host roots may release root exudates that work to inhibit fungal growth, a strategy hosts may use as a means to autoregulate further fungal colonization (Vierheilig *et al.*, 2003).

Lastly, it should be noted that along with the many benefits of *in vitro* culturing, comes certain drawbacks as well. For any lab looking to work with AM fungi under monoxenic

conditions, access to expensive lab equipment (e.g., laminar flow hood, autoclave, incubators, microscopes, etc.) is a requirement (**Figure 1**). Furthermore, setting up and maintaining *in vitro* cultures is laborious, time-consuming, and comes with a high risk of contamination (**Figure 2-3**). However, the most significant drawback of monoxenic cultures is the artificiality of these systems. First off, only a small subset of AM fungi species has thus far been successfully cultured *in vitro* over multiple generations. According to Arthur Schüßler's web page (accessed March 28, 2021; http://www.amf-phylogeny.com/), there is currently a total of 340 species of AM fungi described and validated. However, the true total AMF diversity is estimated to range between 1700 – 2700 species (Öpik & Davidson, 2016). In contrast, two of the largest collections of AM fungi in the world, the Canadian Collection of Arbuscular Mycorrhizal Fungi (CCAMF, Agriculture and Agri-Food Canada; AAFC) and the Glomeromycota *in vitro* Collection – Belgium (GINCO-BEL), currently only maintain five and ten species (respectively) under *in vitro* conditions. Secondly, for those fungal species that are capable of completing their life cycles *in vitro*, it has been reported that certain spore morphological markers, such as size, pigmentation, or spore cell wall thickness, may be altered in comparison to their *in vivo* counterparts (**Figure 4**; Bago and Cano, 2005). Despite these morphological differences, monoxenically produced spores are fully capable of colonizing host roots within soil substrate (Vimard *et al.*, 1999; Cely *et al.*, 2016).

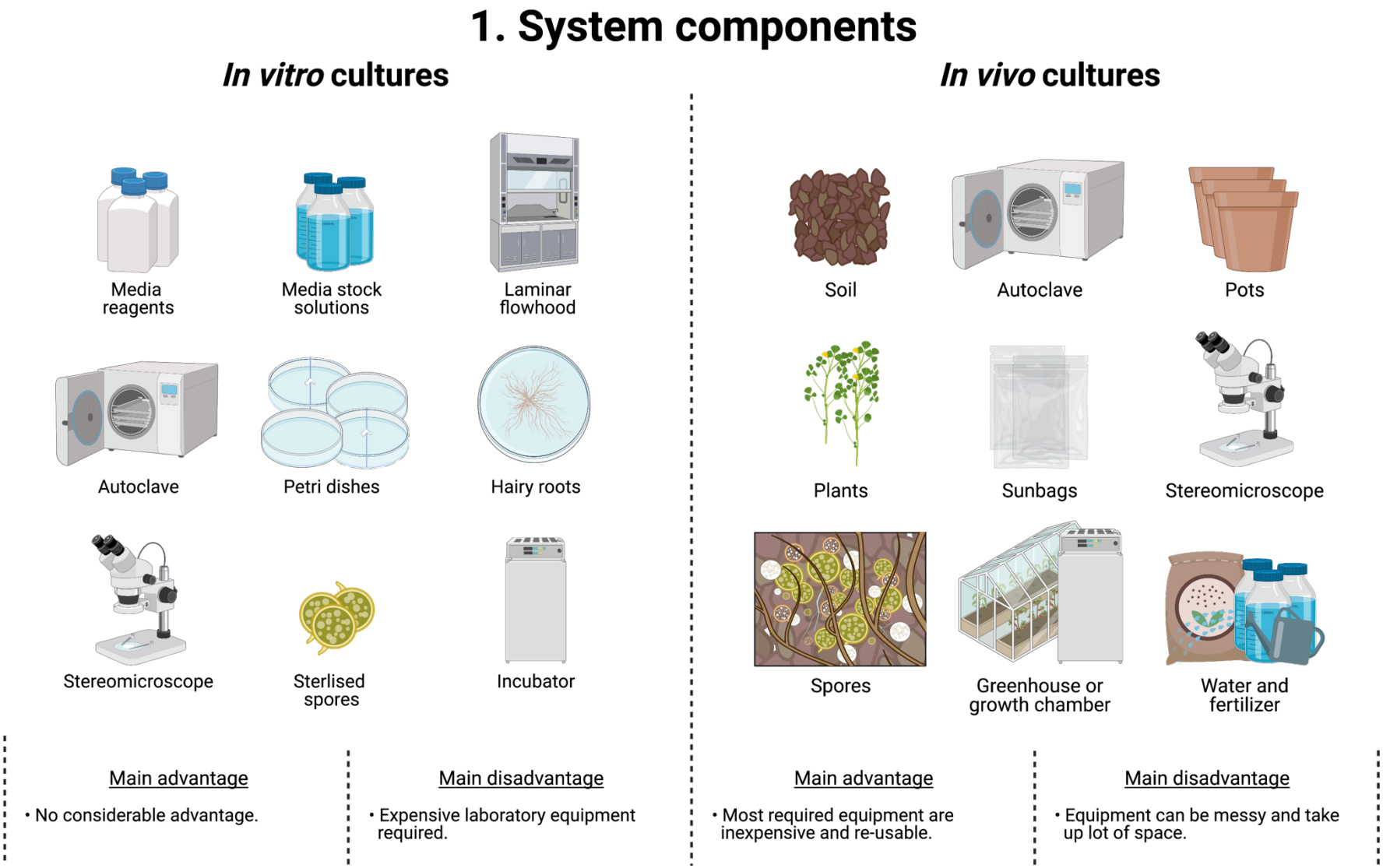


Figure 1. Components necessary to set-up, maintain and obtain data from *in vitro* versus *in vivo* cultures of arbuscular mycorrhizal fungi.

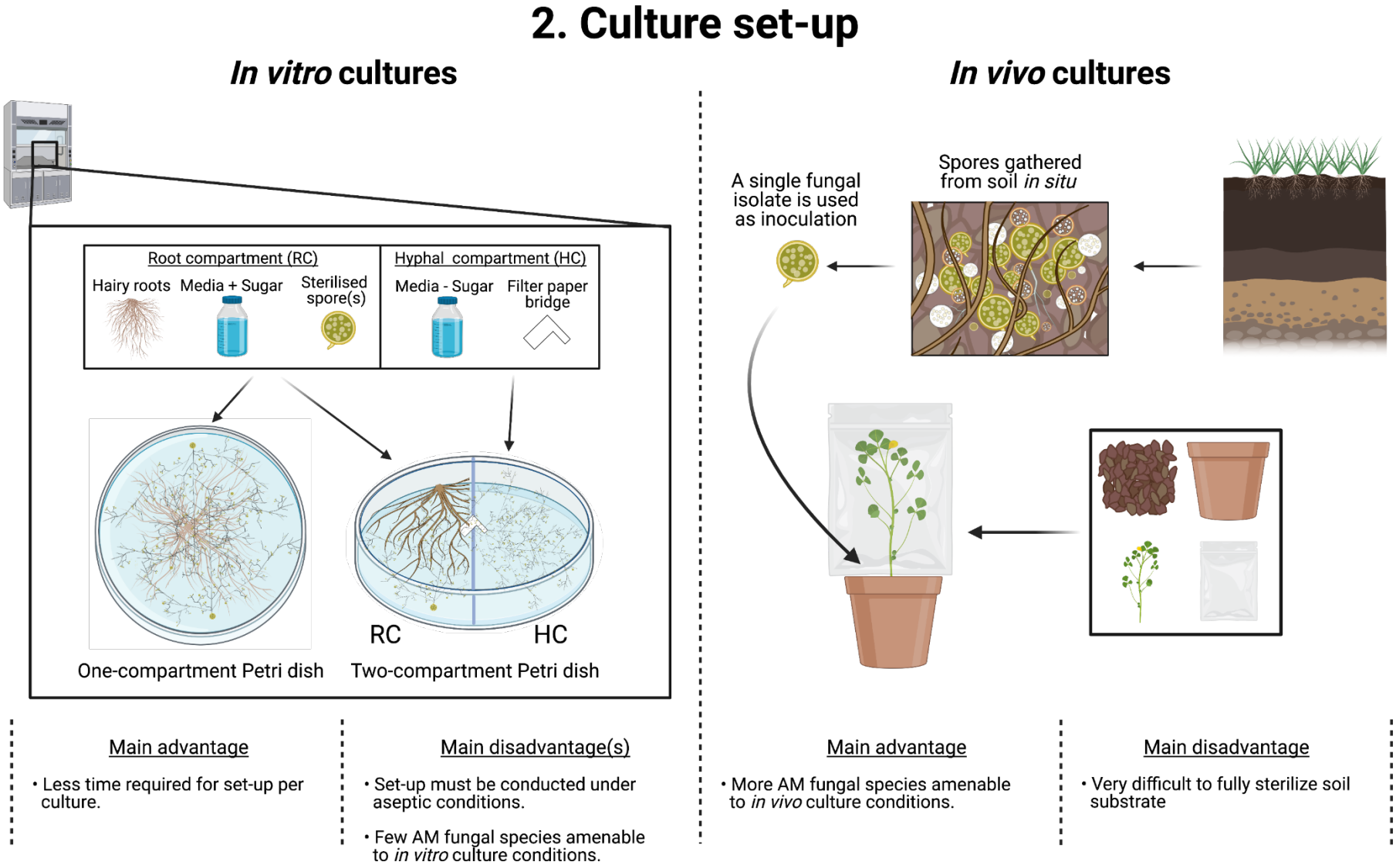


Figure 2. Schematic illustration of the set-up of *in vitro* versus *in vivo* cultures of arbuscular mycorrhizal fungi. RC: root compartment, HC: hyphal compartment.

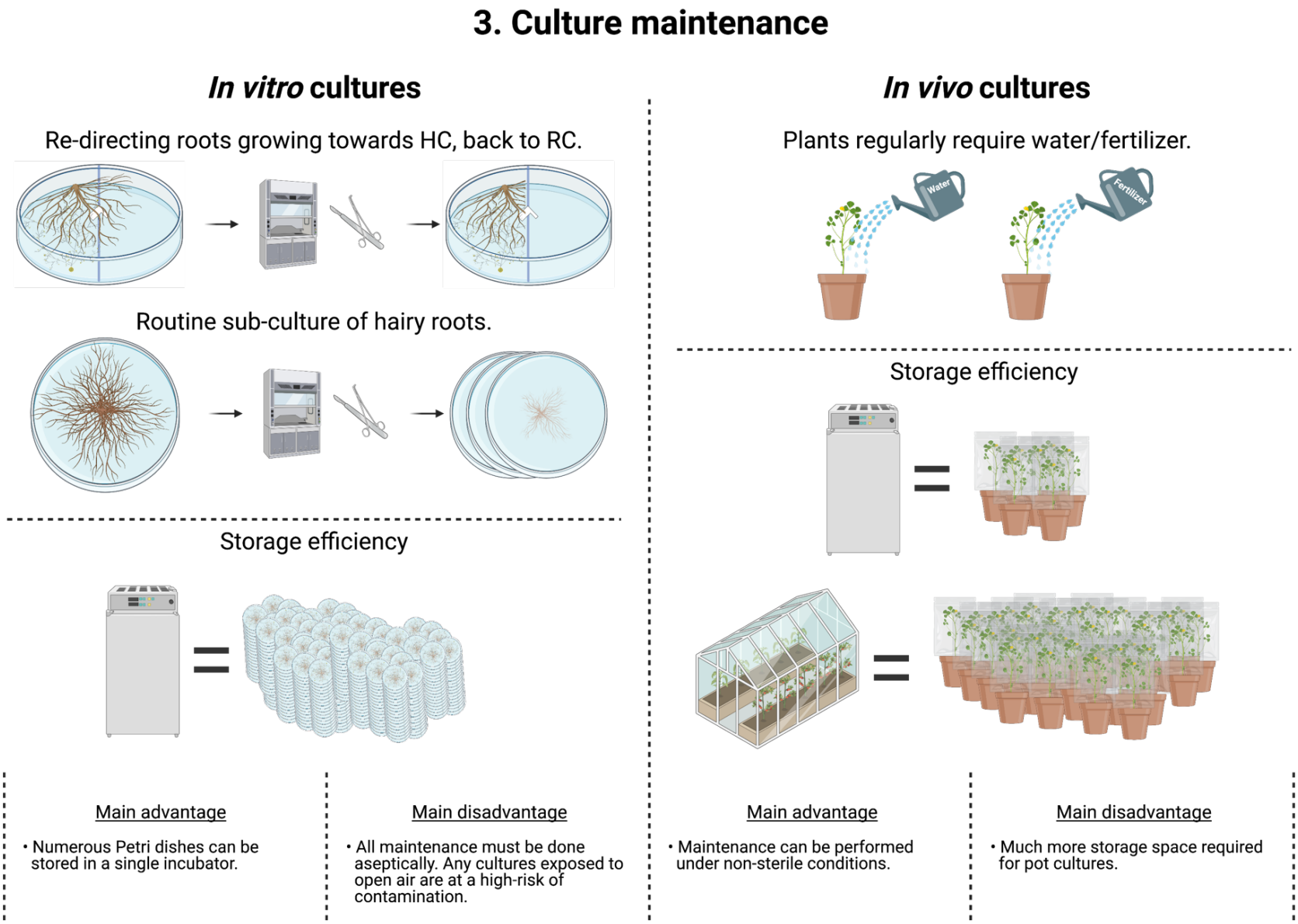


Figure 3. Maintenance requirements and storage efficiency of *in vitro* versus *in vivo* cultures of arbuscular mycorrhizal fungi. RC: root compartment, HC: hyphal compartment.

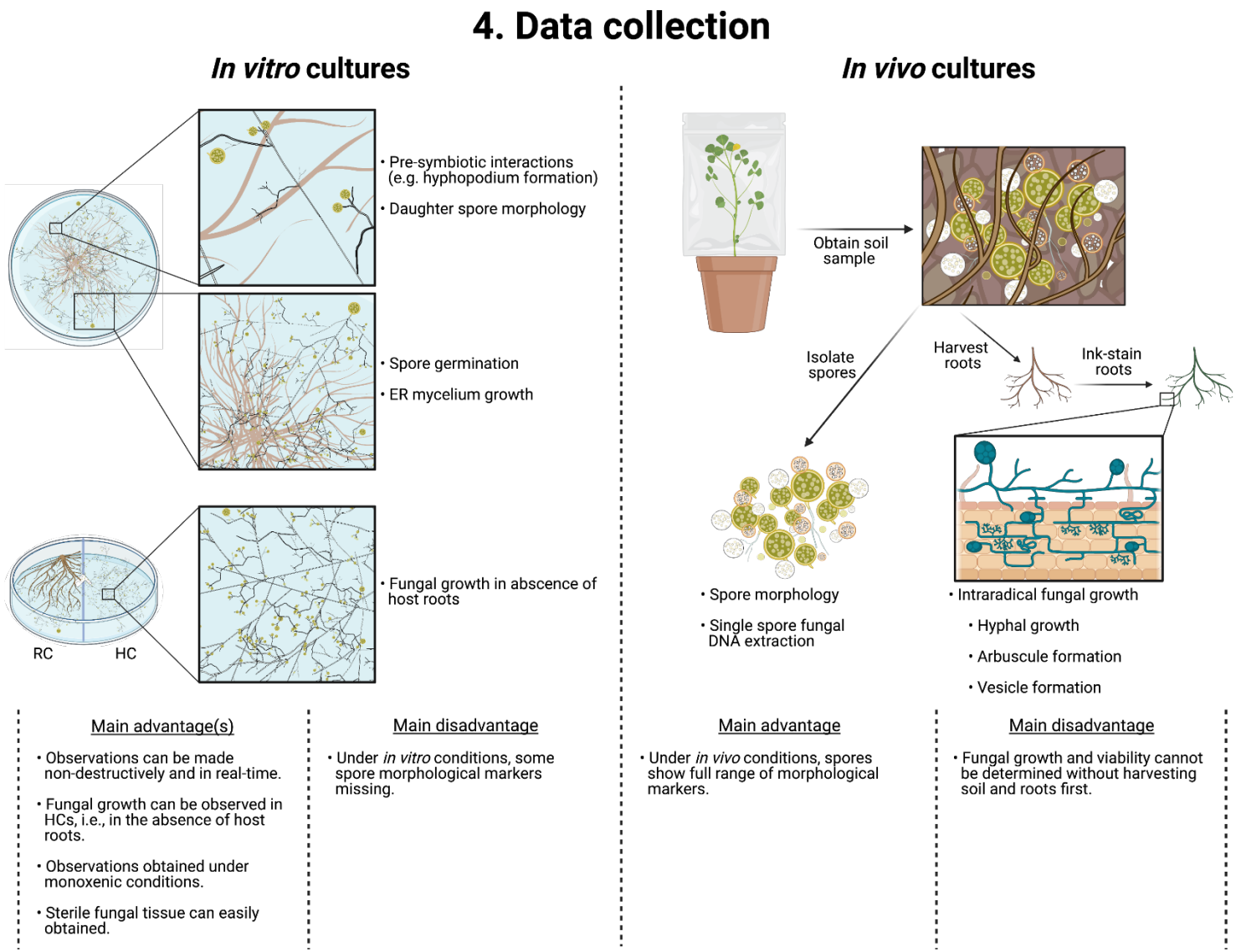


Figure 4. Types of data that can be collected from of *in vitro* versus *in vivo* cultures of arbuscular mycorrhizal fungi. RC: root compartment, HC: hyphal compartment.

1.3.3 Long-term preservation of AM fungi

Regardless of which conditions are used to culture AM fungi, *in vivo* or *in vitro*, all cultures are subject to some form of maintenance (**Figure 3**). Not only can maintenance be laborious and time-consuming, but it can also be detrimental to the long-term preservation of cultures. Routine sub-culture of species under conditions of varying levels of artificiality (e.g., without soil-borne microbiota, without interaction amongst other native plant/AM fungi species, etc.) could potentially lead to physiological or genetic changes over time (Fortin *et al.*, 2002). Cryopreservation may be one possible solution.

Early attempts by Douds and Schenck (1990), involved first immersing isolated spores in cryoprotective agents or air-drying spore-filled soil before placing inoculum directly into -60ºC to -70ºC freezers. In the end, only the thawed spore-filled soil was found to exhibit viability similar to the samples that were not exposed to the cold-treatment. In more recent years, a highly promising method was introduced by Lalaymia et al. (2012) wherein spores from multiple species and strains of *Rhizophagus* were entrapped within alginate beads before undergoing a series of preparatory steps, which ultimately led to freezing at -130ºC. The authors found that after 6 months of cryopreservation, fungal viability was not significantly affected. In the following years, a similar study was conducted (i.e., freezing alginate bead-encased fungal tissue at -130ºC) using several other species of AM fungi produced *in vitro* along with some produced *in vivo* (Lalaymia and Declerck 2014). The outcome was similar in that the spores originally sourced from *in vitro* cultures showed no significant decline in viability. In contrast, the inoculum initially obtained from *in vivo* cultures exhibited mixed results. Ultimately, cryopreservation offers many practical advantages when storing AM fungi long-term and, given recent advances, may one day become common practice in large collections of AM fungi.

1.3.4 Alternative culturing methods

Over the years of culturing AM fungi, there have been many interesting deviations from the traditional pot culture or ROC set-up. Some culturing methods sought to combine elements from both *in vivo* and *in vitro* systems. Voets et al. (2005) introduced a culturing system that made use of autotrophic and sterile hosts in which two-compartment Petri plates were manipulated such that roots were grown *in vitro* along with AM fungi but the still-attached host shoots could grow externally and perform photosynthesis (**Figure 5A**). In another example, Silvani et al. (2019) designed a hybrid culturing method wherein AM fungi and hairy roots grew in two-compartment Petri plates under monoxenic conditions. However the HC substrate was sterile soil, thus replicating more natural conditions (**Figure 5B**).

Designing culturing methods that combine the benefits of pot cultures (which closely resembles natural conditions) and ROCs (which offer a high degree of experimental control and sterile conditions), is a crucial endeavour. A hybrid system may allow for a greater range of AM fungi species to be cultured *in vitro*, while also taking a step towards the goal of large-scale production of high-quality, aseptic fungal inoculum (IJdo *et al.*, 2011).

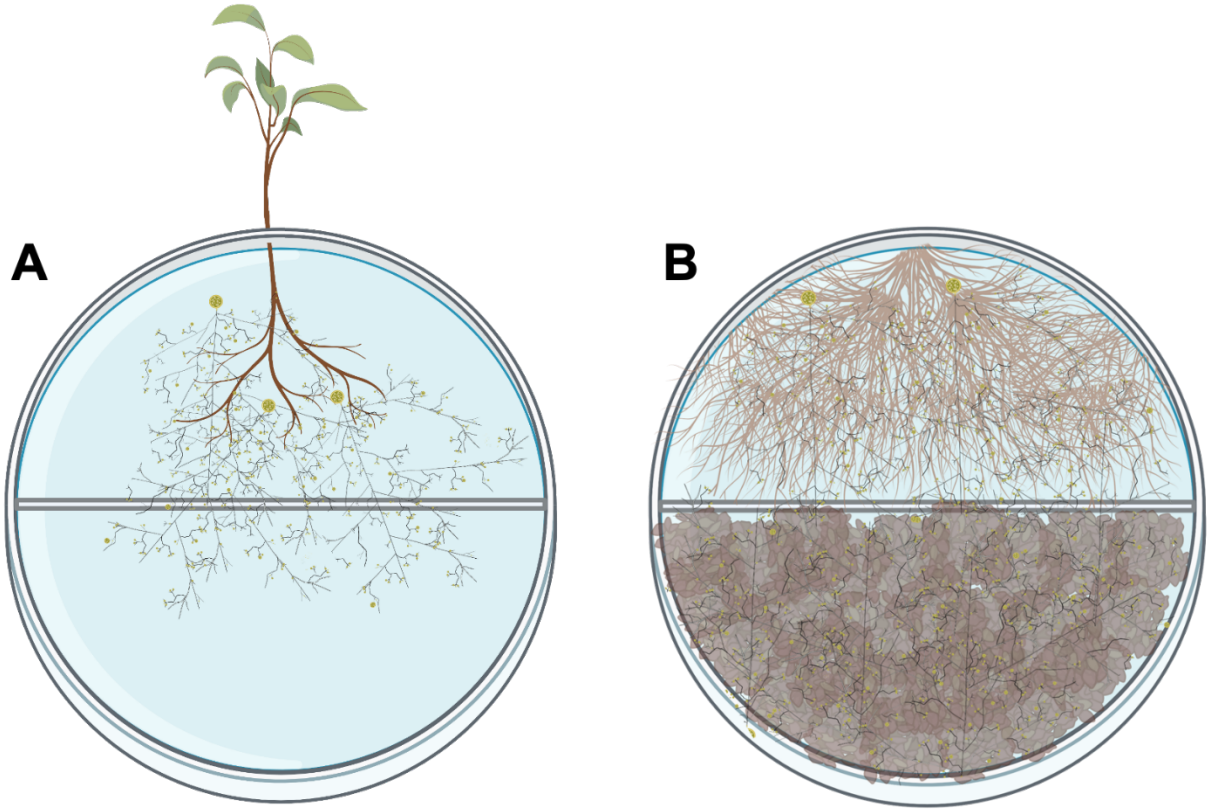


Figure 5. Alternative *in vitro* culturing systems. Voets et. al., (2005) designed a hybrid system wherein arbuscular mycorrhizal fungi are hosted by an autotrophic plant grown such that roots proliferate alongside fungi within sterile conditions while shoots grow externally (A). The culturing system described in Silvani et. al., (2019) resembles that of the traditional two-compartment Petri plate set-up, however the substrate in the hyphal compartment was autoclaved soil as opposed to gelled media (B).

1.4 Host-preference in the AM symbiosis

1.4.1 Non-random distribution of AM fungi amongst different host roots

With approximately 435,000 terrestrial plant species (Enquist *et al.*, 2019), of which about 72% are capable of engaging in AM symbiosis (Brundrett & Tedersoo, 2018), it is all but assured that there are countless redundancies in host species selection by AM fungi. According to "MaarjAM", a global database constructed using Glomeromycota small subunit RNA gene sequences, there is an uneven distribution of Glomeromycota taxa amongst the angiosperm superorders. In fact, some of these taxa were reported to have colonized only a single angiosperm superorder (Öpik *et al.*, 2010). As such, while host selection by AM fungi may not be overly specific, there is an abundance of evidence that under field and greenhouse conditions AM fungi (at the level of species or strain) exhibit a non-random distribution amongst different species of host (Eom *et al.*, 2000; Helgason *et al.*, 2002; Croll *et al.*, 2008; Davison *et al.*, 2011; Torrecillas *et al.*, 2012; Holland *et al.*, 2014). It is likely that preferential selection of host by AM fungi reflects the tendency for plants and AM fungi to induce differential growth responses in the other depending on symbiont identity (Bever, 2002; Klironomos, 2003). Different hosts possess unique life history strategies that can contribute to these differential growth responses. For example, certain root system characteristics correlate with AM colonization levels. Indeed, there is evidence of a negative relationship between host root thickness (diameter) and mycorrhizal colonization (Liu *et al.*, 2015).

1.4.2 Differential growth response of AM fungi amongst different hosts in vitro

Under *in vitro* conditions, there is a similar host-specific effect on the growth and development of AM fungi as highlighted by two studies in particular. Tiwari and Adholeya (2003) found that

the root colonization and sporulation of *R. irregularis* (referred to as *G. intraradices*) was significantly different when co-cultured with different host genotypes, carrot and clover (*Trifolium subterraneum*). Similarly, Ehinger, Koch, and Sanders (2009) worked with *R. irregularis* (though with multiple strains) co-cultured with carrot, *Medicago truncatula,* and *Solanum tuberosum* L. (potato), and found that host identity significantly affected fungal fitness.

In one-on-one *in vitro* co-cultures between different combinations of a single host and fungus pair, any variation in fungal fitness can be directly attributed to the unique interaction between symbiont identities. In fact, a recent study by Kokkoris et al. (2021) demonstrated that the host identity can alter the relative distribution of nucleotypes within dikaryotic AM fungi. It is reasonable then to assume that certain pairs of host and AM fungi genotypes would be more compatible than others. In this context, "compatible" broadly refers to the relative time that symbionts require to progress from pre-symbiotic signalling to symbiosis establishment as well as the resulting host and fungal fitness. For example, if a certain host genotype and fungus genotype are very compatible (relative to other symbiont combinations), then root colonization and nutrient exchange may occur faster. Perhaps then, in a developmental study, fungal growth (e.g., ER mycelium extension and sporulation) may be observed in higher amounts because symbiosis was established sooner.

Prior to colonization, there is a molecular crosstalk where both hosts and fungi actively locate and make contact with one another via chemical signalling. The hosts release several organic compounds all of which induce hyphal growth such as elongation and branching (Nadal & Paszkowski, 2013). Of particular significance are strigolactones, a group of plant sesquiterpenes that elicit vigorous hyphal branching when AM fungi are in close proximity to host roots (Akiyama *et al.*, 2005). Any differences amongst hosts regarding molecular signalling

behaviour (e.g., relative concentrations or timing of root exudate release) would then impact how efficiently nearby fungi can locate and colonize roots.

As AM fungi actively seek out hosts, they constitutively release their own symbiotic signals, lipochitooligosaccharides (LCOs), which stimulate both root branching as well as mycorrhiza formation (Maillet *et al.*, 2011). Moreover, AM fungi secrete numerous effector proteins within roots whose expression is host-dependent (Zeng *et al.*, 2018). While the specific role of many fungal effector proteins remains unknown, it is likely that a subset play a role in the suppression of the plants' defence response, thus facilitating successful symbioses (Kloppholz *et al.*, 2011).

Finally, once resource exchange begins between hosts and AM fungi, the differential fungal growth is a direct result of the relative carbon influx facilitated by different host genotypes. As mentioned above (section 1.4), there is evidence that hosts will adjust the amount of carbon they provide based upon the relative amount of nutrients received from their fungal partner (Kiers *et al.*, 2011).

In summary, there are many ways in which the unique life history strategies of different hosts and AM fungi can directly influence their "compatibility" as partners. Yet, there is still a trend in the field of AM fungi where most publications under *in vitro* conditions involve the same model species.

1.5 Bias towards model species in the study of AM fungi

1.5.1 Fungal symbiont: Rhizophagus irregularis

On the fungal side, *R. irregularis* has been, and continues to be, one of the most studied species of AM fungi (Parniske 2008; Rosikiewicz, 2017). Working with *R. irregularis* comes with a

variety of advantages: they are highly amenable to *in vitro* conditions, they grow relatively fast, and they produce many daughter spores that can be used to quickly establish new cultures (Berruti et al. 2016). In fact, it is precisely these characteristics that have made *R. irregularis* such a promising candidate species for mass-production of large-scale commercial inoculum (IJdo *et al.*, 2011; Berruti *et al.*, 2016; Rosikiewicz *et al.*, 2017).

1.5.2 Host-root symbiont: Daucus carota (carrot)

The widespread use of transformed carrot is likely a reflection of the enormous success achieved with these roots. In 1987, Mugnier and Mosse were the first to achieve the colonization of hairy roots with an AM fungus. They noted that Ri T-DNA transformed carrot roots were a promising host species compared to transformed roots from bindweed, cowpea and potatoes. The year after, Bécard and Fortin (1988) successfully propagated *Gigaspora margarita* using Ri T-DNA transformed carrot roots with more than 100 spores per plate. After their success, many labs followed suit and began to use the same strain(s) of transformed carrot roots as those reported in Bécard and Fortin (1988). In subsequent years, the propagation of AM fungal species using transgenic carrot roots made it possible to study fungal growth and development (Chabot *et al.*, 1992; Declerck *et al.*, 1996; Nagahashi & Douds, 1997), to culture an array of different fungal species (Douds, 1997; De Souza & Berbara, 1999; Dalpé & Declerck, 2002), and to better understand the potential for AM fungi to uptake nutrients such as phosphorus and nitrogen (Pfeffer *et al.*, 1999; Hawkins *et al.*, 2000).

Ultimately, the characteristic that most likely drew so many researchers to selecting carrot as their *in vitro* host is its consistency. Specifically, transformed carrot roots consistently exhibit vigorous primary and lateral root growth on media. They consistently facilitate high

sporulation with select AM fungal species, and all obtained results can be directly compared with countless other studies due to the consistency between chosen hosts (Bècard & Fortin, 1988; Declerck *et al.*, 1996). Moreover, because of the many benefits derived from transgenic carrot roots, curators of *in vitro* culture collections of AM fungi such as the collections maintained at CCAMF and GINCO-BEL use carrot to host the AM fungal species that tolerate these conditions.

1.6 Rationale of the research and objectives

It is clear that in the AM symbiosis, host identity can significantly affect fungal growth and development under both *in vivo* and *in vitro* conditions (sections 3.1 & 3.2). Therefore, studies involving only a single host species could potentially be flawed as the resulting fungal behaviour may simply reflect that which is elicited by a specific host genotype. Yet, over the last few decades only a single transformed root species (carrot) has primarily been used as the sole host in numerous different *in vitro* studies of AM fungi and for maintaining fungal species in long-term culture collections (section 4.2).

Consequently, we hypothesize that under *in vitro* conditions, novel Ri T-DNA transformed roots will: 1) allow for an increased diversity of AM fungal species to be cultivated under *in vitro* conditions, and 2) elicit AM fungal behaviour differing from that typically associated with transformed carrot roots (e.g., consistent fungal growth, high sporulation rates, etc.; section 4.2).

To test our hypotheses, we first developed two new lines of Ri T-DNA transformed roots, *Medicago truncatula* (cv. R108; henceforth "Med") and *Nicotiana benthamiana* (henceforth "Nic"). The Med root system is widely viewed as a model organism in the study of root-microbe

interactions as they are highly amenable to genetic engineering, are diploid and have had their genome fully sequenced, and, as a legume, form symbioses with both nitrogen-fixing rhizobacteria as well as AM fungi (Douglas Cook, 1999; Young *et al.*, 2011; Maclean *et al.*, 2017). Furthermore, pioneering work by Boisson-Dernier et al. (2001) demonstrated the potential for transformed *M. truncatula* roots to be used as *in vitro* hosts to AM fungi.

Similarly, Nic is also regarded as a model species, albeit for slightly different purposes. In the study of plant-pathogen interactions, Nic is often studied as they are highly susceptible to viruses and are also amenable to genetic engineering (Bally *et al.*, 2018). Furthermore, it has also been previously demonstrated that Nic is 1) capable of hosting AM fungi in pot cultures and 2) responsive to *Agrobacterium*-mediated root transformation (Lonoce *et al.*, 2016; Zeng *et al.*, 2018). Finally, as a control, transformed carrot roots (strain P68; henceforth "Dau") were used as well in this study.

To test our first hypothesis, both Med and Nic roots were used to host seven species of AM fungi selected based on their behaviours under *in vitro* conditions (see **Table 1**, Materials and Methods) when associated with transformed carrot roots (Dau). Additionally, we attempted to non-destructively monitor IR fungal growth in real-time by co-transforming Med and Nic roots with both Ri T-DNA and a fluorescent reporter gene system. The rationale behind transforming an additional DNA sequence into hairy roots is that potentially the symbiosis-specific expression of green fluorescent proteins (GFPs) could act as a proxy measurement to assess IR fungal growth.

For our second hypothesis, a factorial experiment was set-up involving nine unique pairs of a single host (either Med, Nic, or Dau) and a single AM fungal species (either *R. irregularis*, *R. clarus*, or *Glomus* sp.) to investigate any potential host-specific effects. Unlike in previous *in*

vitro studies which compared hosts by their ability to facilitate IR fungal growth and sporulation in close proximity to roots, this study measured how host identity influenced the development of AM symbiosis and sporulation in HCs (i.e., at a distance from host roots) over 12 weeks of incubation. To accomplish this, we developed two non-destructive protocols to 1) monitor symbiotic development and 2) quantify sporulation such that we could estimate both the rate and the spatial distribution of spore production by AM fungi when allowed to grow in isolation from hosts.

CHAPTER TWO

Materials & Methods

2.0 Materials and Methods

2.1 Production and maintenance of Ri T-DNA transformed roots

2.1.1 Transforming Agrobacterium rhizogenes

Med and Nic hairy roots were obtained via *Agrobacterium*-mediated co-transformation. Fresh colonies of *Agrobacterium rhizogenes* strain ARqua1 (acquired from Dr. Maria Harrison, Boyce Thompson Institute, Cornell University) were obtained by streaking glycerol stocks onto LB medium (with streptomycin, 200 µg/ml) and incubated at 28˚C. Single colonies of ARqua1 were used to inoculate LB broth (with streptomycin, 100 µg/ml), which were then incubated, shaking at 28˚C. Liquid ARqua1 cultures were harvested via centrifugation (4000 g, 5 minutes, 4˚C) when an optical density$_{600nm}$ reading between 0.5-1 was recorded. ARqua1 cells were spun down four times (4000 g, 5 minutes, 4˚C), first to harvest cells from LB broth, then twice in dH_2O to wash cells, and lastly in 10% glycerol.

ARqua1 cells were transformed via electroporation with one of two expression vectors. These expression vectors contained an *NLS-GFP-GUS*/pKm43GW-RR sequence with either MtPT4 or MtBCP1 promoters to drive expression of the NLS-GFP-GUS cassette. While both promoters are symbiosis-specific, MtPT4 expression is restricted to root cortical cells containing an arbuscule, whereas MtBCP1 activity is more broadly expressed and is active in all root cortical cells that are in contact with hyphae as well as adjacent cortical cells (Pumplin & Harrison, 2009).

Expression vectors described above were created using Gateway® Cloning Technology, specifically via an LR reaction (**Figure 6**). DNA fragments of interest, promoters, NLS-GFP-GUS sequence, and a 35S stop-codon sequence were acquired from Dr. Harrison's lab in the

form of entry clones; MtPT4p/pDONR1.4 (or MtBCP1p/pDONR1.4), NLS-GFP-GUS/pDONR1,2, and 35S Transcription Stop-Codon/pDONR2.3.

The purpose of the expression vectors are two-fold: 1) the destination vector, pKm43GW-RR, contains a red root (RR) sequence which will constitutively express the red fluorescent protein DsRed1 (Limpens *et al.*, 2004), used to screen for hairy roots successfully co-transformed with both an Ri plasmid as well as an expression vector, and 2) the symbiosis-specific promoters should drive the expression of GFPs and potentially allow for non-destructive observations of IR fungal colonization.

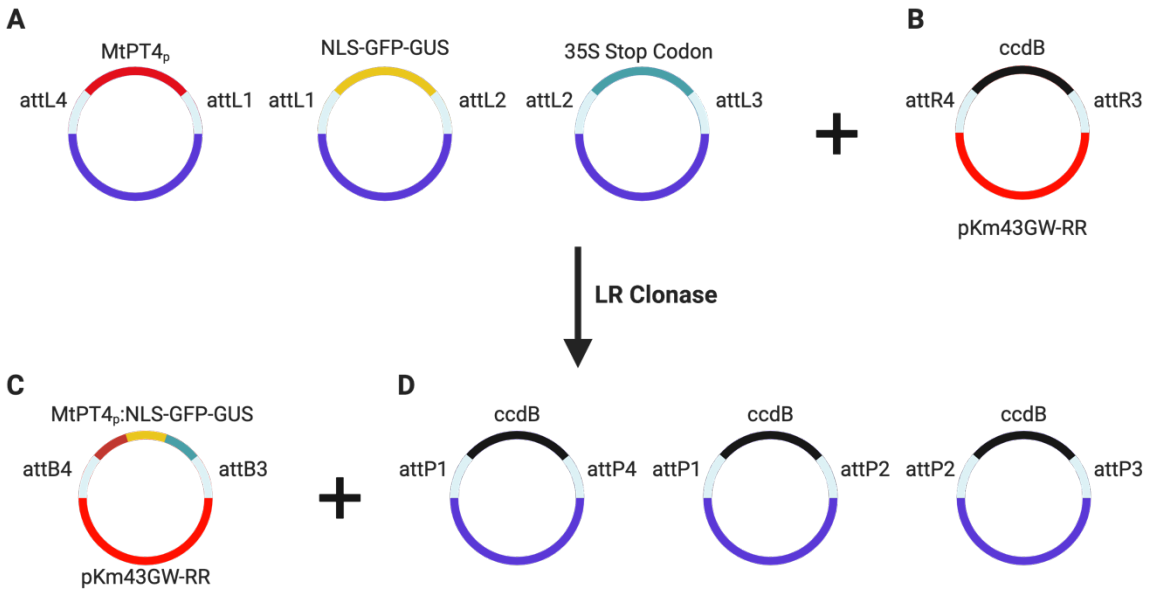


Figure 6. Construction of expression vector MtPT4p: NLS-GFP-GUS/pKm43GW-RR using an LR reaction (Gateway Cloning). Flanking att-sequences are recognized by LR clonase which works to transfer DNA fragments from entry clones (A; MtPT4p/pDONR1.4, NLS-GFP-GUS/pDONR1,2, and 35S Transcription Stop-Codon/pDONR2.3) and the backbone of a destination vector (B; pKm43GW-RR/pENTR4,3) into an expression vector (C). Additional vectors produced by an LR reaction are byproducts (D) which express a toxic ccdB gene used as a positive-selection marker.

2.1.2 Co-transformation of Medicago truncatula roots

The co-transformation of Med (two cultivars, cv. A17 and R108; Boyce Thompson Institute, Cornell University) was performed according to the methods described by Floss et. al. (2013) until section 3.2.3 *Induction of Transgenic Roots*, step 8, where the following modifications were made. Successfully co-transformed roots identified by their DsRed1 expression were excised and aseptically transferred to MRC medium containing 200 μg/ml of cefotaxime to eliminate any residual *A. rhizogenes*. Hairy roots were cultured upon MRC medium for six weeks, sub-culturing onto fresh medium every two weeks to ensure cultures were axenic. Next, A17 and R108 hairy roots were transferred to M-medium (Becard & Fortin, 1988) modified by adding 20 g/L of sucrose and increasing the pH to 6.0, gelled with 0.3% phytagel (Sigma, Aldrich).

2.1.3 Co-transformation of Nicotiana benthamiana roots

To create Nic hairy roots, the protocol established to transform chickpea (*Cicer arietinum* L.; Aggarwal et. al., 2018) was followed as a template with several modifications. Nic seeds (Boyce Thompson Institute, Cornell University) were sown on F-medium (Floss *et al.*, 2013) and allowed to germinate at room temperature on a windowsill under natural light until seedling roots were approximately a centimetre in length. The day prior to seedling co-transformation, transformed ARqua1 was added to LB mixed with spectinomycin (200 μg/ml) for selection of the expression vectors described above and incubated overnight, shaking at 28ºC. The following day, the overnight culture was used to initiate secondary ARqua1 cultures in 50 ml Falcon tubes and incubated (shaking at 28ºC) until an Optical Density$_{600nm}$ reading between 0.5-1 was recorded.

Next, the ARqua1 cells were harvested by centrifuging at a speed of 2,900 g at 28ºC for 10 min. A bacterial suspension was then created by re-suspending the ARqua1 pellet using MS medium (Murashige & Skoog, 1962) and then transferred to one-compartment Petri dishes. To increase *Agrobacterium* virulence, 100 µmol of acetosyringone was added to the ARqua1 suspension. Under a flow hood, Nic root tips were removed from seedlings before they were placed into the ARqua1 suspension oriented such that sectioned roots were entirely submerged. Seedlings were left in the suspension for an hour before they were transferred to F-medium mixed with kanamycin (50 µg/ml). Incubation of Nic seedlings was then performed according to steps 7 and 8 under the 3.2.3 *Induction of Transgenic Roots* section of Floss et. al. (2013). The screening and sub-culturing of Nic hairy roots was conducted identically to that of Med.

2.1.4 Sub-cultivating Ri T-DNA transformed roots

Given the variable nature of hairy root growth, the root segments used for sub-culturing had to be chosen methodically. That is, only root segments which possessed characteristics observed to be associated with relatively consistent and rapid growth were excised. For Nic, sections of medium containing thin, pale roots were removed from stock plates and placed onto new medium such that roots segments were in contact with the medium. Alternatively, thin, pale Med roots possessing many lateral roots resembling a "fish bone" shape (Chabaud et. al., 2002) were selected, cut, and gently pressed onto new medium. As the process of root sub-culturing continued, it became apparent that R108 roots were growing more consistently than A17 under *in vitro* conditions. Therefore, R108 was chosen as the representative cultivar for Med in this study.

The transformed Dau roots (cv. P68) used in this study were obtained from AAFC (Ottawa, ON). The gravitropism of Med and Nic hairy roots was observed to be negative, therefore the cultures were incubated right-side up. Conversely, Dau cultures were inverted before incubation.

2.2 AM fungi

Seven different species of AM fungi were used in this study. These species were chosen as they are representative of three distinct growth profiles when co-cultured with carrot roots under the monoxenic incubation conditions used at CCAMF, AAFC, Ottawa, ON (Claudia Banchini & Franck Stefani, personal communication). The *in vitro* AM fungi growth behaviours described by curators of CCAMF were used to classify the seven species into three categories (**Table 1**).

The first category is defined by AM fungi that grow well in both one and two-compartment Petri dishes and is represented by *Rhizophagus irregularis* (strain 4375/DAOM234181) and *Glomus* sp. (strain GC-4). Specifically, *R. irregularis* and *Glomus* sp. consistently produced an extensive network of ER hyphae along with an abundance of ER spores close to host roots. Moreover, when these fungi were cultured in the RC of two-compartment Petri dishes along with carrot roots, hyphal growth and sporulation were often observed to occur at a distance from host roots in the HC.

The second category is defined by species that do not colonize and propagate in the HC, i.e., they stay always in close proximity to roots. It is represented by *Rhizophagus clarus* (strain GC-5/DAOM234281) and *Claroideoglomus lamellosum,* (strain 4350B).

The third category is defined by species that, to date, have either not been successfully cultured *in vitro,* or have previously been reported to grow under monoxenic conditions but for only a limited number of sub-cultures (abortive spores). This category is represented by

Funneliformis mosseae (strain 2101), *Cetraspora* cf. *gilmorei* (strain: 4398), and *Gigaspora margarita* (strain: 4292).

Table 1. Species of AM fungi used in this study, categorized based on *in vitro* growth behaviour with Ri T-DNA transformed carrot roots (strain: P68).

Category	Species	Strain / DAOM No.	Origin	Notes
1	*Rhizophagus irregularis*	4375 / DAOM234181	Magdalens Islands, QC	Grows well and produces many spores
	Glomus sp.	GC-4 / DAOM240160	Angers, France	
2	*Rhizophagus clarus*	GC-5 / DAOM234281	La Palma, Cuba	Does not colonize and propagate in the hyphal compartment
	Claroideoglomus lamellosum	4350B	St-Sixte, QC	
3	*Funneliformis mosseae*	2101	Fort Erie, ON	Few abortive spores
	Cetraspora cf. *gilmorei*	4398	Magdalens Islands, QC	Few abortive spores and auxiliary cells
	Gigaspora margarita	4292	Ottawa, ON	

2.3 Screening Med and Nic for AM symbiotic compatibility

2.3.1 In vivo cultures

Symbiotic compatibility between hosts Med and Nic, and the AM fungi used in this study was first screened under more natural conditions using pot cultures. Med seeds (Boyce Thompson Institute, Cornell University) were removed from seed pods before chemically scarifying seed coats in sulphuric acid. After nine minutes, the seeds were immediately rinsed with water five times. They were then placed into a 10% bleach and 0.1% Tween® 20 solution for 10 minutes, shaking. Under a flow hood, the seeds were washed five times with sterile water, placed into Petri dishes lined with damp filter paper, and stored for three days in the dark at 4 ºC for cold stratification. Then the seeds were placed into a dark, room temperature drawer to germinate overnight. The following day, seedlings were transferred to a windowsill to grow under sunlight at room temperature. Med seedlings were ready to be sown into soil once their cotyledons developed. Previous tests determined that no prior steps were required to grow Nic seedlings other than to sow seeds directly onto the soil. Two Med seedlings were sown one centimetre deep into the soil, while four Nic seeds were sown on top of the soil and thinned out to two seedlings after germination.

Plants were grown in cone-tainers filled with a 1:1 mixture of autoclaved sand and turface. A marble was placed at the bottom of each cone-tainer to prevent soil from spilling out, while also ensuring adequate drainage. AM fungal species belonging to categories 1 and 2 were previously maintained *in vitro* meaning that inoculum from these species came in the form of mycorrhizal roots and spores (fungal propagules) embedded in sterile medium. Alternatively, inoculum for fungal species belonging to category 3 were received as soil filled with fungal

propagules. All fungal inocula was mixed into the soil approximately 2-3 cm below the surface. In total, five cone-tainers for each combination of host and AM fungus species were set-up.

Plants were incubated in a growth chamber with a 16/8h, 25/22˚C, light/dark regime with a light intensity of 220 μM/m^2s. Med were watered once a week and fertilized every three weeks with half-strength Hoaglands solution (Hoagland & Arnon, 1950). Nic followed a similar watering cycle to that of Med, but with half-strength Hoaglands solution twice a week.

All plants were harvested after 10 weeks of growth. They were removed from cone-tainers, their stems were cut, and roots were carefully cleansed of soil by gently massaging the roots under water. The roots were then placed into 50 ml Falcon tubes filled with a 50% ethanol solution. After a 15-minute incubation at room temperature, the ethanol solution was removed and replaced with a 20% KOH solution before the tubes were placed into a 65˚C incubator overnight. The next day, the tubes were drained of 20% KOH before the roots were washed three times with phosphate buffered saline (PBS), replacing with fresh PBS and shaking for 10 minutes each time. After the final PBS wash, the tubes were once again drained. Next, a fluorescent dye, wheat germ agglutinin (WGA) Alexa Fluor ™ 488, was mixed with PBS (0.2 ng/ml) and was added to each tube such that all roots were completely submerged in the dye. WGA Alexa Fluor ™ 488 was used to facilitate the visualization of fungal structures both within and along harvested roots. After at least 24 hours of incubation in the fluorescent dye at 4˚C in the dark, the roots were ready for microscopy. The presence of fungal colonization was then assessed using a fluorescent stereomicroscope.

2.3.2 In vitro cultures

Monoxenic *in vitro* cultures were set-up to test whether newly co-transformed Med and Nic hairy roots were symbiotically compatible with the species of AM fungi used in this study. Under a flow hood, actively growing root segments were excised and transferred to one-compartment (100 mm× 15 mm) Petri dishes filled with M-medium. See section 2.1 for details regarding root sub-culturing and modifications to M-medium. Plugs of MSR medium (Declerck et. al, 1998) filled with fungal propagules belonging to *R. irregularis, Glomus* sp*., R. clarus,* and *C. lamellosum* were cut out, placed near sub-cultured root segments, and smeared into a jelly-like consistency to maximize the number of spores that could be in close contact with hosts. Spores belonging to the species of AM fungi that belong to category 3, *F. mosseae, Cetraspora* cf. *gilmorei,* and *G. margarita* were first isolated from bulk soil via wet sieving. Next, the spores were separated out from fine soil particles under a stereomicroscope and placed into a PCR tube. Finally, under a flow hood, the spores were sterilised according to Bécard and Fortin (1988). Following sterilisation, the spores were incubated at room temperature in the dark and monitored daily. Once germ tube growth was observed under a stereomicroscope, individual germinating spores were transferred to Petri dishes and positioned no further than a cm away from root segments. Monoxenic cultures were incubated in the dark at room temperature and observed every two weeks to monitor symbiosis development (SD). Select host and AM fungus pairs whose symbioses yielded newly produced daughter spores were graduated to a two-compartment Petri dish.

2.4 Symbiosis assays in a two-compartment Petri dish

2.4.1 In vitro assay set-up

Two-compartment Petri dishes (Corning™ or Kord Valmark™, 100 mm × 15 mm) were used in this study so that a designated RC and HC could be studied independently of one another as they are separated by a built-in plastic barrier. In each two-compartment Petri dish, RCs and HCs were filled with M-medium (Becard & Fortin, 1988) but modified as follows: pH was adjusted to 6.0, phytagel (Sigma Aldrich) was used as a gelling agent, RC medium was filled with 20 g/L of sucrose, and HC medium lacked sucrose. To facilitate the fungal colonisation of the HC, autoclaved filter paper bridges (3 cm × 1 cm) were placed over the centre of the plastic barrier dividing each two-compartment Petri dish, with each end of the bridge partially embedded into RC and HC media (Dalpé & Seguin, 2010).

To test whether an effect of host species exists in the *in vitro* cultivation of AM fungi, we conducted a factorial experiment using the *in vitro* system described above. The factors were host (three levels: Nic, Med and Dau) and AM fungi (*R. irregularis, R. clarus,* and *Glomus* sp.), which translated into nine unique pairs of host and AM fungus species. Experimental replicate consisted of a culture in a two-compartment Petri dish containing a section of freshly sub-cultured hairy roots from a single host and twenty daughter spores of the same fungal species, both components placed in the same location of the RC across all replicates (**Figure 7A**). The number of replicates per host and AM fungus pair varied since any observation of contamination resulted in the contaminated replicate getting removed from the assay (**Table 2**). For each trial, all replicates received fungal inoculum that was sourced from a single *in vitro* culture and that was solubilised using citrate buffer (Doner & Bécard, 1991). For example, when setting up a trial using *R. clarus*, the spores from a single Petri-plate were used to inoculate multiple replicates for

Nic, Med, and Dau. Inoculation took place under a flow hood where spores were aseptically manipulated using a stereomicroscope and syringe needles such that groups of 20 spores could be transferred to the RC via pipette.

2.4.2 Symbiosis development

To track the SD between host roots and AM fungi, six SD stages were first defined and then observed over 12 weeks (**Figure 7**). As mentioned above, each replicate was initially set-up with hairy roots and 20 spores in the RC (**Figure 7A**). At the time of set-up, the spores were examined such that upon subsequent observation, any new hyphal growth (**Figure 7B**) could be distinguished and progression to stage 1 could be recorded. As both symbionts grew into their surrounding area in the RC, eventually the second stage was reached and both roots and hyphae came into close contact (**Figure 7C**). This second stage is a rough estimate of when pre-symbiotic interactions via root and fungal exudates is first observed to occur based upon both symbionts growing in close proximity (approximately ≤ 1mm between roots and hyphae). The third stage, new daughter spores in RC, was scored at the first observation of any new spores in the RC, thus marking the completion of the AM fungi life cycle (**Figure 7D**). As the network of ER hyphae becomes more extensive, hyphae were soon observed to cross the hyphal bridge and into the HC (fourth stage, **Figure 7E**). The fifth stage, new daughter spores in HC (**Figure 7F**), is similar to that of stage 3 differing only in which compartment the new daughter spores were first observed. Finally, the sixth stage was determined once at least 30 spores were counted in the HC (**Figure 7G**). The rationale behind a 30-spore cut-off is similar to that of typical bacteria colony forming units (CFUs) plate counting methods. That is, researchers score the number of plates which exhibit 30-300 CFUs as this interval represents a statistically significant amount

(Breed & Dotterrer, 1916). In this study, plates which exhibit over 30 daughter spores in the HC were selected for the spore quantification method outlined below.

Each of the six SD stages was chosen as they are easily identified under a stereomicroscope and are distinct from one another. Moreover, developmental stages are associated with ordered numbers of increasing value as each stage number indicates a progression from previous stages with lower values. In other words, to progress to the final stage (stage 6), the symbiosis must first progress through stages 1-5. Observations were made every two weeks post inoculation (wpi) so that the most advanced stage reached by each replicate could be recorded.

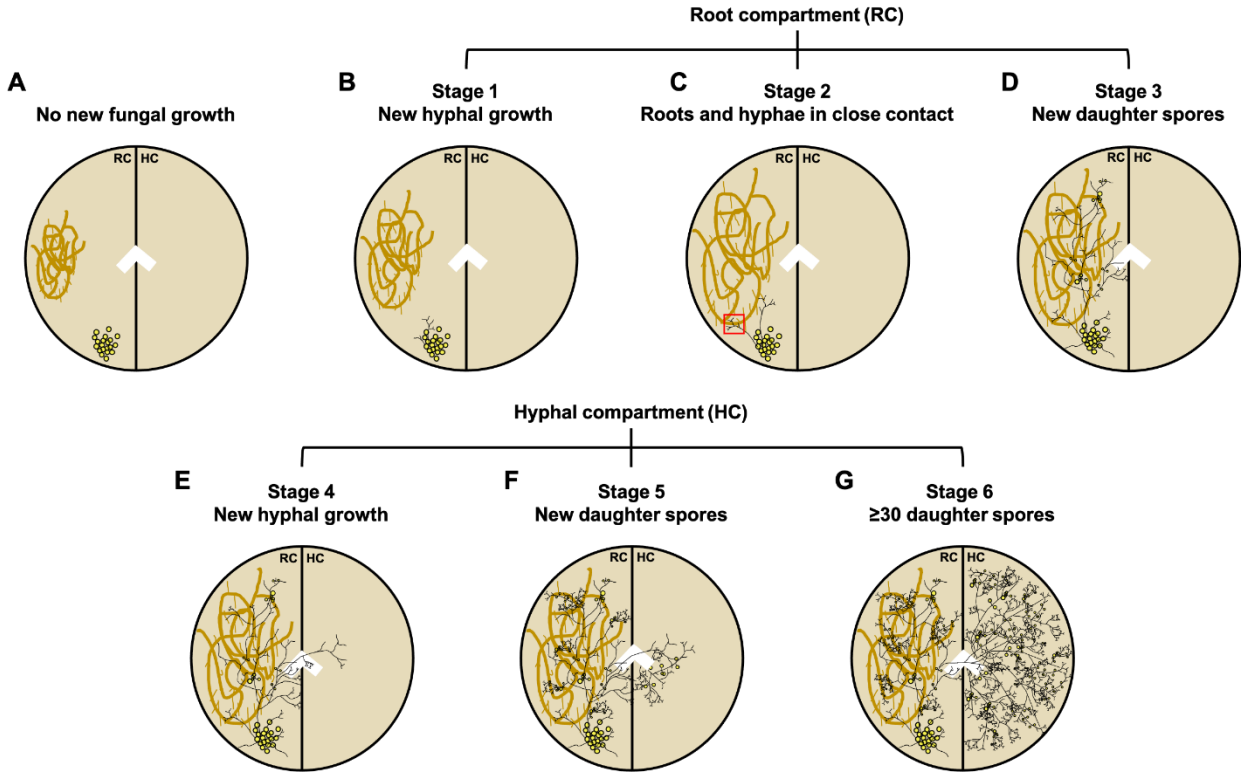


Figure 7. Stages of symbiosis development under in vitro conditions (two-compartment Petri dish). A) each Petri dish replicate was set-up with a segment of hairy root and 20 AM fungi spores, B) stage 1: new hyphal growth in the RC, C) stage 2: roots and hyphae in close contact are often observed at the same time but the latter (denoted by a red square) suggests that pre-symbiotic host and AM fungus interactions were taking place, D) stage 3: new daughter spores observed in the RC, E) stage 4: hyphal propagation over the hyphal bridge and into the HC, F) stage 5: new daughter spores produced in the HC, G) stage 6: more than 30 new daughter spores observed in the HC.

2.4.3 Quantifying spore production in HCs

Replicates of host and AM fungi pairs that exhibited highly successful symbioses in the SD assay (section 2.4.2) were further characterized by quantifying their HC sporulation. In other words, cultures that had progressed to the final SD stage at either 8, 10, or 12, wpi were selected for observation (**Table 2**). The protocol used in this study yielded an estimate for the number of spores present in any given 5 mm^2 area in HC. The two-compartment Petri dishes were inverted so that their bottom layer could be fitted to a 3D-printed grid-mount. The grid-mount was designed in collaboration with the University of Ottawa's Centre for Entrepreneurship and Engineering Design (**Figure 8**). The purpose of the grid-mount was to overlay projector paper printed with a 5 mm^2 grid on top of the HC and lock all components into a consistent position for observation.

Selection of which squares in the grid were to be observed was conducted methodically in order to ensure that all areas, regardless of position, had equal representation. Controlling for any sampling bias as a result of varying square locations required measuring distances from the centre of each square to the centre of the bottom layer of a typical 100 mm× 15 mm Petri dish. Since hyphal bridges were placed in the centre of each two-compartment Petri plate, this centre location represented approximately the first point of contact that hyphae would have into the HC. Two-compartment Petri dish dimensions were measured so that a reference image could be created on PowerPoint using accurate measurements. Next, the reference image was imported to FIJI, an image analysis software, so that using the appropriate dimensions for scale, distances from each square to the centre of the reference image could be measured (Schindelin *et al.*, 2012).

The 5 mm^2 grid was then divided in two different ways (**Figure 9**). First, two semicircular lines were drawn onto the HC, dividing the 5 mm^2 grid into three spatial zones (**Figure 9A**), each representing a different range of distance from the theoretical hyphal bridge location, i) 0-20 mm, ii) 20-30 mm, and iii) 30-40 mm. The rationale behind semicircular lines was to accommodate the radial pattern that AM fungi exhibit when growing into the HC. If a square was intersected by a semicircular line, it was only made available for observation if at least 80% or more of the squared area resided in a single spatial zone.

After adjusting for intersecting lines, a total of 76 different squares qualified to be selected. Next, the 76 squares present in the three spatial zones were further divided into three sections with a similar number of squares. This resulted in nine areas which, when observed together, provided a representative snapshot of sporulation inside a HC. At the time of observation, a set of nine different numbers was randomly generated using R (**Figure 9B**) and the corresponding squares were then selected accordingly for observation (**Figure 9C**).

A unique set of numbers was generated for each of the bi-weekly observations of all trials (≥3 trials/AM fungal species), and the corresponding squares made up the nine observational 5 mm^2 areas examined for all of the host and AM fungus replicates (**Table 2**) belonging to each trial. In other words, for all trials at 8, 10, and 12 wpi, the steps outlined in **Figure 9B-C** were carried out prior to observations. Under a stereomicroscope, the selected squares were consistently observed at the highest magnification where the field of view still included the entire outline of a single square. For example, with a Zeiss Discovery. V8 stereomicroscope with CL 1500 ECO, a 32× magnification was used. All spores in each square were counted.

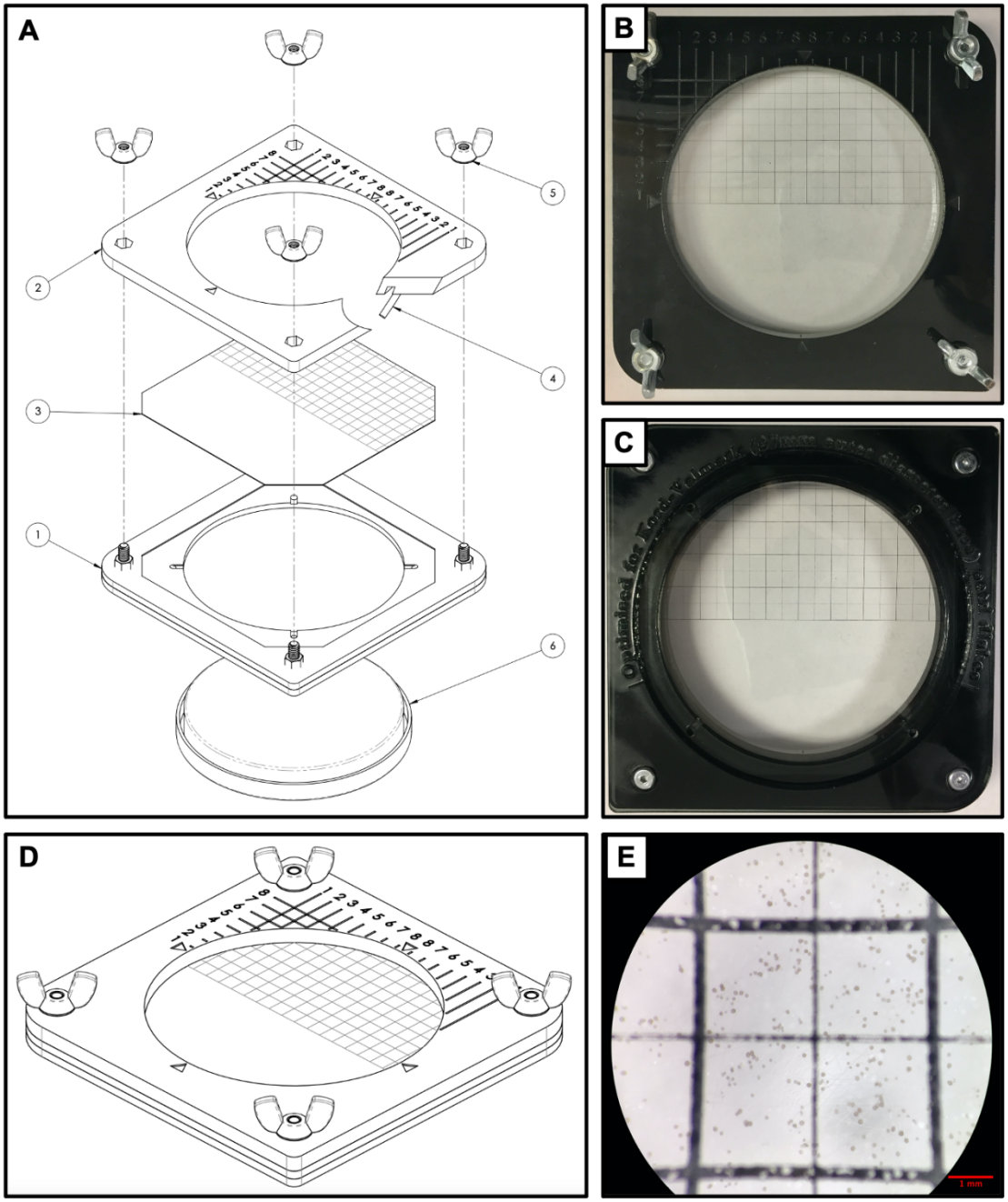


Figure 8. 3D-printed Petri dish grid-mount used for quantifying spores. Individual components (A) are 1) base layer where grid-mount is placed upon Petri dish, 2) top layer of grid-mount, 3) laser-cut projector film with a 5mm^2 grid printed on, 4) rubber O-ring to ensure grid does not slide, 5) wingnuts, and 6) Petri dish inverted, oriented such that HC is overlaid with grid. Actual printed design, top layer (B) and bottom layer (C) as compared to the design mock-up in (D). Photograph of a single 5mm^2 grid square viewed through ocular lens of a Zeiss Discovery. V8 stereomicroscope with CL 1500 ECO at 32× total magnification (E).

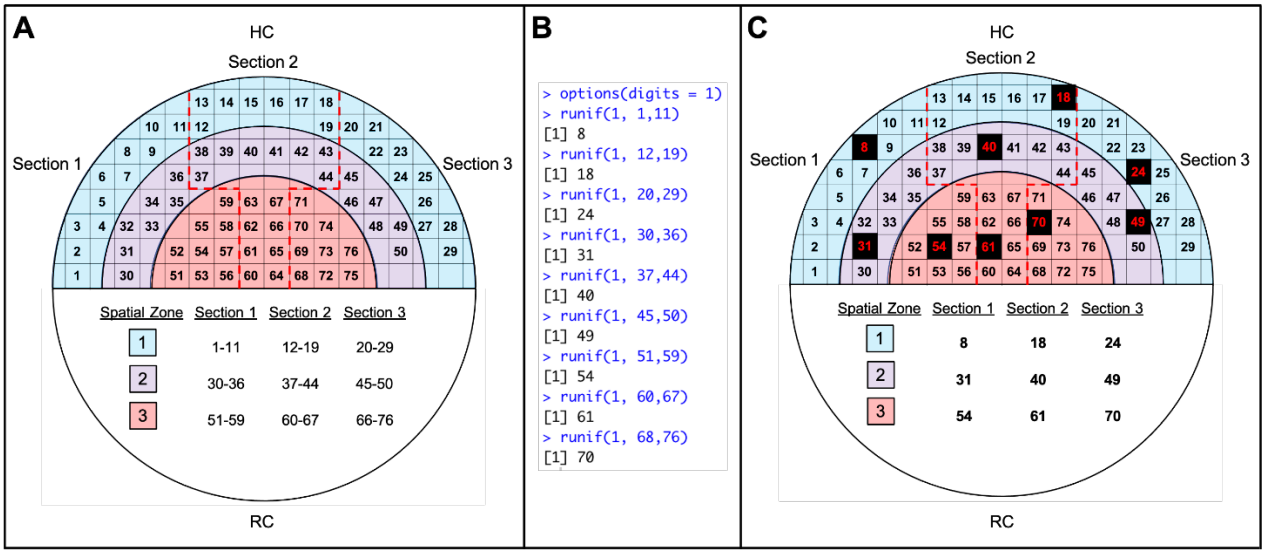


Figure 9. Hyphal compartment overlayed with 5mm^2 grid (A) divided into three growth zones (blue, purple, and red) and three different sub-sections (separated by dashed red lines), resulting in 9 distinct areas. Each area in the grid contains multiple squares, each associated with a number. One number from each of the nine areas was randomly selected using an R script (B), marked for reference (C), and then the total number of spores present in each of the corresponding nine squares were then counted.

Table 2. Sample size for each host and AM fungus pair. Replicates refer to the number of two-compartment Petri dishes that remained monoxenic at 8-, 10-, and 12-weeks post inoculation. Following the spore quantification method described above (section 2.4.3), each replicate yielded nine observations.

		Nic		**Med**		**Dau**	
Species	Week	Replicates	Total No. of observations	Replicates	Total No. of observations	Replicates	Total No. of observations
	8	1	9	10	90	14	126
R. irregularis	10	1	9	16	144	21	189
	12	1	9	21	189	26	234
	8	1	9	4	36	7	63
R. clarus	10	1	9	5	45	11	99
	12	0	0	3	27	8	72
	8	0	0	4	36	0	0
***Glomus* sp.**	10	0	0	6	54	1	9
	12	0	0	6	54	3	27

2.5 Statistical analysis

All statistical analyses were performed using the R software (version 4.0.3; R Development Core Team, Vienna Austria).

2.5.1 Progression through symbiotic developmental stages

To test whether there was a host-specific effect on the development of AM fungi, each of the nine unique host and AM fungus pairs was compared by the rate at which they progressed through the defined SD stages (described above). Fungal development was tested as a function of a three-way interaction between the following predictor variables: host species, AM fungal species, and time (in weeks). Fungal development was also measured as an ordinal factor (i.e., progressing through the different development stages was dependent on first completing all preceding stages), therefore a multinomial linear regression (MLR) was fitted using the *polr* function from the MASS package (Venables & Ripley, 2002). A Type III analysis of variance (ANOVA) was used to determine whether the predictor variables (independently or through interaction) conferred a significant effect ($p \leq 0.05$) on the progression of the cultures (replicates) through the SD stages. Finally, a post-hoc pairwise comparison was used to determine if the rate at which specific host and AM fungus pairs progressed through SD stages was significantly different.

2.5.2 HC Spore quantification

To test whether there was a host-specific effect on spore production, a two-compartment Petri dish set-up was used and all sporulation in the HC was quantified and compared for all hosts and AM fungi pairs at 8, 10, and 12 wpi (section 2.4.3). A linear mixed model (LMM) was fitted to

test spore production as a function of a three-way interaction between host species, AM fungal species, and time (in weeks) as fixed effects. Furthermore, it was predicted that as time progressed, sporulation would occur further into the HC. This represented a longer distance from the hyphal bridge (i.e., initial point of contact between fungal hyphae and HC) and therefore an interaction between distance and time was also included as a fixed effect for sporulation. To control for repeated measurements over multiple time points, replicates were assigned unique IDs (Petri dish number, trial number, host species, and AM fungi species), which were then included as a random effect. Due to poor fungal development in cultures involving *Glomus* sp. (strain GC-4), few replicates exhibited sporulation. As a result, *Glomus* sp. data was excluded from the first model used.

To determine whether the trends observed in the first model was consistent amongst either *R. irregularis* and *R. clarus* data independently, two additional models were fit using AM fungi-specific subsets of data, (i.e., either only *R. irregularis* data or *R. clarus* data) using the same fixed effects (minus AM fungal species) and random effect (replicate ID). Only with R108 did an acceptable number of *Glomus* sp. replicates (n = 6) exhibit HC sporulation. A fourth model was fit using spore production as a function of the following fixed effects: AM fungal species, time, and a time × distance interaction. Again, replicate ID was used to account for random effects. All models were followed by Type III ANOVAs to determine whether any of the fixed effects (independently or through interaction) conferred a significant effect on spore production. Each ANOVA was followed by a Tukey HSD *post hoc* test for pair-wise comparisons between unique pairs of hosts and AM fungi. Finally, all graphs were generated using the function *allEffects* from the R package “effects” version 4.0.2 (Fox & Weisberg, 2019).

CHAPTER THREE

Results

3.0 Results

3.1 Co-transformation and maintenance of newly Ri T-DNA transformed roots

While the co-transformation of both Med and Nic roots was successful, transformation efficiency for the latter was much lower. Each Med seedling subjected to co-transformation typically yielded at least one hairy root (**Figure 10A**). In contrast, of the 14 tested Nic seedlings, only a single Nic root fluoresced DsRed1 (**Figure 10B**). However, hairy roots from both plant species grew continuously and continued to fluoresce red within Petri plates (**Figure 10C**). Over time, stock plates were accumulated through regular sub-culture (**Figure 11**).

The morphology of sub-cultured Med hairy roots was found to vary depending upon the morphology of the root used to start a new culture. When fast growing, white "fish bone" shaped Med segments were sub-cultured, the roots tended to grow in a similar fashion, and the resulting morphology would resemble that seen in **Figure 11A**. Once growing ceased after approximately 4 weeks, the roots would darken from their initial pale, white colour to a brown-yellow colour. When thicker, brown-yellow Med roots were sub-cultured, the resulting cultures grew similarly with growth ceasing as early as 2-3 weeks.

Nic roots tended to show little variation in morphology between sub-cultures. Newly growing roots would appear pale, almost translucent, and their entire length would be coated in countless fine root hairs with a diameter comparable to that of fungal hyphae (**Figure 11B**).

Depending on plant species, hairy root cultures differed in their growth rate and the relative accumulation or location of condensation; both characteristics were directly related to the frequency of contamination observed for each species. Despite working aseptically, the cultures that required the most maintenance exhibited the highest likelihood of becoming

contaminated. Furthermore, when roots did require re-direction, the presence of condensation within cultures exacerbated the contamination rate.

During the first four weeks of culturing all three hairy root species, (Med, Nic, and Dau), Med and Nic roots grew the fastest. In the subsequent weeks, Med growth slowed down considerably while Nic continued to grow at a consistent pace until 8-10 weeks post sub-culture. Dau roots grew slower than the other root species initially but while they slowed down, Dau growth outpaced Med and Nic by continuing to grow at a similar rate throughout all 12 weeks of observation.

AM symbioses culturing in two-compartment Petri dishes required routine maintenance, which involved working aseptically to manually re-direct any hairy roots growing towards the HC, back to into the RC (**Figure 3**). Moreover, the weekly maintenance requirements for Med, Nic, and Dau were directly related to each of their respective growth rates.

All hairy roots initially required re-direction 2-3 times a week before their respective growth rates slowed down, i.e., re-directions occurred 2-3 times a week for approximately 4, 8, and 12 weeks, for Med, Nic, and Dau, respectively. Regardless of species, once a root culture growth began to slow down, maintenance was typically once a week at the most.

Our incubation conditions inevitably caused condensation to form in all cultures along the inner surface of the Petri dish lids. As a result, the positioning of condensation relative to the symbionts culturing on or in medium was dependent upon how the Petri dishes were oriented. Whereas the right-side-up orientation of Med and Nic cultures during incubation positioned the Petri dish lids and condensation above the roots, the opposite was observed for Dau cultures whose orientation was inverted. Any slight disturbance to Med or Nic cultures caused moisture to come into contact with roots, thus increasing the potential for contamination.

Ultimately, Med root cultures had the lowest likelihood of becoming contaminated as they required the least maintenance overall. Both Nic and Dau cultures were found to possess relatively high likelihood of contamination. Nic roots posed the greatest risk of contamination because whenever the Petri dishes were opened, the numerous root hairs from the Nic roots increased the potential for contact with airborne contaminants. Dau root cultures were difficult to maintain as they required the most re-directions and consistently accumulated the most condensation amongst all root species.

3.2 Newly produced hairy roots exhibited non-symbiosis specific activity

Both symbiosis-specific promoters, MtPT4 and MtBCP1, were active in Med (**Figure 12**) and Nic (not shown) roots growing in the absence of AM fungi. Non-specific promoter activity was confirmed by GFP expression in cortical cells along the length of non-mycorrhizal hairy roots. Therefore, it was not possible to use the MtPT4p/MtBCP1p:NLS-GFP-GUS constructs to monitor IR fungal growth within *in vitro* cultures non-destructively.

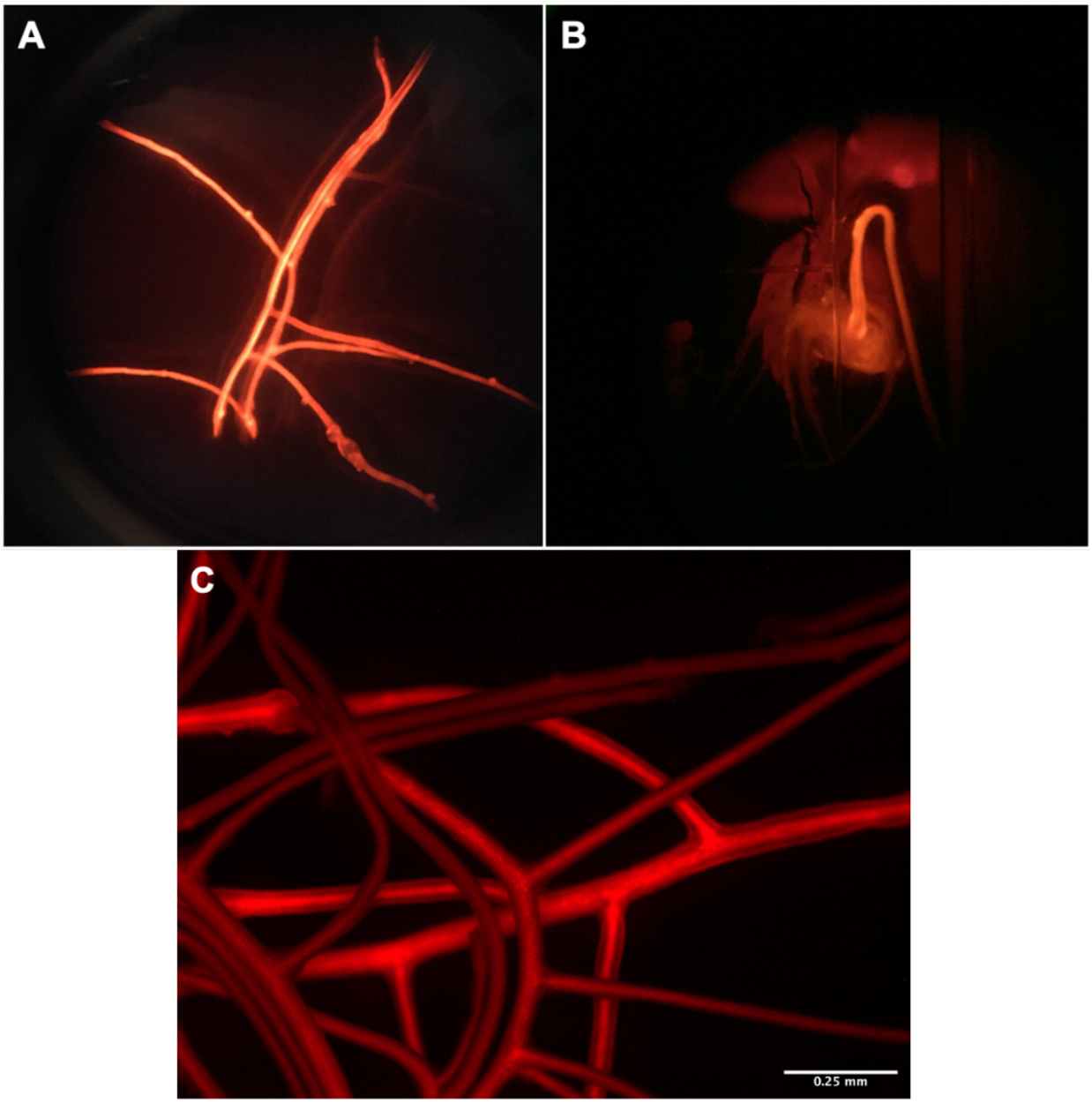


Figure 10. DsRED1 expression in Ri T-DNA transformed roots. Photographs taken through a red plastic filter of excised Med roots (A) and a single Nic hairy root still attached to shoot (B). Micrograph of a Med ROC taken with Zeiss AxioZoom.V16 (C).

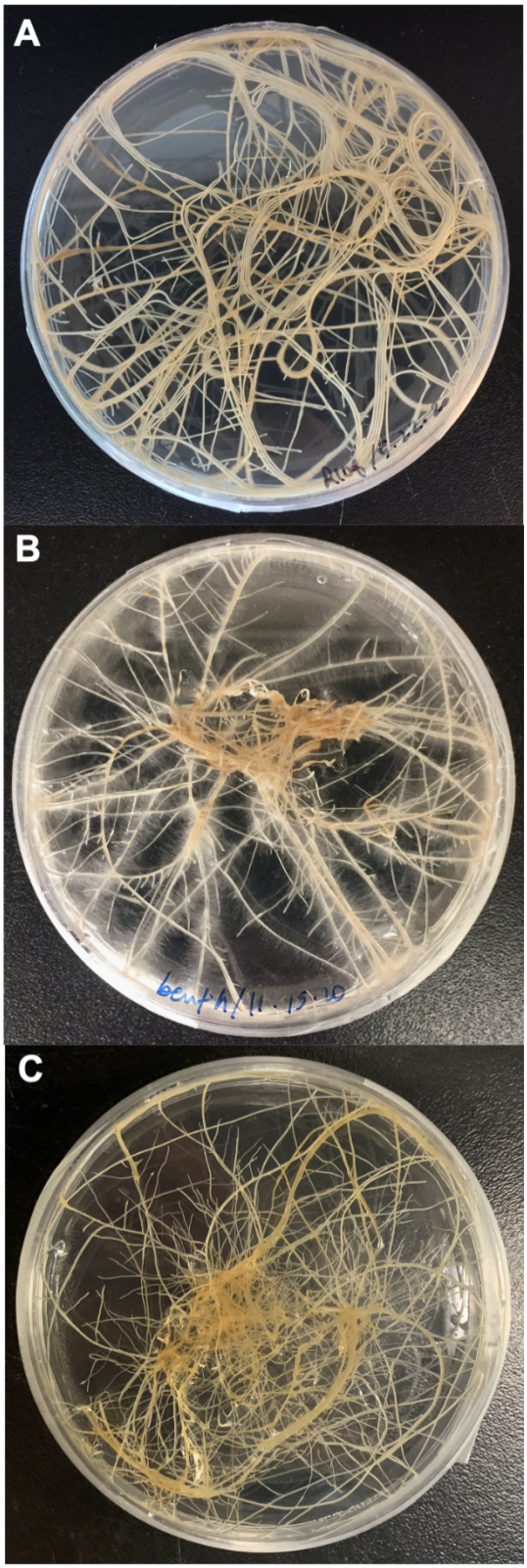


Figure 11. Ri T-DNA transformed roots of (A) Med (cv. R108), (B) Nic, and (C) Dau (strain P68).

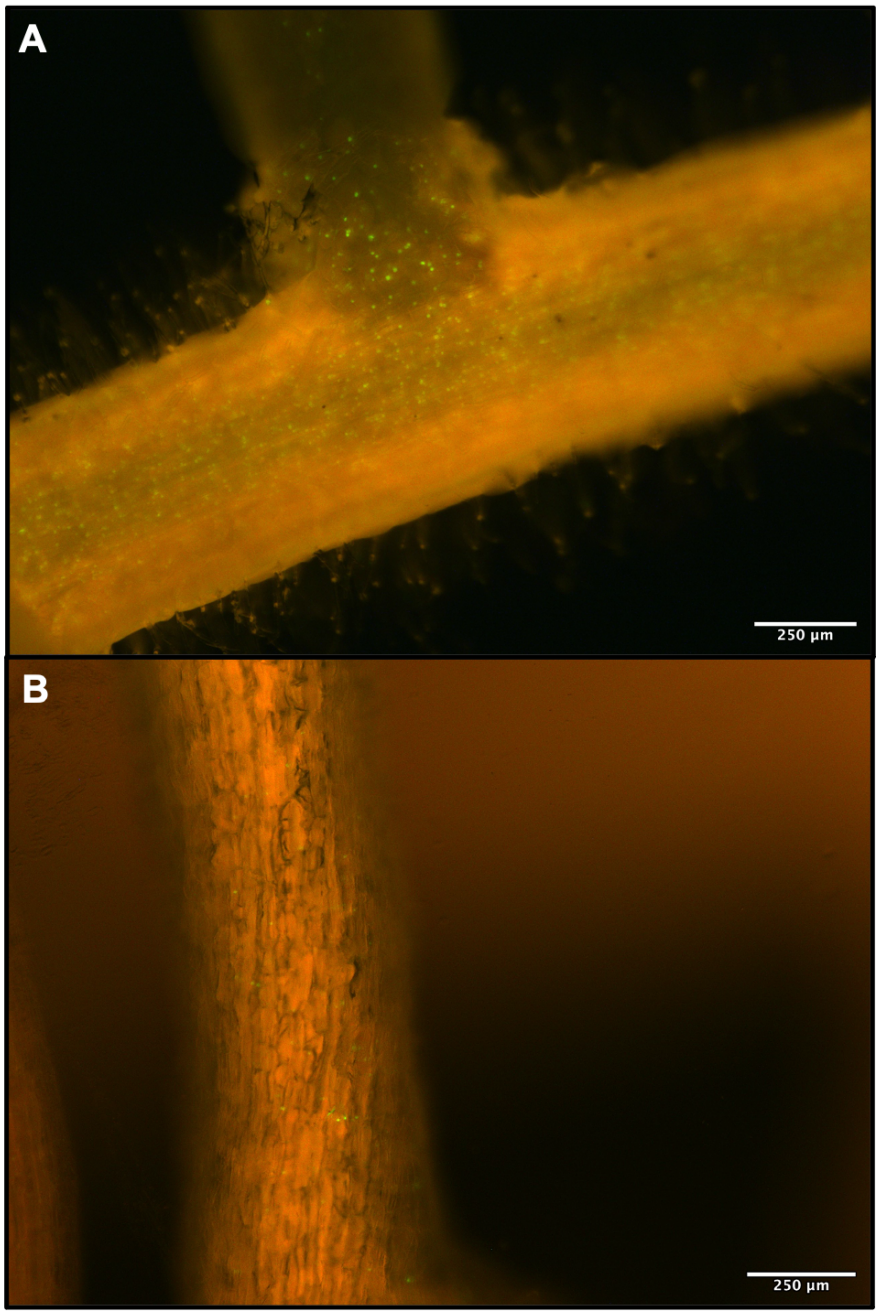


Figure 12. Green fluorescence protein expression in non-mycorrhizal *M. truncatula* hairy roots. A) Med (cv. A17) roots co-transformed with MtPT4p:NLS-GFP-GUS, and B) R108 roots co-transformed with MtBCP1p:NLS-GFP-GUS. Micrographs taken with Zeiss AxioZoom.V16.

3.3 Med, Nic, and Dau hosted a similar range of AM fungal species

Both Med and Nic roots were found to be colonized by all seven tested AM fungi species when co-cultured in pot cultures. While the degree to which either host was colonized by each of the tested AMF species differed, the presence of IR fungal structures such as hyphae and arbuscules in all host and AM fungus combinations provided enough evidence that co-culturing these symbionts *in vitro* was achievable (data not shown).

When using the presence of new daughter spores as the sole metric for a compatible host and AM fungus pair, it was found that Med and Nic possessed an identical *in vitro* host-range as Dau for the species of AM fungi used in this study (**Table 3**).

Under monoxenic conditions, all three hosts facilitated the production of new daughter spores in one-compartment Petri dishes inoculated with *R. irregularis, Glomus* sp.*,* and *R. clarus*. Conversely, when Med or Nic were grown alongside AM fungi species belonging to category 3, those that were shown not to have completed their life cycle with Dau *in vitro*, yielded no new daughter spores. Although auxiliary cells were observed when co-culturing *G. margarita* or *C.* cf. *gilmorei* with Med or Nic (**Figure 13**).

No data was collected for *C. lamellosum* or for Dau-hosted *F. mosseae*, *G. margarita* or *C.* cf. *gilmorei* co-cultures due to difficulties with contamination, which resulted in limited access to sufficient fungal inoculum for setting up new trials.

Table 3. Summary of all *in vitro* screening of all host and AM fungus pairs when co-cultured in one-compartment Petri dishes over at least 12 weeks. At least 5 replicates prepared for each pair. Untested pairs were due to limited access to fungal inoculum.

Category*	Species	Strain / DAOM No.	Nic	Med	Dau
1	*R. irregularis*	4375/DAOM234181	AM fungal species yielded new daughter spores with all three hosts		
	Glomus sp.	GC-4/ DAOM240160			
2	*R. clarus*	GC-5/DAOM234281			
	C. lamellosum	4350B	Lost all co-cultures due to contamination		
3	*F. mosseae*	2101	No progression past pre-symbiotic interactions		Not tested
	C. cf. *gilmorei*	4398	Symbiosis development halted after production of auxiliary cells		
	G. margarita	4292			

*Category numbers refer to *in vitro* fungal growth behaviour outlined in Materials and Methods, Table 1.

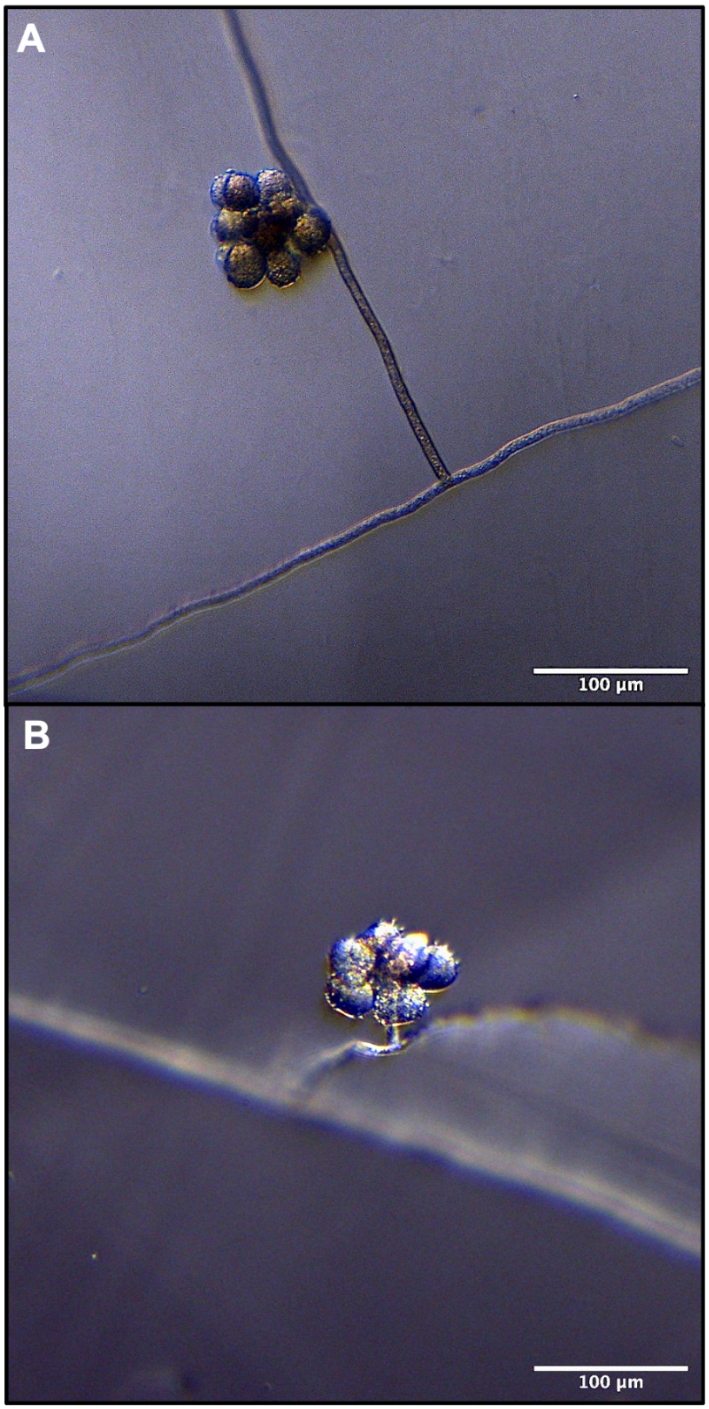


Figure 13. *Gigaspora margarita* auxiliary cells formed in co-culture with Nic (A) and Med (B) under *in vitro* conditions. Micrographs taken with Zeiss AxioZoom.V16.

3.4 Impact of host, fungal species and growing time on the development of AM symbiosis

The probabilities associated with each host and AM fungus pair and the progression of their symbioses over time was modelled using an MLR model. In other words, if one were tracking any single two-compartment Petri dish used in this study, our model predicted the probability that the fungal culture within said Petri dish would have progressed to any one of the six SD stages at each recorded time point (**Figure 7**).

While a host-specific effect was indeed found to significantly affect the development of AM fungi, so did both AM fungi identity as well as a three-way interaction between host species, AM fungal species, and time (**Table 4**). Therefore, the factors influencing fungal development were more complex than just host-effect alone.

3.4.1. Symbiotic monoxenic cultures inoculated with R. irregularis DAOM234181

Nic, relative to Med and Dau, was a poor host for *R. irregularis*. Progression through the SD stages was significantly slower for Nic/*R. irregularis* when compared to Med (p = 2.15e-06) and Dau (p = 3.97e-09). At 12 wpi, there was an 89% probability that fungal growth would stay restricted to the RC in Nic/*R. irregularis* replicates (**Figure 14A**). Furthermore, *R. irregularis* exhibited relatively low viability when hosted by Nic. The MLR model predicted that at 12 wpi, there was only a 36% probability that the 3rd SD stage (new daughter spores) would be reached. In contrast, at 12 wpi the probability of *R. irregularis* sporulating in the RC within Med and Dau-hosted cultures was as high as 96% and 97%, respectively (**Figures 14B and 14C**). The rate at which *R. irregularis* began to sporulate in the RC when hosted by Med and Dau outpaced that of Nic as early as 4-6 wpi, where the probabilities of RC sporulation increased from 33% to 57% for Med and 31% to 57% for Dau. Further similarity in *R. irregularis* development between Med

and Dau-hosted cultures was observed when comparing fungal growth in the HC. Throughout the 12-week duration of the SD assay, the probability of *R. irregularis* progressing to the final stage (> 30 daughter spores) was nearly doubled every week in both Med and Dau replicates. At 8, 10 and 12 wpi, the probability of Med/*R. irregularis* reaching the final developmental stage was 26%, 49%, and 71%, and 28%, 53%, and 77%. for Dau/*R.irregularis* cultures, respectively. The difference in the rates at which *R. irregularis* advanced through the SD stages was not significant (p = 0.47) between Med and Dau cultures.

3.4.2. Symbiotic monoxenic cultures inoculated with R. clarus DAOM234281

Of all the host and AM fungus combinations, none showed as little development as that of Nic*/R. clarus* (**Figure 14D**). When hosting *R. clarus*, Nic facilitated the slowest progression through SD stages in comparison to Med (p = 1.63e-06) and Dau (p = 3.48e-14). At 12 wpi, our MLR model predicted that in Nic-hosted replicates, there is a 99% probability that *R. clarus* would not grow into the HC and only a 5% probability that new spores would be produced in the RC. Furthermore, there was a 17% probability that Nic*/R. clarus* replicates would not exhibit any fungal growth in either compartment at 12 wpi.

There were significant differences (p = 1.63e-06) between the other two hosts, where *R. clarus* exhibited further advancement through the SD stages in cultures hosted by Dau (**Figure 14F**) as opposed to Med (**Figure 14E**). At 12 wpi, Med/*R. clarus* cultures had a 42% probability of reaching stages 4-6, of which only 25% of those would reach stage 6; Dau-hosted cultures were shown to have a probability of 82% of reaching stages 4-6, nearly doubling that of Med cultures. When looking at stage 6 specifically, Dau/*R. clarus* cultures have a 69% probability of reaching the final developmental stage at 12 wpi. Thus, far more fungal growth in the HC was

likely to be observed when *R. clarus* was hosted by Dau as opposed to Med. The disparity between Med/*R. clarus* and Dau/*R. clarus* was also observed in the RC where the probability of sporulation was predicted to be 76% and 96% for Med and Dau at 12 wpi, respectively.

3.4.3. Symbiotic monoxenic cultures inoculated with Glomus sp. (strain GC-4)

The weakest host-effect was observed in *Glomus* sp. cultures as neither of the three hosts facilitated very much symbiotic progression as compared to that seen with the symbiotic monoxenic cultures inoculated with *R. irregularis* or *R. clarus*. Regardless of host, at 12 wpi *Glomus* sp. replicates had less than 17% probability of reaching the HC (**Figure 14G-I**). As a result of the small amount of fungal growth in the HC, *Glomus* sp. was the least represented species in the spore quantification protocol.

Furthermore, fewer than 50% of all replicates were predicted to even exhibit the completion of *Glomus* sp. life cycle, as the probability of reaching the 3rd SD stage (new daughter spores) at 12 wpi was 47%, 46%, and 47% in Nic, Med, and Dau, respectively. There were similarities found in the MLR's predicted SD probabilities amongst all three hosts. Notwithstanding, significant difference was found between Med and Dau ($p = 0.03$), indicating that *Glomus* sp. progressed through the SD stages faster with Dau roots than with Med roots.

Table 4. Multinomial linear regression (MLR) comparing the progression of nine unique host (Nic, Med, and Dau*)* and AM fungus (*R. irregularis, R. clarus,* and *Glomus* sp.) pairs through six distinct symbiosis development stages as a function of a three-way interaction between host species, AM fungal species, and time (0-12 weeks). Estimates and standard error (SE) obtained from MLR, and Chi square values, degrees of freedom (Df), and *p* value (Pr(>Chi square)) obtained via type III ANOVA. Significance denoted by asterisk 0^{***}, 0.001^{**}, 0.01^{*}.

Coefficients	Estimate	SE	Chi square	Df	p-value
AMF	—	—	13.66	2.00	1.08e-3 **
Glomus sp.	1.17	0.45	—	—	—
R. irregularis	1.48	0.42	—	—	—
Host	—	—	12.80	2.00	1.66e-3 **
Dau	0.82	0.42	—	—	—
Med	1.51	0.42	—	—	—
Week	0.08	0.05	2.62	1.00	0.11
AMF × Host	—	—	14.01	4.00	7.25e-3 **
Glomus sp. × Dau	-1.73	0.62	—	—	—
R. irregularis × Dau	-1.37	0.56	—	—	—
Glomus sp. × Med	-1.33	0.61	—	—	—
R. irregularis × Med	-1.78	0.57	—	—	—
AMF × Week	—	—	4.45	2.00	0.11
Glomus sp. × Week	0.14	0.06	—	—	—
R. irregularis × Week	0.07	0.06	—	—	—
Week × Host	—	—	47.82	2.00	4.13e-11 ***
Week × Dau	0.43	0.06	—	—	—
Week × Med	0.22	0.06	—	—	—
AMF × Host × Week	—	—	27.63	4.00	1.49e-05 ***
Glomus sp. × Week × Dau	-0.36	0.09	—	—	—
R. irregularis × Week × Dau	-0.05	0.09	—	—	—
Glomus sp. × Week × Med	-0.24	0.09	—	—	—
R. irregularis × Week × Med	0.12	0.09	—	—	—

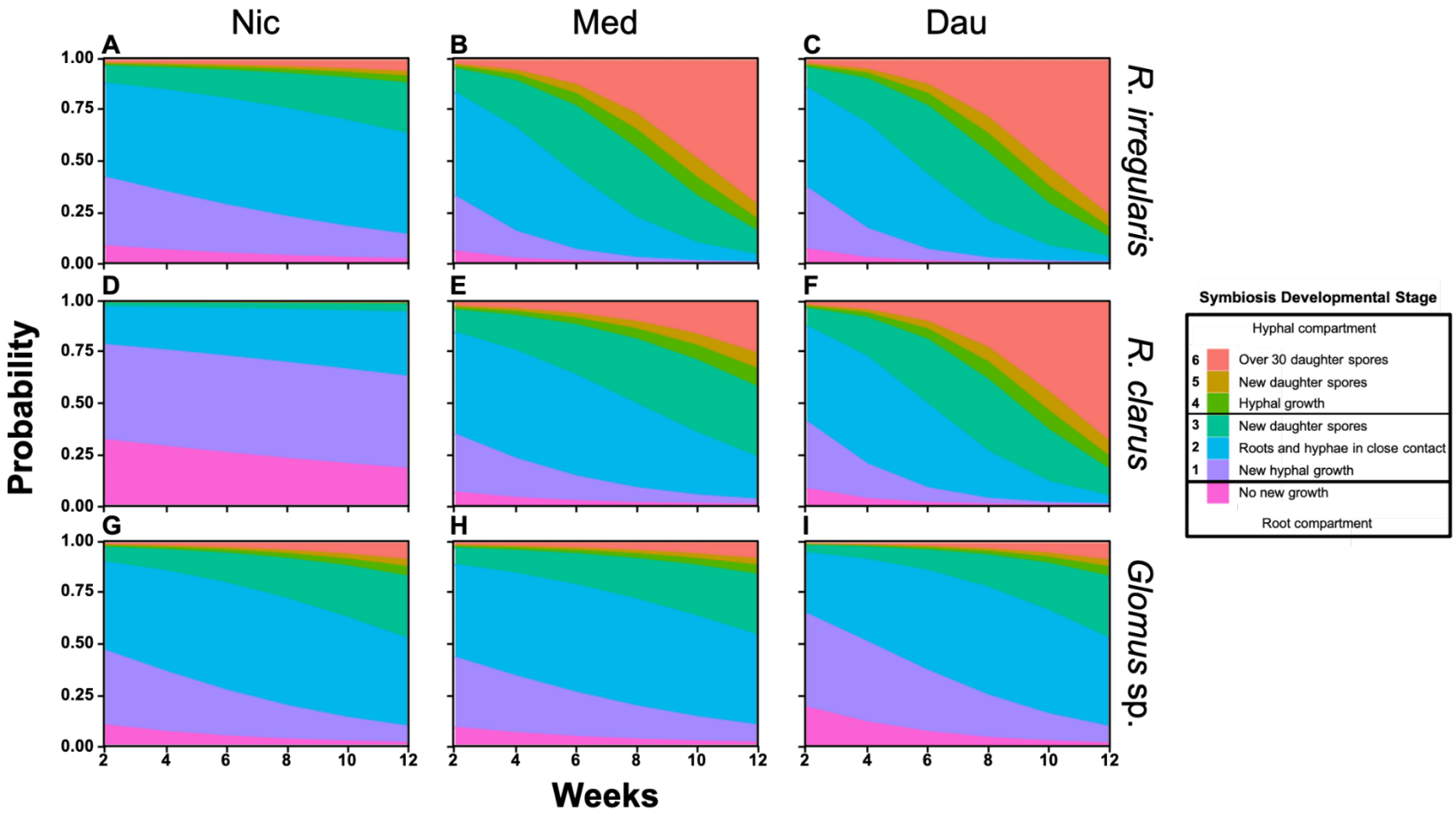


Figure 14. Stacked probabilities of each pair of host and AM fungal species, with host roots corresponding to each column (left to right: Nic, Med, and Dau) and AM fungi corresponding to each row (top to bottom: *R. irregularis, R. clarus,* and *Glomus* sp.). Each colour corresponds to a specific symbiotic developmental stage (pink: no new fungal growth, purple: **stage 1**/ new hyphal growth in root compartment (RC), blue: **stage 2**/ roots and hyphae in close contact in the RC, light green: **stage 3**/ new daughter spores in RC, dark green: **stage 4**/ new hyphal growth in the hyphal compartment (HC), dark yellow: **stage 5**/ new daughter spores in the HC, and red: **stage 6**/ over 30 daughter spores in HC. Distribution of coloured polygons correspond to the probability distribution of any pair host and AM fungus replicate reaching a developmental stage at any given time during the 12-week assay duration. Probabilities were generated by testing fungal development as a function of a three-way interaction between host identity, fungal identity, and time (in weeks) using a multinomial linear regression.

3.5 Three-way interaction between host species, AM fungal species, and time

LMMs were used to examine the effect of predictors: host species, AM fungal species, time (in weeks), and distance (from each grid square to hyphal bridge) on spore production in HCs.

3.5.1 HC sporulation patterns in monoxenic AM fungal cultures is dependent upon host species, fungal species, and time (IC model)

While a host-specific effect was found to influence the rate and distance at which AM fungi sporulated in the HC of two-compartment Petri plates, host identity was not the only predictor to have a significant effect.

This was first demonstrated using a LMM of all data involving *R. irregularis* and *R. clarus* (henceforth "IC" model; **Table 5**), while excluding *Glomus* sp. data (due to low sporulation). Of the four tested predictors (host species, fungal species, time, and distance), only distance independently had a significant effect, i.e., regardless of symbiont species involved or time at which a replicate was observed, the number of spores present in any 5 mm^2 area of a HC was significantly affected by the distance observed from the hyphal bridge. However, a significant two-way interaction between distance and time was observed as well. As time progressed, the relationship between distance and time shifted from strongly negative to positive (**Figure 15A**). In other words, from 8-11 wpi the IC model predicted that higher sporulation would occur closer to the hyphal bridge. However, at 12 wpi this trend reversed and more spores were predicted to be observed further into the HC, and away from the hyphal bridge. The other three predictors, host species, AM fungal species and time, only had a significant effect on HC spore production as a three-way interaction. In other words, each unique combination of host, AM fungi, and time (weeks) was observed to affect spore production differently (**Figure 15B**).

Pairwise comparisons between pairs of hosts and AM fungi in the IC model revealed a host-specific effect only on the sporulation of *R. irregularis* (**Table 6**). The Dau/*R. irregularis* pair sporulated at a significantly higher rate in HCs as compared to Med/*R. irregularis*, Dau/*R. clarus* and Med/*R. clarus* (**Figure 15B**.). Although there were trends suggesting that the spore production of Dau/*R. irregularis* also outpaced both Nic-hosted pairs, this was not found to be significant. However, this was likely due to only a small sample size of Nic cultures being available as many of the cultures were lost due to contamination (**Table 2**).

Table 5. IC linear mixed model comparing the rate of hyphal compartment (HC) sporulation exhibited by six unique host (Nic, Med, and Dau*)* and AM fungus (*R. irregularis* and *R. clarus*) pairs after 8 weeks post inoculation as a function of a three-way interaction between host species, AM fungal species, and time (weeks). Intercept refers to the HC sporulation of the Nic/*R. clarus* pair at 8 wpi. Estimate and standard error (Std. Error) obtained from IC model, and associated Chi square values, degrees of freedom (Df), and p-values (Pr(>Chi square)).

Fixed Effects	Estimate	Std. Error	Chi square	Df	p-value
(intercept)	0.65	2.17	0.09	1.00	0.77
Distance	-0.07	0.00	624.07	1.00	< 2.2e-16***
Week	0.02	0.18	0.02	1.00	0.90
AMF	—	—	0.24	1.00	0.62
R. irregularis	1.30	2.62	—	—	—
Host	—	—	0.64	2.00	0.73
Dau	1.43	2.21	—	—	—
Med	1.74	2.27	—	—	—
Distance× Week	0.01	0.00	578.02	1.00	< 2.2e-16***
AMF× Host	—	—	0.07	2.00	0.96
R. irregularis × Dau	-0.65	2.67	—	—	—
R. irregularis× Med	-0.73	2.72	—	—	—
Week× AMF	—	—	0.00	1.00	0.98
Week× *R. irregularis*	-0.01	0.19	—	—	—
Week× Host	—	—	0.48	2.00	0.79
Week× Dau	-0.10	0.18	—	—	—
Week× Med	-0.09	0.18	—	—	—
Week× AMF× Host	—	—	51.18	2.00	7.69e-12***
Week× *R. irregularis*× Dau	0.22	0.19	—	—	—
Week× *R. irregularis*× Med	0.02	0.19	—	—	—

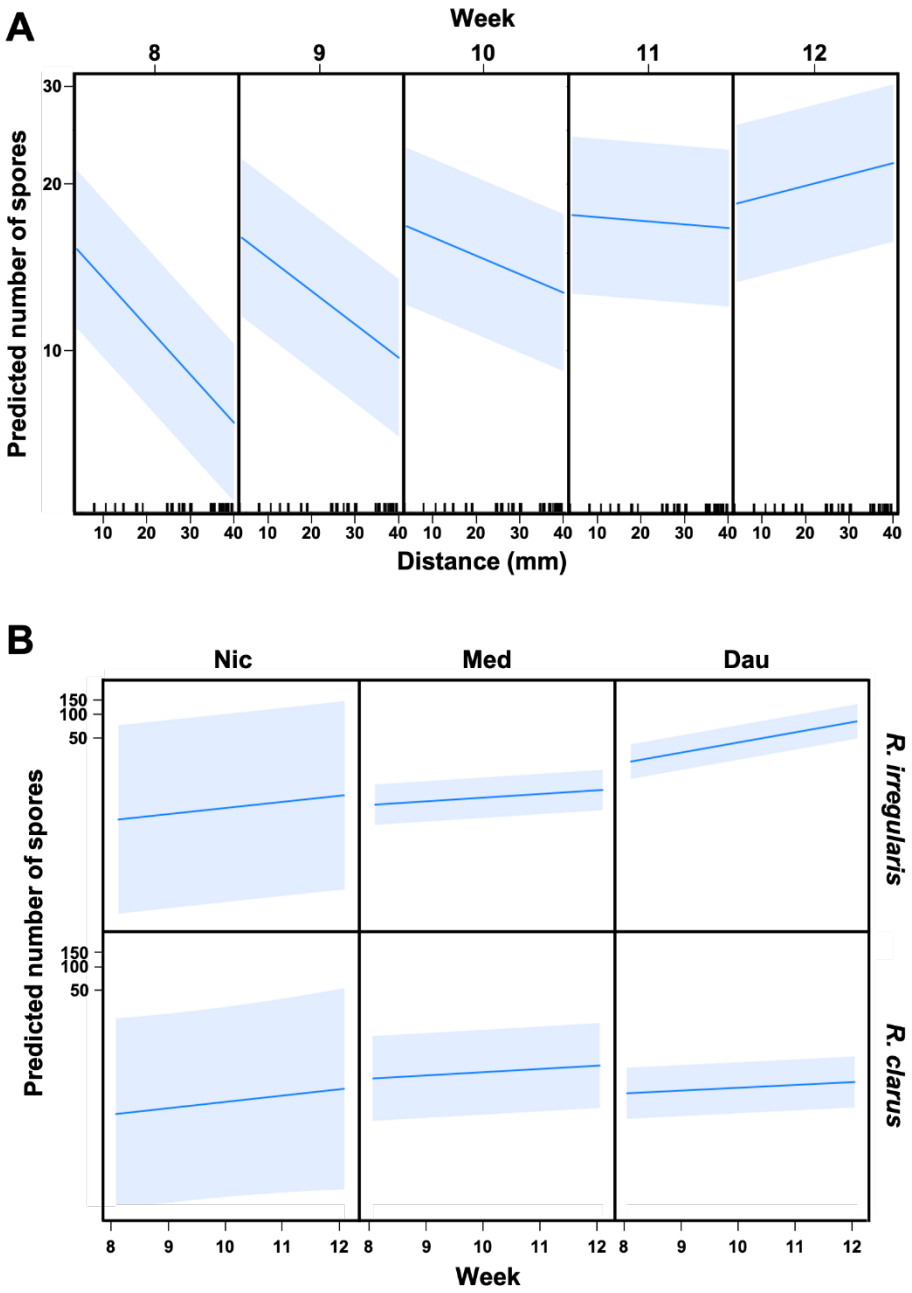


Figure 15. Mixed model linear regression ("IC" model) with four predictor variables, host (Dau, Med, Nic), AM fungi (*R. irregularis* and *R. clarus*), time (8, 10, and 12 wpi), and distance (mm) (location of grid squares in relation to hyphal bridge) and their effect on spore production (dependant variable). Petri dish ID (individual replicates) were used as random effect. Two-way interaction between distance and week (A) along with a three-way interaction between week, AM fungi, and host (B) were plotted. Slope of each individual graph indicates a relationship between predictors and spore production, while shaded area illustrates standard error.

Table 6. Post-hoc pairwise comparisons between all host and AM fungus pairs used in the IC linear mixed model. Estimates, standard error (SE), degrees of freedom (Df), and p-values obtained from Tukey HSD tests.

Pairwise comparison							
Host 1	**Fungus 1**	**Host 2**	**Fungus 2**	**Estimate**	**SE**	**Df**	**p-value**
Nic	*R. clarus*	Nic	*R. irregularis*	-1.24	1.99	Inf	0.99
Nic	*R. clarus*	Dau	*R. clarus*	-0.39	1.47	Inf	0.99
Nic	*R. clarus*	Dau	*R. irregularis*	-3.21	1.44	Inf	0.22
Nic	*R. clarus*	Med	*R. clarus*	-0.82	1.55	Inf	0.99
Nic	*R. clarus*	Med	*R. irregularis*	-1.51	1.45	Inf	0.90
Nic	*R. irregularis*	Dau	*R. clarus*	0.85	1.44	Inf	0.99
Nic	*R. irregularis*	Dau	*R. irregularis*	-1.98	1.41	Inf	0.73
Nic	*R. irregularis*	Med	*R. clarus*	0.42	1.52	Inf	0.99
Nic	*R. irregularis*	Med	*R. irregularis*	-0.27	1.42	Inf	1.00
Dau	*R. clarus*	Dau	*R. irregularis*	-2.83	0.45	Inf	<1.0e-4*
Dau	*R. clarus*	Med	*R. clarus*	-0.44	0.73	Inf	0.99
Dau	*R. clarus*	Med	*R. irregularis*	-1.13	0.48	Inf	0.17
Dau	*R. irregularis*	Med	*R. clarus*	2.39	0.68	Inf	5.5e-3*
Dau	*R. irregularis*	Med	*R. irregularis*	1.70	0.39	Inf	2.0e-4*
Dau	*R. clarus*	Med	*R. irregularis*	-0.69	0.69	Inf	0.92

Inf: infinite degrees of freedom

3.5.2 Host species differently affected HC sporulation of R. irregularis and R. clarus (irregularis model & clarus model)

To confirm the findings of our IC model (section 3.5.1), *R. irregularis* and *R. clarus* data was separated into their own individual models (henceforth "*irregularis*" and "*clarus*" models, respectively). A significant effect of host species was only found in the *irregularis* model, suggesting that host identity alone was sufficient to predict HC sporulation for *R. irregularis*, but not for *R. clarus*.

Furthermore, the time × distance interaction on HC sporulation observed in the IC model (**Figure 15A**) was fungus specific. Only the *irregularis* model (**Figure 16A**) exhibited a similar time × distance interaction to that of the IC model, while the *clarus* model did not (**Figure 16B**), suggesting that across all three hosts, only *R. irregularis* tended to sporulate further and further into the HC over time. In contrast, *R. clarus* consistently sporulated close to the hyphal bridge and spore production did not extend any further regardless of the host.

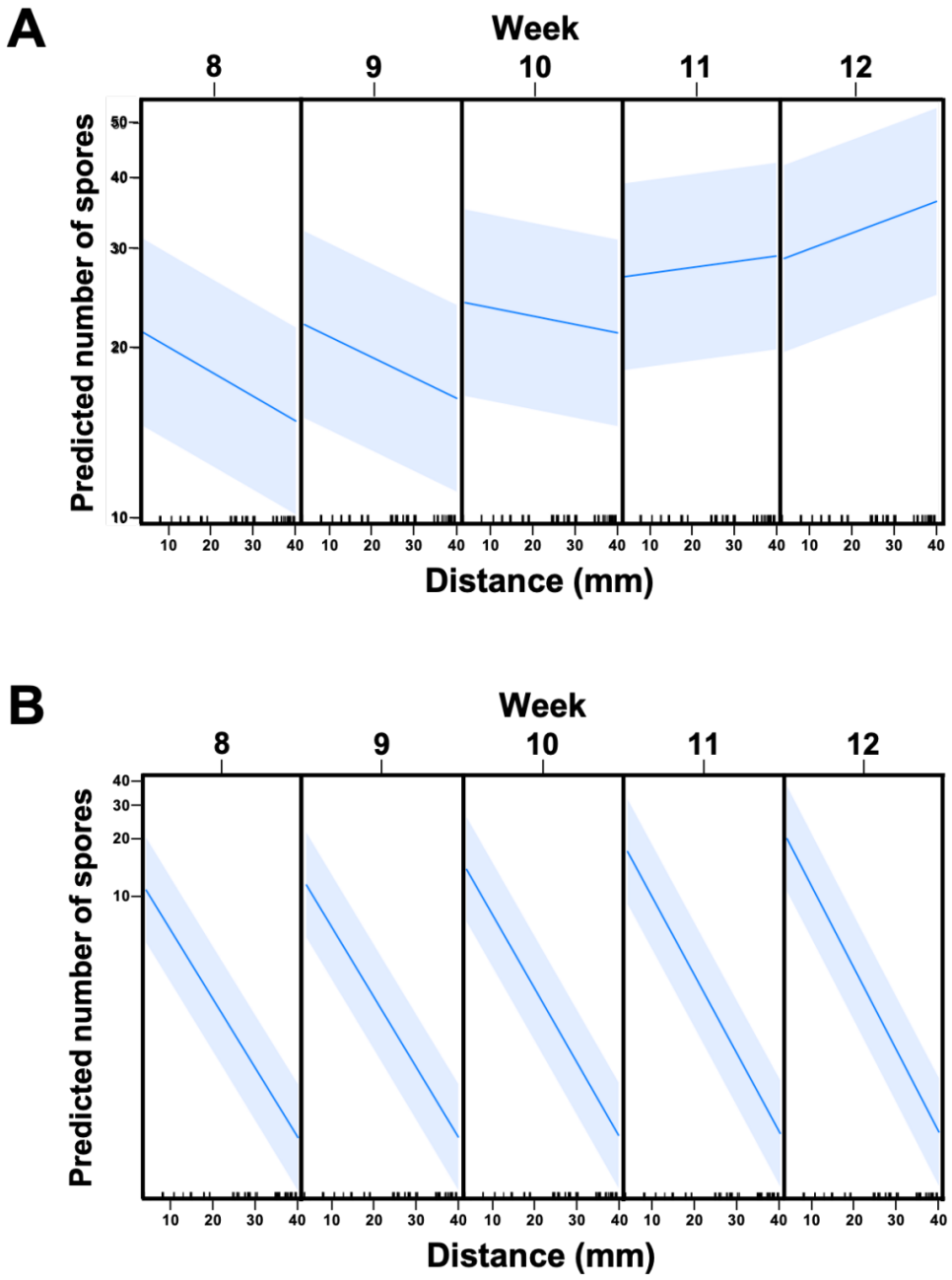


Figure 16. Effect of interaction between distance (from observation square to hyphal bridge in mm) and time (8-12 weeks post inoculation) on the number of spores recorded in any given 5 mm^2 area within the hyphal compartments. Regression lines were obtained using a model with data from three predictor variables, host (Nic, Med, Dau), time (8, 10, and 12 wpi), and distance (location of grid squares in relation to hyphal bridge) and their effect on *R. irregularis* (A) or *R. clarus* (B) spore production. Data for weeks 9 and 11 were not recorded, therefore the data shown are slopes predicted by the model. Petri dish ID (individual replicates) were used as random effect.

3.5.3 Across all tested AM fungal species, HC sporulation associated with Med roots exhibited both a positive and negative relationship with time and distance, respectively (Medicago model)

The SD assay found that regardless of host, *Glomus* sp. exhibited very little fungal growth in the HC, resulting in a very limited dataset that was available for an in-depth analysis of spore production. Recall from section 3.4.3, that at 12 wpi *Glomus* sp. only has a ~17% predicted probability of growing into the HC for all three hosts. Yet, with Med we were able to obtain a sufficient number of replicates (n = 6) for analysis suggesting that there may have been a host-specific effect on the co-culture of *Glomus* sp. Specifically, *Glomus* sp. produced more spores in the HC when associated with Med than with either Nic or Dau. Since Med was the only host with a sufficient sample size of replicates for all three AM fungi, a model excluding Nic and Dau data was generated (henceforth referred to as the "*Medicago*" model; **Table 6**).

The *Medicago* model found that both time (weeks) and distance (mm) significantly affected spore production in Med replicates. When plotted, time was found to have a positive relationship with spore production (**Figure 17A**) indicating that Med tends to facilitate an increase in spore production over time. In contrast, a negative relationship existed between distance and spore production (**Figure 17B**), suggesting that sporulation in Med-hosted cultures was more likely to occur closer to the hyphal bridge and not further into the HC.

Table 7. Linear mixed model (model "*Medicago*") comparing the rate of hyphal compartment (HC) sporulation exhibited by three unique host (Med) and AM fungi (*R. irregularis, R. clarus,* and *Glomus* sp.) pairs after 8 weeks post inoculation (wpi) as a function of a three-way interaction between host species, AM fungal species, and time (weeks). Intercept refers to the HC sporulation of the Med/*R. clarus* pair at 8 wpi. Estimates and standard errors (SE) obtained from *Medicago*, and Chi square values, degrees of freedom (Df), and p-value (Pr(>Chi square)) obtained via Type III ANOVA. Significance denoted by asterisk 0^{***}, 0.001^{**}, 0.01^{*}.

Fixed Effects	Estimate	SE	Chi square	Df	p-value
(intercept)	17.77	51.95	0.12	1.00	0.73
AMF	—	—	8.01	2.00	1.82e-2 *
Glomus sp.	-109.10	52.91	—	—	—
R. irregularis	1.71	45.75	—	—	—
Week	3.61	4.90	0.54	1.00	0.46
Distance	-0.85	1.30	0.43	1.00	0.51
AMF× Week	—	—	19.51	2.00	5.8e-05 ***
Glomus sp. × Week	15.26	4.78	—	—	—
R. irregularis× Week	0.36	4.18	—	—	—
Week× Distance	-0.08	0.12	0.44	1.00	0.51

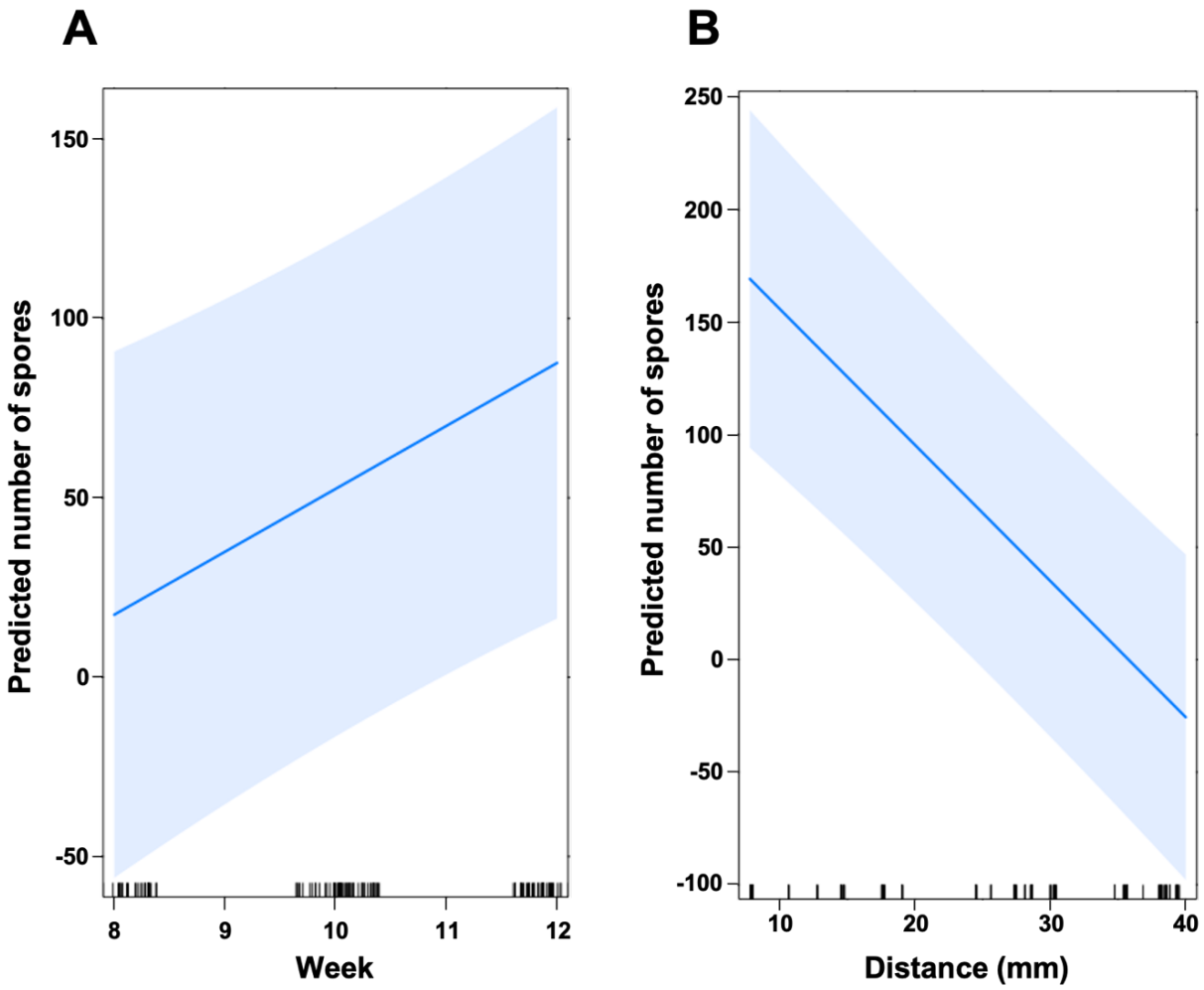


Figure 17. A) effects of time (weeks) and B) distance (mm) on the number spores found in any given 5mm^2 area within a two-compartment Petri plate HC. Regression lines were obtained using a model with data from three predictor variables, host (Med), AM fungi (*R. irregularis, Glomus* sp., and *R. clarus*), time (8, 10, and 12 wpi), and distance (location of grid squares in relation to hyphal bridge) and their effect on spore production. Petri dish ID (individual replicates) were used as random effect.

3.5.4 Overall effect of distance on HC sporulation

To complete the model data above, every individual spore count obtained from each host and AM fungus pair from 8-12 wpi was plotted against the distance variable (**Figure 18**). Thus, the four predictors (host identity, AM fungi identity, time, and distance) and their effect on sporulation in the HC could be visualized all at once.

Only a single host and AM fungus pair, Dau/*R. irregularis,* exhibited a positive relationship between distance and spore count (**Figure 18C**). At 12 wpi, significantly higher numbers of spores were counted further away from the hyphal bridge in Dau/*R. irregularis* cultures, suggesting that Dau facilitated *R. irregularis* growth further away from roots than any of the other tested host and AM fungus combination. The spatial distribution of independent spore counts illustrated in **Figure 18** confirmed the significant negative relationship between distance and spore production in the HC for all Med and Nic replicates, across all time points. These findings were consistent even when controlling for any slight variation in hyphal bridge placement (i.e., not perfectly centered in Petri dish; **Supplemental Figure 1**)

Lastly, **Figure 18** illustrates that the distance at which sporulation tended to occur within the HC was dependent upon a three-way interaction between host identity, AM fungi identity and time (weeks), as found by the IC model. The distance of sporulation from the paper bridge in the HC is therefore unlikely to be predicted by host identity alone.

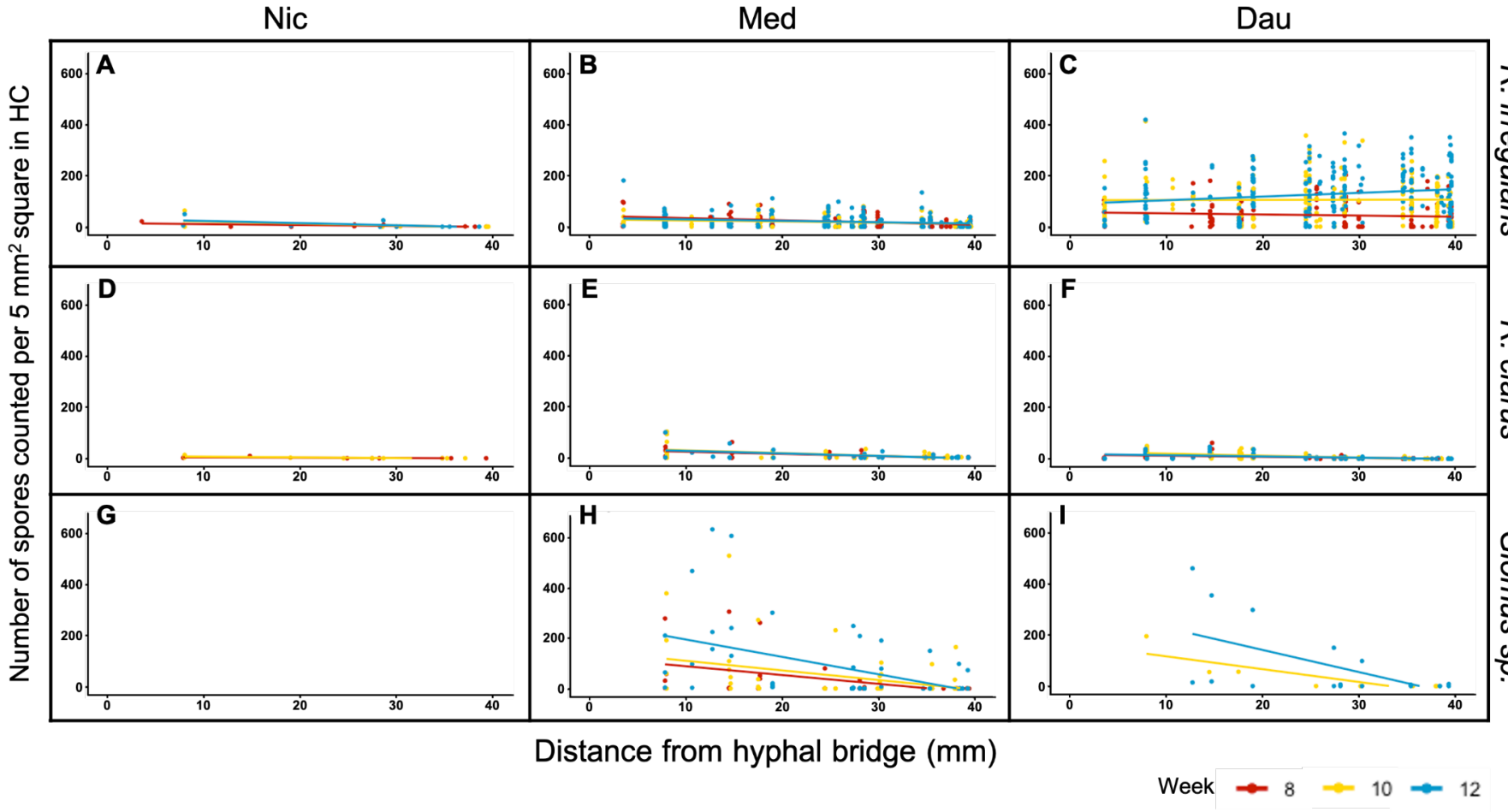


Figure 18. Distribution of spore counts scored in 5 mm^2 grid squares in relation to the distance each square was located from hyphal bridge placed in the centre of a two-compartment Petri plate. Regression lines were fit to spore counts recorded at 8 wpi (red), 10 wpi (yellow), and 12 wpi (blue). Panel columns arranged by host (left to right: Nic, Med, and Dau) and rows arranged by AM fungi (top to bottom: *R. irregularis, R. clarus,* and *Glomus* sp.)

CHAPTER FOUR

Discussion

4.0 Discussion

4.1 Limitations of in vitro culture

Presently, the obligate biotrophic nature of AM fungi is one of the most considerable challenges in their study as these fungi are not amenable to axenic culture (Lanfranco *et al.,* 2016). Furthermore, under *in situ* conditions, these fungi are highly promiscuous and can interact with an array of other organisms such as in common mycorrhizal networks linking multiple different host and AM fungal species, as well as with soil-borne microbiota (Lekberg, et. al., 2010; Verbruggen, van der Heijden, Rillig, & Kiers, 2013). With so many possible confounding variables under *in situ* and *in vivo* conditions, it is critical that *in vitro* methods of culturing AM fungi are optimized. Laboratory conditions can distill the AM symbiosis down to a single host and a single fungal strain, thereby allowing for the highest level of experimental control currently possible.

Unfortunately, our understanding of cultivating AM fungi *in vitro* is still quite limited. Of the 340 species of AM fungi currently described and validated, only 10 are continuously cultivated under *in vitro* conditions (such as CCAMF and GINCO-BEL). What is it that makes those 10 AM fungal species amenable to *in vitro* conditions while so many other fungal species are not? Host identity is a strong possibility. Presently, there is little to no variation in the species of hairy roots chosen to maintain and/or study AM fungi under *in vitro* conditions. The host species used is typically carrot, however chicory roots are used as well but to a much lesser extent. Lack of alternative hairy root species is a concern as there is an abundance of evidence that host identity can significantly impact AM fungal behaviour (Tiwari & Adholeya, 2003; Ehinger *et al.*, 2009; Mateus *et al.*, 2019; Kokkoris *et al.*, 2021). Between the current limitations of culturing AM fungi *in vitro* (e.g., lack of host variation), and the overwhelming evidence of

host-specific effects on the AM symbiosis, it is critical then that new hairy root species are developed and rigorously tested under controlled laboratory conditions.

4.2 Similar host-range amongst Nic, Med, and Dau

In this study we generated two new lines of hairy root species, Med and Nic. We then tested them under monoxenic conditions to determine if they were capable of facilitating daughter spore production from fungal species that were previously unsuccessful with carrot (Dau). In all, seven different fungal species (**Methods, Table 1**) were tested and no differences in host-range was found amongst all three host species (Med, Nic, and Dau). In other words, the species that produced new daughter spores with Dau (*R. irregularis, R. clarus* and *Glomus* sp. from category 1 and 2), did so with Med and Nic as well. Conversely, *F. mosseae, S. gilmorei,* and *G. margarita* (category 3) which were previously reported to be unable to complete their life cycle with Dau, failed to do so as well with either Med or Nic. Therefore, in the context of this study and the species of AM fungi we selected, no support was found for our first hypothesis and the limited number of AM fungal species currently cultivated *in vitro* cannot be specifically attributed to the host-range of Dau. This is not entirely surprising as there is a substantial gap between the number of environmental stimuli present under *in situ* conditions, in which all AM fungal species are able to proliferate, and the artificial conditions of *in vitro* culture. For example, the latter removes any interaction with other microbes which are known to influence AM fungi via auxiliary symbioses for improved nutrient mobilisation/uptake, or through FA signals which can elevate fungal oxidative state, elicit hyphal branching, and stimulate secondary spore production (Zhang *et al.*, 2016; Kameoka *et al.*, 2019). Conversely, AM fungi can shape their surrounding bacterial community in the mycorrhizosphere via hyphal exudates with either

stimulatory or inhibitory effects on neighbouring fungi and bacteria (Filion *et al.*, 1999; Rillig *et al.*, 2006). Taken together, it is clear that the life history of AM fungi is intimately connected with other microbiota. Therefore, it is possible that the current limited host-range of *in vitro* culture collections may be a reflection of the absence of soil-borne microbial communities. The scope of this study only included two new host species. As such, it cannot be dismissed that of the hundreds of thousands of terrestrial plant species that engage in AM symbiosis, there exists unidentified host species with the potential to facilitate life cycle completion of recalcitrant species of AM fungi under *in vitro* conditions.

4.3 Host-specific effects are inconsistent across AM fungal species

We hypothesized that the fungal behaviour typically associated with Dau under *in vitro* conditions is a host-specific effect, and that novel host roots would elicit differing fungal behaviour. To test our hypothesis, nine unique pairs consisting of a single host root (Med, Nic, or Dau) and AM fungus (*R. irregularis, R. clarus,* or *Glomus* sp.) were compared by their rate of SD and by their rate of sporulation within the HCs as well. However, because two host and AM fungus pairs failed to exhibit any HC sporulation at all (Dau/*Glomus* sp. and Nic/*Glomus* sp.), the remaining data was analysed using two separate LMMs: 1) the "IC" model consisting of all data except for replicates involving *Glomus* sp*.,* and 2) the "*Medicago*" model consisting only of data involving Med roots.

4.3.1 Rate of HC sporulation

The IC model found that co-cultures between the two model species, Dau and *R. irregularis*, exhibited a rate of sporulation in the HC that significantly increased over time, past 8 wpi. These

findings were entirely unique compared to all other pairs of hosts and AM fungi. In other words, the rates of sporulation recorded in the HCs of the other pairs of hosts and AM fungi: 1) did not change from 8-12 wpi, 2) did not differ amongst each other, and 3) were all significantly lower than the HC sporulation rate of Dau/*R. irregularis.*

Of all the host and AM fungus pairs we tested, the symbiosis between different cultivars/strains of carrot and *R. irregularis* is among the most commonly used under *in vitro* conditions (see **Table 1** for species/strain information). When these two symbionts are co-cultured, they have been reported to produce as many as 700 spores per single compartment Petri plate every four months (Chabot et. al., 1992); in two-compartment Petri dish, *R. irregularis* can produce upwards of 15,000 spores per HC and up to 65,000 spores if carbon supply is replenished (St-Arnaud *et al.*, 1996; Douds, 2002). Moreover, with Dau roots, the rate of *R. irregularis* sporulation has also been characterized as following a sigmoidal curve with a lag, a log, and a stationary phase (Declerck et.al., 2001). The results of our study are consistent with the publications mentioned above, as the Dau and *R. irregularis* pair were found to significantly increase in HC sporulation over time. This increase in sporulation was expected as Declerck et al. (2001) found that *R. irregularis* sporulation can continue to increase until around 15-16 weeks of co-culture with carrot roots.

To explain the high HC sporulation rates of our Dau/*R. irregularis* replicates, we must consider the total dependence of AM fungi on host-derived carbon. Consequently, any differences in sporulation when an AM fungus is associated with different hosts may be looked upon as a proxy measurement for host carbon allocation. In other words, the significant increase in HC sporulation observed in Dau/*R. irregularis* may be interpreted as a significant increase in carbon transferred from Dau to *R. irregularis.*

When plants host AM fungi, carbon allocation to their partners may be modified for several reasons: 1) hosts' access to carbon (Zheng *et al.*, 2015), 2) hosts' dependence on symbionts for nutrient uptake (e.g., under nutrient limiting conditions; Tawaraya, 2003), and 3) hosts' ability to reward partners that are beneficial (Bever *et al.*, 2009; Kiers *et al.*, 2011) or sanction non-cooperative partners (Grman, 2012). However, the first two points cannot explain the high HC sporulation of Dau/*R. irregularis* because all hairy roots were cultured on sucrose-rich M-medium and little to no *R. clarus* or *Glomus* sp. sporulation was observed in the HCs of Dau-hosted replicates. Therefore, it is unlikely that the high HC sporulation rate of Dau/*R. irregularis* is a result of host identity alone, but rather, a result of the interaction between symbiont genotypes.

If Dau roots are capable of discerning the variation in their carbon transfer, then this could suggest that either Dau roots struggle to restrict carbon allocation to levels similar to that seen in other host and AM fungus pairs, or that the Dau genotype identifies the *R. irregularis* genotype as highly cooperative and is offering an increased amount of carbon as a reciprocal reward (Kiers *et al.*, 2011).

Our findings are similar to that of St-Arnaud et al., (1996) as the authors observed significantly more *R. irregularis* spores in HCs separated from Dau roots as opposed to the number of spores in RCs. Although the Dau and *R. irregularis* genotypes differed between our studies (see **Table 1**), the symbiont species were similar which suggests that the high HC sporulation of Dau/*R. irregularis* could be a result of the interaction between symbionts at the species level. While, St-Arnaud et. al., (1996) postulated that sporulation is enhanced if AM fungi are grown in the absence of host-imposed growth suppression, however, the results from

our study would suggest that this was only the case for the Dau/*R. irregularis* pair and cannot be generalized to other combinations of host and AM fungi.

Contrary to Dau/*R .irregularis*, the IC model did not predict any significant change in HC sporulation for all other host and AM fungus pairs. While our work took into account the change in sporulation over time, a static measurement by Ehinger *et al.* (2009) also found that when compared to Med roots, Dau facilitated higher *R. irregularis* spore densities close to the roots. Conversely, Engelmoer & Kiers (2015), using real-time PCR compared the ER abundance of *R. irregularis* in a root-free compartment between Med and Dau and found no difference between hosts.

The rate of sporulation in the HC for *R. clarus* remained steady at a relatively low rate for all three hosts. This result is in line with the culturing experience of *R. clarus* from the work at CCAMF, which observed that *R. clarus* ER growth tends to stay in close proximity to host roots (**Table 1**, Materials and Methods). Given the similarity in *R. clarus* HC sporulation between Med, Nic, and Dau roots, it is likely that host-identity had little effect for this fungal species.

4.3.2 Spatial distribution of HC sporulation

To our knowledge, this was the first time a test for the effect of host identity on the spatial distribution of fungal sporulation in the HC of a two-compartment Petri dish was conducted. Essentially, by measuring the distance of each observation area to the hyphal bridge (i.e., first point of contact into HC for fungi), we were able to determine whether any of the hosts tended to facilitate sporulation closer or further away from their roots.

Only the Dau/*R. irregularis* pair was found to produce significantly more spores at distances further away from the hyphal bridge location. This is an important distinction because

it suggests that rather than *R. irregularis* clustering sporulation near to the hyphal bridge and closer to Dau roots, the strategy of the fungus appears to be that of colonizing the entire HC and maximizing the distribution of their spores. Such a strategy must be energetically costly as this would require an extensive ER mycelium to transport from the host roots the carbon necessary for sporulation at a distance from host roots. This suggests that *R. irregularis* could receive a high amount of carbon from Dau roots in comparison to all the other tested host and AM fungus pairs.

The spatial distribution of fungal sporulation may be important ecologically because while such a life history strategy would carry a high carbon cost for hosts, it could possibly increase AM fungal fitness. Distant germinating spores would be well positioned to come into contact with other hosts and take on additional sources of carbon (Denison & Kiers, 2011) or anastomose with nearby, compatible mycorrhizal networks to extend overall ER mycelium surface area (Giovannetti *et al.*, 2004; Voets *et al.*, 2006; Mikkelsen *et al.*, 2008).

In summary, we did not find that host identity alone was a very strong predictor of HC sporulation. Aside from Dau/*R. irregularis*, no notable differences were observed amongst host and AM fungus pairs.

4.3.3 Symbiosis development

Of the three hosts, only Nic elicited a similar rate of progression through SD stages for all three of the AM fungal species with which they were co-cultured. Regardless of fungal species, all Nic-hosted replicates exhibited much poorer progression through the SD stages relative to replicates involving Med or Dau.

In particular, the combination of Nic and *R. clarus* was the least compatible as even after 12 wpi, only 83% of replicates were likely to germinate. Furthermore, since all spore inoculum for each trial was sourced from a single culture, the lack of pre-symbiotic *R. clarus* growth was specific to cultures with Nic roots. Regarding viability, AM fungi had the lowest probability of completing their life cycle when cultured alongside Nic roots. Only one previous study has reported the behaviour of Nic roots in an AM context; Kokkoris et al. (2021) co-cultured multiple strains of *R. irregularis* along with Nic roots (same strain as used in this study) and the authors also obtained little to no new daughter spores.

Of the three hosts, only Nic roots were covered in an abundance of fine root hairs which likely allowed for a nutrient acquisition strategy that was the least dependent upon AM fungi for assistance (Schweiger *et al.*, 1995). Root hairs allowed Nic roots access to a greater volume of medium than what might be accessible with primary and lateral roots alone. Under the phosphorus limited conditions of M-medium (Bécard & Fortin, 1988), root hair growth and elongation are stimulated, which subsequently assist roots with phosphorus and nitrogen mobilisation and uptake (Bates & Lynch, 1996; Gilroy & Jones, 2000). It is possible that for Nic roots the benefits of AM symbiosis may be redundant to that already gained from their root hairs.

In an *in vivo* study comparing multiple barley (*Hordeum vulgare*) mutants with differing root hair length phenotypes, an inverse relationship was found between root hair length and root length AM colonization (Brown *et al.*, 2013). In contrast, another study reported that Nic roots were equally mycorrhized relative to Med and chives (*Allium schoenoprasum*) when grown in phosphorus-limited soil (Zeng et al., 2018). These authors' findings along with our work, suggests that plant species may not always be consistent in their potential to host AM fungal species across *in vivo* and *in vitro* conditions. To our knowledge, there are no reports on the

effect of Ri T-DNA transformed root hairs on the AM symbiosis under *in vitro* conditions. Future studies looking to test novel hairy root species should first consider the plant's nutrient acquisition strategy as this could impact their hosting ability under *in vitro* conditions.

Unlike Nic, the rate of progression through SD stages for symbioses involving either Med or Dau roots differed according to the identity of the fungal symbiont. Based on the HC sporulation data, it was expected that the Dau/*R. irregularis* pair would progress through the SD stages at a much higher rate than Med/*R. irregularis*. Surprisingly, the SD of *R. irregularis* cultures were nearly identical between Med- and Dau-hosted replicates. For *R. clarus*, the opposite trend was observed. Specifically, while no significant difference in *R. clarus* HC sporulation was observed amongst all hosts, the SD of Dau/*R. clarus* pair outpaced that of Med/*R. clarus* as early as 4 wpi. A possible explanation for the discrepancy between trends observed in SD data and that seen in HC sporulation data, is that within Dau cultures *R. clarus* appeared to prioritize the construction of a very extensive hyphal network as opposed to producing spores (data not shown).

The lack of consistency in host-effects exhibited by Med and Dau across *R. irregularis* and *R. clarus* highlights how important the identity of both symbionts can be in determining symbiotic outcome. Oftentimes, host identity is not enough to explain the growth response or morphology of their fungal partner (Cavagnaro et. al., 2001; Koch et. al., 2017). This is not surprising as recent studies have found that the identities of both symbionts can play a critical role in the resulting AM symbiosis through a genotype × genotype interaction (Mateus et. al. 2019; Kokkoris et. al., 2021).

Our work highlights the need to continue to develop and test new host root species under *in vitro* conditions, as novel hairy root species may be more compatible with recalcitrant AM fungal species than the hairy roots currently used.

4.3.4 Glomus sp. exhibited consistent poor development

We found that replicates involving *Glomus* sp. were the least affected by host identity. Regardless of host, *Glomus* sp. developed at the same pace over 12 weeks of co-culture, concluding with all replicates having a 45-47% probability of exhibiting any new daughter spores. Research at CCAMF has found that when a volume of inoculum (>500 spores) that is ~10-fold greater than that used in this study (20 spores) is added to cultures containing Dau roots, thousands of *Glomus* sp. (GC-4) spores can be harvested from a single HC (Claudia Banchini, personal communication). The inoculation method used at CCAMF, specifically the placement of gel plugs filled with numerous aseptic spores directly on or near host roots, is common practice for initiating monoxenic AM cultures. As such, it is likely that the low inoculum quantity used in our study is what caused the discrepancy between the observed *Glomus* sp. development in our study and what is observed at CCAMF.

Another possible explanation could be related to the size of *Glomus* sp. spores (diameter 33-79 µm; CCAMF). AM fungal spores can be comprised of anywhere between 45% to 95% of lipids, which make up the carbon reserves available to metabolize and support hyphal germination (Gaspar *et al.*, 1994; Bago *et al.*, 2002). After a certain point, if no host root exudates are detected, AM fungal spores execute a survival strategy wherein growth is arrested and hyphae are retracted before spore reserves are depleted (Giovannetti *et al.*, 2010). Consequently, it is reasonable to predict that in the absence of a host, when smaller spore AM

fungal species germinate, the duration of hyphal growth before retraction should be shorter relative to larger spore species (Aguilar-Trigueros *et al.*, 2019). Compared to the spores of *R. irregularis* (diameter 70-165 µm; INVAM, 2021a) and *R. clarus* (diameter 100-260 µm; INVAM, 2021b), *Glomus* sp. spores were clearly the smallest. Therefore, of the three fungal species, *Glomus* sp. may have been the most sensitive to the initial spore placement when setting up co-cultures. Recall from **Figure 7** that all our two-compartment Petri plate cultures were set-up such that spores and host roots were at a distance from one another. It is possible that our AM culture set-up (i.e., spore placement and quantity) exacerbated the already low likelihood of *Glomus* sp. locating a host due to their limited capacity to support asymbiotic growth. Moreover, the placement of a high quantity of *Glomus* sp. spores in close proximity to Dau roots by CCAMF researchers, likely offset the inefficient growth of each spore and facilitated eventual fungal life cycle completion.

The decision to set-up all AM cultures with spores placed in a similar location was chosen as it controlled for the effect of distance from hairy roots. However, this spore placement may have affected our AM fungal species differently.

Ultimately, we found that across the nine unique pairs of host roots and AM fungi, only two symbiont species elicited consistent behaviour from their partners, Nic roots and *Glomus* sp. Furthermore, the fungal behaviour facilitated by Dau roots differed greatly depending on the AM fungal species. Therefore, our second hypothesis was rejected: host specificity is not the only effect as the interaction between host and AM fungal species seems to influence the outcome of the fungal behavior.

4.4 Novel methods

4.4.1 HC sporulation

In this study we employed a novel protocol to quantify HC sporulation *in vitro*. Our approach incorporated aspects from previously described methods which facilitated non-destructive, repeated observations of monoxenic AM cultures. Namely, we used a 5 mm^2 grid (Declerck *et al.*, 1996) and randomized our selected observational areas (Koch *et al.*, 2004; Ehinger *et al.*, 2009). We then we built upon these procedures with two key modifications. First, we opted to observe sporulation strictly in HCs because of the seminal work by St-Arnaud et. al., (1996) which suggested that HC sporulation may be significantly higher than that observed in RCs. Additionally, HC observations are not obstructed by roots growing between spores and microscope objective lens. The second modification was to make the observation areas more dynamic by re-randomizing their positions within nine evenly distributed sections in the HC. In doing so, we greatly reduced the potential for sampling bias and were able to gather enough data to model the relative spatial distributions of HC sporulation for each host and AM fungus combination.

A 3D printed two-compartment Petri plate grid mount was designed and used throughout the HC sporulation assay thereby reducing any potential error from inconsistent relative positioning of the two-compartment Petri dishes and a 5 mm^2 grid. Design files and materials used to construct our two-compartment Petri plate grid-mount are available upon request. For future studies, it would be beneficial to include hyphal measurements as some fungal taxa such as *Gigasporaceae* tend to prioritize hyphal growth over sporulation (De Souza et. al., 2005). Hyphal measurements could complement the spore counts obtained from each of the 5 mm^2 areas selected, thus providing a more comprehensive understanding of fungal behaviour.

Measurements could be taken by first imaging observation areas and then using digital analysis tools such as NeuronJ or *HyLength,* (Meijering *et al.*, 2004; Cardini *et al.*, 2020).

4.4.2 Symbiosis development

Tracking the progression of AM fungi through our selected stages of SD was conducted such that we could compare the compatibility between different host and AM fungi species. In doing so, we gained a rough estimate of important events throughout symbiosis such as: the duration prior to symbiosis establishment (stages 0-2, before first observed sporulation event), viability (stages 3+, sporulation of any kind, in RC or HC), ER mycelium growth (stages 4+, HC growth of any kind), and sporulation in the HC (stage 6, HC sporulation). Ultimately, tracking the progression of each unique host and AM fungus combination through stages of SD was successful in finding that while the host-effect imposed by Nic roots is consistent, Med and Dau roots elicited mixed fungal behaviour.

For future studies, there is plenty of room for improvement. We sacrificed more detailed observations for a higher sample size (n = 21-68, for any host and AM fungus pair at any time point). This was done because AM fungal growth is often variable and a higher sample size would work to correct for any inconsistencies stemming from a smaller number of replicates. Specifically, we would have liked to replace “roots and hyphae in close contact” (SD stage 2) with a visual confirmation of hyphopodium and to also quantify RC spore production. These changes would allow for a more accurate timeline of when IR colonization began (Genre et. al., 2005) and RC sporulation data would further illustrate any host-specific differences in overall sporulation dynamics thereby giving a better indication of carbon competition amongst symbionts. Of course, measuring IR fungal growth is also important when gauging carbon flux in

the AM symbiosis. However, obtaining such measurements is typically destructive and does not allow for continued observation of SD.

4.4.3 Non-destructive tracking of IR AM fungal growth

Using fluorescent markers whose expression is driven by symbiosis specific promoters may be a feasible method to non-destructively monitor IR colonization. In this study we made several attempts to do so using either MtPT4p or MtBCP1p to drive the expression of an NLS-GFP-GUS sequence, in addition to a constitutively expressed red fluorescent protein (DsRED1; Limpens *et al.*, 2004). Both Nic and Med roots passed screening as they fluoresced red, indicating that Ri T-DNA and our destination vector were inserted into the nuclear genomes of both hosts. Despite confirmation that Nic and Med were successfully co-transformed, neither MtPT4p nor MtBCP1p exhibited symbiosis-specific expression. It should be noted that *Agrobacterium*-mediated co-transformation is an imprecise process as there is no controlling where in a genome a DNA fragment of interest is inserted (Visser *et al.*, 1989). As such, it is possible that our destination vector was inserted onto a genetic locus downstream of a constitutively expressed promoter with strong activity, such that GFP was observed in non-mycorrhizal hairy roots. Alternatively, the uncharacteristic expression of these promoters may be due to the fact that both tested promoters are native to the genomic DNA sequences of A17, a cultivar of *M. truncatula* (Harrison *et al.*, 2002; Ivanov & Harrison, 2014), and foreign to the genomes of Nic and Med (cv. R108).

Despite our failed attempts, the use of symbiosis-specific promoters to non-destructively monitor IR fungal growth could be a powerful research tool. If successful, one would be able to observe every stage of AM fungal growth, both IR and ER under *in vitro* conditions. For future studies, we suggest performing several independent co-transformation attempts using A17 roots

and MtPT4p/MtBCP1p as it is possible that at least one of the resulting hairy root lines will express symbiosis specific GFP expression.

4.5 Med hairy roots offer practical advantages for long-term AM fungi culture collections

Based on the host-specific effects reported in our study as well as in the literature, it is strongly recommended that alternative hairy root species outside of Dau roots are used over long-term *in vitro* culture of AM fungi. We demonstrated in this study that across *R. irregularis, R. clarus,* and *Glomus* sp., Med roots can facilitate sporulation in both RCs (close to the roots) and in HCs (at a distance from host roots). Furthermore, the *Medicago* model predicted that HC sporulation in Med replicates tended to increase over time. Overall, Med roots facilitated a comparable amount of fungal growth to that seen with Dau roots except for two circumstances, 1) *R. irregularis* HC sporulation, and 2) progression of *R. clarus* through SD stages. Regardless, the key benefit of introducing Med as an alternative host is to allow species/strains of AM fungi to interact with and complete their life cycle with more than a single host genotype, thus limiting possible fungal evolution (Kokkoris & Hart, 2019). Alternating between Med and Dau may be somewhat analogous to the practice of host passage where routinely associating symbiotic organisms with different compatible host genotypes could alleviate any potential bottleneck effects (Marx, 1981).

One of the challenges that exist when overseeing a large culture collection is finding balance between the size of the collection and the practicality of maintaining said collection. In other words, it is not feasible for curators to increase the number of cultures in their collection if their staff are then unable to properly cultivate a corresponding number of specimens. For collections of AM fungi, specifically co-cultures residing in two-compartment Petri dishes,

maintenance (e.g., re-directing roots growing towards HC back towards the RC) is time-consuming and often results in a loss of co-cultures to contamination. By reducing the time necessary for culture maintenance, Med roots should allow for a greater number of cultures to be maintained under *in vitro* conditions.

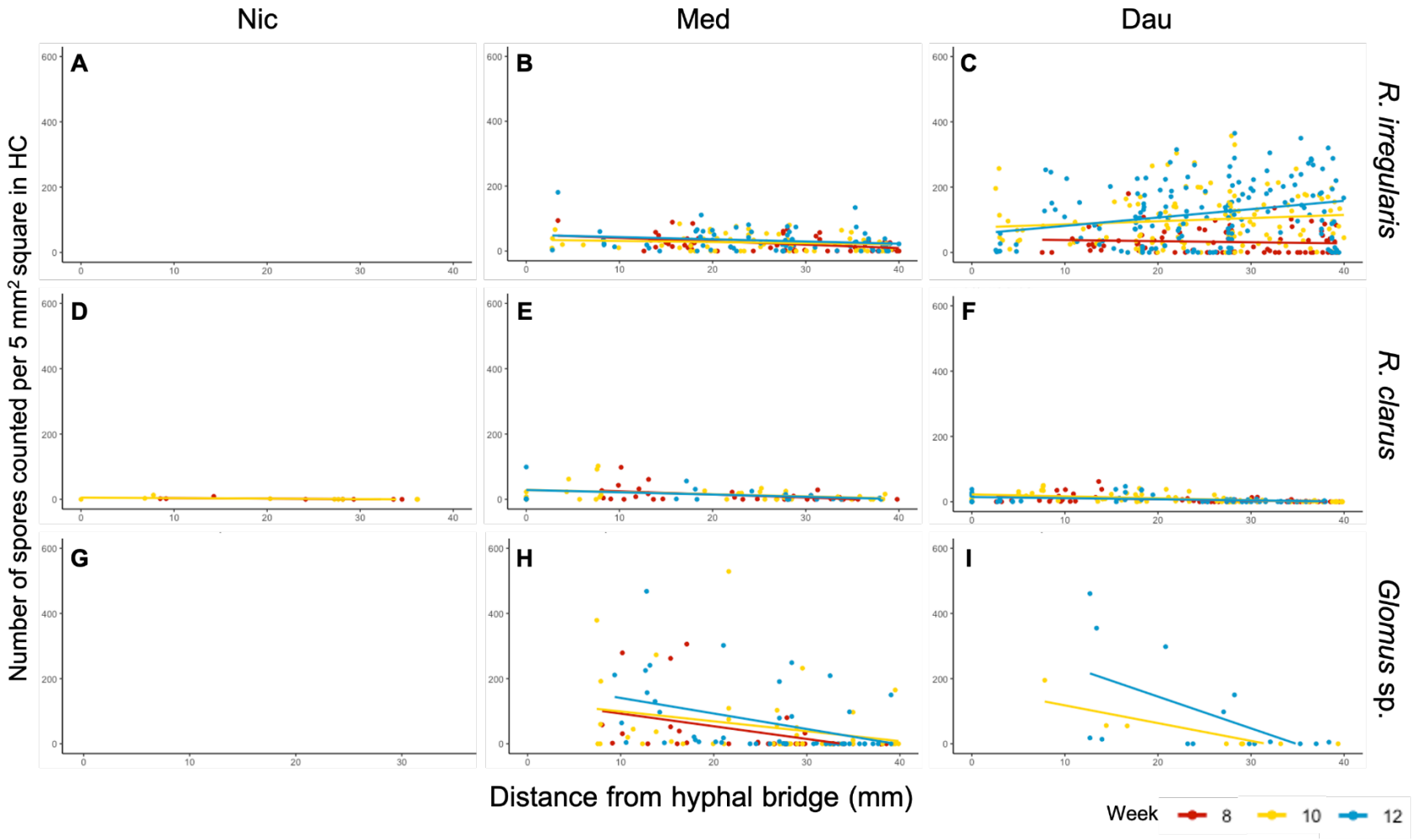
In this study it was found that of the three hairy root species, Med-hosted cultures required the least amount of maintenance over a 12-week period, and as a result, had the highest likelihood of remaining monoxenic. In contrast Dau, the traditionally used as the *in vitro* host at CCAMF and GINCO-BEL, and Nic-hosted co-cultures were lost quite frequently as their roots grew much quicker than Med roots and therefore needed to be re-directed far more often.

4.6 Conclusion

While there is a clear effect of host on the growth and development of AM fungi under *in vitro* conditions, host identity alone is not a consistent indicator of fungal growth across multiple fungal species. We found that specific hosts and AM fungi pairs exhibited unique SD timelines and HC sporulation patterns that could not be broadly categorized according to a behaviour typical of a specific symbiont. Continuing to study the interaction between the same host genotype and AM fungi neglects the potentially enormous genetic variation which results from each unique genotype × genotype interaction (Mateus *et al.*, 2019). This has implications for large *in vitro* collections of AM fungi such as the collection at CCAMF and GINCO-BEL. Continuing to maintain much of the collection over many generations hosted only by Dau roots may inadvertently be driving fungal evolution (Kokkoris & Hart, 2019). Under natural conditions, AM fungi are exposed to a wide variety of host species, often simultaneously. Such host diversity is almost certainly necessary for many species of AM fungi to complete their life

cycle. Therefore, it is critical that *in vitro* collections of AM fungi continue to develop and test new hairy root species, either under monoxenic conditions or possibly using multiple hosts to simulate *in situ* conditions. Such an effort will make it possible for *in vitro* AM fungi collections to maintain the genetic integrity of the species they currently cultivate, while opening up the possibility of cultivating new species.

Supplementary Information



Supplemental Figure 1. Distribution of spore counts scored in 5 mm^2 grid squares in relation to the distance each square was located from hyphal bridge. Exact hyphal bridge position measured to control for slight variation from the centre position of each Petri plate. Regression lines were fit to spore counts recorded at 8 wpi (red), 10 wpi (yellow), and 12 wpi (blue). Panel columns arranged by host (left to right: Nic, Med, and Dau) and rows arranged by AM fungi (top to bottom: *R. irregularis, R. clarus,* and *Glomus* sp

Supplemental Table 1. Cultivar, DAOM, or strain information from the publications mentioned above that reported using either *R. irregularis* or *Daucus carota.*

Host/fungal species	Cultivar/DAOM/Strain	Publication
Rhizophagus irregularis	Unknown	Chabot et. al, 1992
	DAOM 181602	St-Arnaud et. al., 1996 Douds, 2002
	MUCL 41833	Declerck et. al., 2001
	A4, B3, C3, D1, and C2	Ehinger et. al., 2008
	A4, G1, SL1, and A5	Kokkoris et. al, 2021
	DAOM234181	Goh et. al., 2021
Daucus carota	DC1 or DC2	Becard & Fortin, 1988[1,2] Douds, 2002[1] St-Arnaud et. al., 1996* Ehinger et. al., 2008*
	P68	Kokkoris et. al., 2021 Goh et. al., 2021

*Authors did not state which *Daucus carota* strain from Becard & Fortin, (1988) was used.
[1]Authors used DC1 strain of *Daucus carota*
[1,2]Authors used both DC1 and DC2 strains of *Daucus carota*

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