In vitro Propagation of Arbuscular Mycorrhizal Fungi May Drive Fungal Evolution

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Transformed root cultures (TRC) are used to mass produce arbuscular mycorrhizal (AM) fungal propagules in vitro. These propagules are then used in research, agriculture, and ecological restoration. There are many examples from other microbial systems that long-term in vitro propagation leads to domesticated strains that differ genetically and functionally. Here, we discuss potential consequences of in TRC propagation on AM fungal traits, and how this may affect their functionality. We examine weather domestication of AM fungi has already happened and finally, we explore whether it is possible to overcome TRC-induced domestication.

Keywords: arbuscular mycorrhizal fungi, fungal domestication, fungal evolution, in vitro propagation, transformed root cultures

INTRODUCTION

Domestication of plants and animals has been a hallmark of the Anthropocene (Zeder, 2006), resulting in altered morphology, decreased genetic diversity, altered behavior, and altered function in the domesticant. Such far reaching changes are necessary to maintain the domesticated state but can present a risk to food security (Chen et al., 2015; Whitehead et al., 2017; Egan et al., 2018), and in some cases, to the status of the domesticated species (Pearce, 2003; Ploetz, 2003). For example, domestication of wild bananas led to a sterile and genetically homogeneous cultivar that now faces extinction (Pearce, 2003; Ploetz, 2003). Similarly, decreases in genetic diversity as a result of domestication has been well documented in many crops, including common bean (Bitocchi et al., 2013), rice (Ram et al., 2007), wheat (Reif et al., 2005), soybean (Hyten et al., 2006), and pear (Nishio et al., 2016). This decrease in genetic diversity is often associated with losses of functional traits such as herbivore resistance (Chaudhary and Bhupendra, 2013), reduced immune function (e.g., Honey bees, López-Uribe et al., 2017) or even behavioral changes such as loss of immigration ability in monarch butterflies (Tenger-Trolander et al., 2019).

The effect of domestication on ecological competence is not novel in mycology (Jinks, 1952; Roper et al., 2011); plant pathogens lose pathogenicity when kept in culture for extended periods (Naiki and Cook, 1983), and domestication of Saccharomyces cerevisiae created yeasts without the ability to reproduce sexually or survive outside of laboratory conditions (Gallone et al., 2016). Aspergillus oryzae diverged from a pathogen to become a commercially important fermenter and subsequently lost genes related to pathogenicity (Machida et al., 2005). Such losses may result from bottleneck effects and environmental selection, especially if the system used in cultivation does not represent the conditions under which they originally evolved (see review by Douglas and Klaenhammer, 2010).

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs that participate in an ancient symbiosis with plants (Smith and Read, 2008; Brundrett and Brundrett, 2009). Through this symbiosis, AM fungi provide plants increased access to soil resources in return for carbon in...
the form of sugar and lipids (Luginbuehl et al., 2017). Besides the nutritional benefit to the plants, AM fungi can also increase plant tolerance to environmental stress [e.g., water (Ruiz-Lozano and Aroca, 2010), salinity (Porcel et al., 2012), and heavy metals (Díaz et al., 1996)]. AM fungi are known to stimulate plant photosynthetic activity (Boldt et al., 2011) and enhance plants’ disease resistance (Pozo and Azcón-Aguilar, 2007; Jung et al., 2012). Because of these benefits, considerable effort has focused on finding ways to propagate and study these fungi for potential applications including agriculture, landscaping, and landscape restoration (Sawers et al., 2008; Berruti et al., 2016). One of the most successful methods of propagating clean material employs the use of transformed root cultures (TRC) (see sidebar1 for information on TRC) (Mosse and Hepper, 1975; Bécard and Fortin, 1988; Stockinger et al., 2009; Rosikiewicz et al., 2017). While this method is efficient for producing uncontaminated propagules, it represents a highly artificial environment and could potentially lead to domesticated AM fungal strains (Figure 1).

Currently, commercial AM fungal inocula are used both in horticulture and field applications (Berruti et al., 2016). Many of the fungal propagules used in commercial products originate from TRC. The effect of TRC on the evolution of "domesticated" AM fungi is not clear (Plenchette et al., 1996). Here, we argue that commercial production of AM fungi via TRC represents strong selection pressure on fungi and represents a form of domestication, through changes to nutrient limitations, microbial consortia, and reduced host variation. Such selection pressure may lead to reduced genetic diversity and mutualistic quality.

**LUXURIOUS NUTRIENT CONDITIONS**

Although AM fungi form functional mycorrhizas in TRC, the unique nutritional strategy of hairy roots may affect the quality of the symbiosis. In association with a normal plant, AM fungi grow in tandem with roots which fluctuate daily

**Abbreviations**: AM, Arbuscular mycorrhizas/arbucular mycorrhizal; BLOs, Bacterial like organisms; ECM, Ectomycorrhizas/ectomycorrhizal; MRE, Mollicutes/mycoplasma-related endobacteria; MR, Mycorrhizal response; N, Nitrogen; P, Phosphorus; Ri, Root inducing; TRC, Transformed root cultures.
in intraradically and mycorrhizal response (MR) in hosts (Menge et al., 1978; Thomson et al., 1986; Breuillin et al., 2010; Bonneau et al., 2013), which may lead to less beneficial associations. For example, increased N levels (via nitrogen fertilization) can select for rhizobia (Weese et al., 2015), and AM fungi (Johnson, 1993) that provide reduced benefit to the host plants. It is therefore possible that a highly eutrophic environment, such as TRC, may promote selection for less mutualistic AM fungi.

**TALK BETWEEN MICROBIAL NEIGHBORS**

The monoxenic environment of TRC lacks much of the hyphosphere/rhizosphere microbial consortia that play an important role in the AM symbiosis. Co-existing microbes engaged in antagonistic or synergetic interactions produce bioactive compounds which can be used in defense, to confer stress tolerance or boost metabolic activities for the producers (Ola et al., 2013). Such compounds are not produced when bacteria (Koskiniemi et al., 2012) and fungi (Naik and Cook, 1983; Gallone et al., 2016) are maintained under axenic conditions due to lack of appropriate environmental stimuli from neighboring microbes (Marmann et al., 2014). The pathways for these signaling compounds can be lost permanently via selective gene deletion over generations of continuous propagation in vitro.

Similar to other microbes, AM fungi have antagonistic and synergetic relations with other AM fungi (Wilson, 1984; Engelmoe et al., 2014) and other soil microbes (Mar Vázquez et al., 2000). For example, it was recently demonstrated that AM fungi have the ability to indirectly increase the nitrogen (N) uptake by plants via association with soil microbes (Hestrin et al., 2019). Therefore, growing in an environment that inhibits these interactions could reduce the effectiveness of such strains in natural conditions.

In addition to the selection pressure resulting from lack of microbial cross talk, reduction or even elimination of fungal endobacteria and bacteria that reside on the hyphal or spore surface in TRC, can affect fungal function (Dearth et al., 2018) and mutualism performance (Vannini et al., 2016). Establishing AM fungi in TRC requires surface sterilization and antibiotics in order to eliminate surface bacteria (Bécard and Fortin, 1988). However, AM fungi naturally comprise a community of bacteria that reside in, and on, hyphae and spores. Abundant rhizobia and pseudomonads have been found attached on spore and hyphal surface (Biancotto et al., 1996b; Roesti et al., 2005; Agnolucci et al., 2015), but also bacterium-like organisms (BLOs) (Biancotto et al., 1996a; Naumann et al., 2010) and Mollicutes/mycoplasma-related endobacteria (MRE) (Desirio et al., 2014; Torres-Cortes et al., 2015; Naito et al., 2017) were detected within the cytoplasm. Some of these bacteria possess chitinolytic abilities (Roesti et al., 2005; Agnolucci et al., 2015) and their abilities to degrade spore walls can play a crucial role in spore germination (Mayo et al., 1986). Of course, the presence of such bacteria can also benefit the colonized plants via a cascade of gene activation and chemical signals (Artursson et al., 2006). Long-term in vitro culturing could negatively affect the interaction between AM fungi and their own beneficial mutualists (Lumini et al., 2007).

**PLANT IDENTITY**

There is increasing evidence that plant genotype can significantly affect the symbiosis (Chialva et al., 2018; Mateus et al., 2019). In the case of TRC propagation, fungi are exposed to dramatically reduced host diversity [most commonly carrot (Daucus carota) or tomato (Solanum lycopersicum)]. While gene activation in the early stages of colonization are preserved among hosts (Delaux et al., 2014), the progression of the symbiosis can be significantly altered depending on host identity both regarding the plant (Angelard et al., 2010) and fungal response (Cavagnaro et al., 2001; Koch et al., 2017). Mateus et al. (2019) observed large differences in the expression between fungal isolates growing on multiple cassava cultivars, but the differences were influenced largely by the genotype of the cultivar host. The reduction in host genetic diversity to a single genotype in TRC may lead to genetic drift and unused gene deletion for the AM fungus (Muller's ratchet in host restricted lineages, see Moran et al., 2008). Recently, Sugiu et al. (2019) identified myristate, a fatty acid, as a usable carbon source from Rhizoglomus irregularare during the asymbiotic growth that can promote hyphal growth to the production of daughter spores in a host-free culture. While such information advances our knowledge in AM fungal metabolism, such a mechanism could also lead to host-free AM fungal propagation systems, with unknown effects on the efficacy of the symbiosis. Culturing symbionts in host-free environments has been shown to reduce symbiotic quality (Marx and Daniel, 1976; Speakman, 1982).

**IS THERE EVIDENCE OF DOMESTICATION ON ARBUSCULAR MYCORRHIZAL FUNGI?**

Given all the opportunities for deleterious selection on AM fungi growing in TRC, is there any evidence that domestication has happened? Evidence for domestication would require reduced genetic variation as well as morphological and functional changes.

**Reduced Genetic Variation**

There is evidence that controlled conditions such as TRC can lead to loss of genetic diversity among some AM fungal isolates. For example, Wyss and Bonfaante (1993) showed genotypic changes among isolates of a single species (Funneliformis mosseae BEG12) when maintained under long-term lab conditions. In addition, there is evidence of sequence loss in spores of an isolate of Glomus coronatum when maintained in cultures compared to field originated spores (Clapp et al., 2001) and reduced allelic variation in spores of Claroideoglomus etunicatum compared to the parent isolate following single spore inoculations (Boon et al., 2013).

**Morphological and Functional Alterations**

Regardless the mechanism leading to genotype changes, there is evidence that in vitro cultivation affects AM fungal functional traits. In vitro cultivation has led to increased germination rates (Kokkoris et al., 2019b) and reduced in propagule size (Pawlowska...
et al., 1999; Calvet et al., 2013). Plenchette et al. (1996) found that in vitro produced spores of *Glomus versiforme* were significantly less infective, even only after three successive generation in vitro. Calvet et al. (2013) observed that in vitro colonization of AM fungi reduced host nutritional benefit. Similarly, Kokkoris and Hart (2019) showed that in vitro propagation resulted in a trade-off between spore production and phosphorus (P) benefit. Copious spore production over nutritional benefit is a trade-off that seems to be preserved even when this isolate is grown in pots with a variety of different hosts (Kokkoris et al., 2019a).

**Loss of Endobacterial Symbionts**

*In vitro* cultivation may affect the endocellular bacteria associated with fungal spores. *Candidatus* Glomeribacter gigasporarum is a bacterium that resides in spores of *Gigaspora margarita*. *In vitro*, this bacterium experiences population dilution and eventually disappears leading to “pure” spores over successive generation *in vitro* (Lumini et al., 2007). Although the bacterium is not required for *G. margarita* to complete its life cycle, its absence alters spore’s morphology and negatively affects germination and growth (Lumini et al., 2007) and can significantly alter the fungal activity (including protein expression, and quality and quantity of lipidic profile) (Salvioli et al., 2010, 2016).

**Incompatibility Between Isolates?**

One potential consequence of TRC cultivation may affect hyphal fusion among compatible fungi. For example, *in vivo* cultivation for 20 years led to vegetative incompatibility for *F. mosseae* (Sbrana et al., 2018). If long-term culturing in TRC inhibits the ability of anastomosis, then domesticated isolates might be unable to interact with other isolates in nature. Incompatibility could even lead to a permanent homokaryotic stage, preventing genetic information exchange between compatible isolates and thus adaptation to novel conditions (see sidebar#2 for the importance of anastomosis on AM fungi). It could also pose a survivorship disadvantage for such an isolate if used for field inoculations due to the isolation from the natural hyphal network (Sbrana et al., 2011). Loss of anastomosis might be the reason why *in vitro* produced strains often fail to establish and persist in natural environments post inoculation (Corkidi et al., 2004; Farmer et al., 2007; Tarbell and Koske, 2007).

**CAN WE OVERCOME TRANSFORMED ROOT CULTURES-INDUCED DOMESTICATION?**

If TRC propagation of AM fungi produces inferior mutualists, it is reasonable to wonder whether specific practices in TRC production could prevent such unwanted changes. Such practices, like co-cultivation, medium modifications and re-association with natural hosts, already exist and applied in other microbial systems.

**CO-CULTIVATION WITH MICROBES**

Axenic, and in case of AM fungi, monoxenic growing conditions can reduce the chemical diversity of the produced compounds due to lack of environmental stimuli. Co-cultivation of microbes can activate silent gene clusters of the microbial partners (Brakhage et al., 2008), protecting fungi against genetic drift and gene deletion. For example co-culture of bacteria and fungi (*Fusarium tricinctum Bacillus subtilis*) showed a 78-fold increase in fungal metabolite production compared to the pure culture of the fungus (Ola et al., 2013). Similarly, a fungal co-culture (*Coprinopsis cinerea and Gongronella sp.*) produced 900 times increased oxidation activity compared to pure cultures (Pan et al., 2014). Growing AM fungi with two *Paenibacillus validus* bacterial isolates increased fungal growth even in absence...
of a host (Hildebrandt et al., 2002). Co-cultivation of AM fungi with diverse microorganisms may be a way to maintain genetic variation and function by activating AM fungal genes that would otherwise be silent, due to reduced environmental stimuli, and prone to deletion if maintained long term in TRC.

CULTURING CONDITIONS

The most commonly used TRC medium is the M medium proposed by Bécard and Fortin (1988). While no major modifications have been made on M medium, addition of simulative chemical molecules could compensate for lack of microbial associates and trigger gene activation for secondary metabolite production. For example, the addition of fatty acids (signal from P. validus, see previous section) can induce colonization ability and stimulate the spore production of AM fungi (Kameoka et al., 2019). In addition, chemical effectors responsible for promoting hyphal branching, mycorrhization, and the efficiency of the symbiosis have been identified (e.g., strigolactones) (Akiyama et al., 2005; Besserer et al., 2006), which may lead to increased gene activation and help maintaining the genetic and functional variation in TRC.

Additional changes in the medium or in the growing conditions may stimulate recombination in AM fungi and encourage the production of novel genotypes. For example, in Coprinus congregatus, a Basidiomycete, the quantity of arginine in the medium controls the expression of the mating type genes and ultimately the growth of the fungus as a homo or dikaryon (Ross et al., 1991). We need to identify environmental controls of AM fungal mating behavior to optimize growing conditions that will choose for efficient symbionts and not just copious spore producers.

RE-ASSOCIATION WITH COMPATIBLE HOSTS

Ectomycorrhizal (ECM) fungi can lose their symbiotic ability and eventually fail to colonize plant roots if maintained in vitro long term (Marx and Daniel, 1976). Growing strains via host passage (association with a compatible host) every 4 years alleviates this bottleneck (Marx, 1981). The re-isolated strain from the colonized roots shows increased colonization ability but also increased symbiotic quality compared to solely in vitro retained strains (Thomson et al., 1993). Similarly for pathogenic fungi, pathogenicity can be lost with long-term in vitro cultivation and “passaging” the strains through a compatible host and re-isolating and can revitalize their infective abilities (Speakman, 1982). Furthermore, the “asexual” yeast Candida albicans was stimulated to mate when injected into a mammalian host (Hull et al., 1999) showing the significant role an appropriate host can have even for sexual reproduction. While the presence of a root system is a prerequisite for AM fungal cultures, the important differences between TRC and real plant system may alter the AM fungal function. Passage through real host or even community of hosts could retain the functionality of the domesticated strains.

CONCLUSIONS

There is clear evidence that continuous in vitro propagation alters AM fungal morphology, genetics, and functioning, meaning that domestication of such strains is in progress or has occurred. While mass production of AM fungal propagules is needed for a sustainable inoculant industry, in vitro propagation may bring unwanted changes to the cultured isolates. If domestication reduces the isolate’s ability to anastomose, these fungi would have a fitness disadvantage in the field. Alternatively, if the unnatural environment of TRC creates strains that are less beneficial in natural conditions, but these isolates are still able to anastomose with native fungi, such isolates may impact negatively on the gene pool of natural populations. It is important to further examine the effects of domestication on AM fungi and predict how changes could greatly affect the environment following inoculation with such strains.

DATA AVAILABILITY STATEMENT

No datasets were generated or analyzed for this study.

AUTHOR CONTRIBUTIONS

MH and VK conceptualized the work and shared the writing and revision of the MS. MH and VK approved the publication of the MS in its current form. MH and VK agreed to be accountable for all aspects of the work including accuracy or integrity of any part of the work.

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

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