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The role of *in vitro* cultivation on asymbiotic trait variation in a single species of arbuscular mycorrhizal fungus

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ABSTRACT

Cultivating arbuscular mycorrhizal (AM) fungi *in vitro* is an efficient way to produce material for industry and research. However, such artificial growing conditions may impose selective pressure on fungi grown *in vitro* over many generations. We hypothesized that isolates subjected to long term propagation *in vitro* may develop increasingly ruderal traits. We proposed a predictive framework for the effect of *in vitro* cultivation on asymbiotic AM fungal traits. Using photomicrography and image processing, we analyzed morphology and growth traits for 14 isolates representing an *in vitro* cultivation gradient from 0 to >80 generations *in vitro*. We investigated the range of trait variation among asymbiotic growth of arbuscular mycorrhizal (AM) fungus isolates (*Rhizoglomus irregulare*). Spore dormancy was strongly associated with *in vitro* cultivation. We observed extremely high levels of inter-isolate variation for most fungal traits, but this was not related to time *in vitro*. Our results indicate that intra-specific diversity may have a strong ecological role in AM fungal communities.

1. Introduction

Obtaining pure cultures of arbuscular mycorrhizal (AM) fungi for research and industry is difficult as they are obligate root biotrophs. The most effective method for obtaining pure cultures of AM fungi is through *in vitro* propagation using transformed root cultures (TRC) (Mosse and Hepper, 1975). These *in vitro* cultures consist of genetically modified roots that grow without a shoot, allowing the symbiosis to be grown in sterile conditions. TRC have been used for decades to both study and produce sterile fungal inoculum (Mosse and Hepper, 1975; Mugnier, 1987; Bécard and Fortin, 1988; Fortin et al., 2002; Stockinger et al., 2009; Rosikiewicz et al., 2017).

While TRC are useful for observing the symbiosis under controlled conditions, they represent artificial growing conditions (Fortin et al., 2005). Such conditions likely exert selective pressures on fungi that select for traits advantageous for growth *in vitro*. There is some evidence that *in vitro* cultivation leads to smaller spores and lower colonization rates compared to the same isolates *in vivo* (Calvet et al., 2013; Jin et al., 2013; Pawlowska et al., 1999). However, we know little about how *in vitro* propagation influences traits involved directly in the symbiosis.

Given that fungi grown *in vitro* experience little disturbance, stress or competition, *in vitro* propagation may select for more ruderal traits among AM fungi (Fortin et al., 2002). A ruderal life history strategy is one which has high growth rates and reproductive output, but little competitive ability or stress tolerance. It is one of three strategies (ruderal, stress-tolerant, and competitive) proposed by Grime (1977) for the classification of plant life history strategies and has since been used to describe fungi (Chagnon et al., 2013; Hart et al., 2001). Ruderal fungal traits align closely with traits for advantageous *in vitro* propagation, such as high growth rates, early and copious production of small spores, rapid hyphal turnover, and fusion or healing of broken hyphae (Chagnon et al., 2013; van der Heijden and Scheublin, 2007).

An unintended consequence of *in vitro* growth may be reduced trait diversity (Wyss et al., 2016). This phenomenon is well known among domesticated organisms, from fungi (Gibbons et al., 2012) to plants (Doebley et al., 2006; Burger et al., 2008). The consistent, controlled environment of *in vitro* propagation can lead to decreased trait diversity, as traits not related to successful growth *in vitro* may be lost through genetic drift.

Whether directly or indirectly, ruderal traits have been prioritized during the development of *in vitro* cultivation methods starting with Bécard and Fortin (1988) and St-Arnaud et al. (1996) whose innovations led to increased spore production, among other changes (Berruti et al., 2016).

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Based on the unique environment created during *in vitro* propagation, we predict trait changes are likely to occur over time (Fig. 1). There is some evidence for such changes in the literature. For example, reduced spore size has been lined to *in vitro* propagation (Calvet et al., 2013), which can result in reduced stored resources. Smaller spores may ultimately lead to reduced total hyphal length and hyphal diameter.

Hyphal anastomoses may also be affected by *in vitro* cultivation. The ability of AM fungi to anastomose can increase the survival of germlings in the absence of a host (Sbrana et al., 2011) and can increase the interconnectedness of the hyphal network (Pepe et al., 2016). For one species in particular, *Rhizoglomus irregulare*, anastomosis serves as a disturbance response mechanism allowing for rapid wound healing (Chagnon et al., 2013; De Boulois et al., 2006; de la Providencia et al., 2005; van der Heijden and Scheublin, 2007). Considering the lack of disturbance during *in vitro* propagation we expect decreased frequency of anastomosis *in vitro* propagation.

Dormancy may also be affected by *in vitro* growing conditions. For *in vitro* cultures, dormancy may not represent a selective advantage, and in fact may be selected against and ultimately lost from the population. We predict that cultures exposed to long term *in vitro* cultivation will express reduced, or no, dormancy. Additionally, stable *in vitro* conditions may result in reduced stress responses such as such as septa formation (Baar, 2008; Logi et al., 1998; Smith et al., 2008).

Finally, hyphal branching and the distance a hypha is able to grow from its origin represent fungal foraging strategies. Given the stable and uniform conditions of *in vitro*, cultivars reared under these conditions may have reduced branching as well as reduced exploration ability.

In this study we asked if *in vitro* propagation has resulted in trait differentiation among isolates of a single AM fungal species. We hypothesized that isolates that have been propagated *in vitro* over many generations exhibit more ruderal traits than those that have been propagated for fewer generations. We observed trait variation in the asymbiotic stage, given that it involves a suite of traits necessary for successful symbiosis establishment.

2. Materials and methods

2.1. AM fungi

We used 14 isolates of *R. irregulare* (Schenck and Smith), varying in the number of generations propagated *in vitro* from wild (no *in vitro* propagated generations) to highly cultivated (over 80 *in vitro* propagated generations) (Table 1). *R. irregulare*

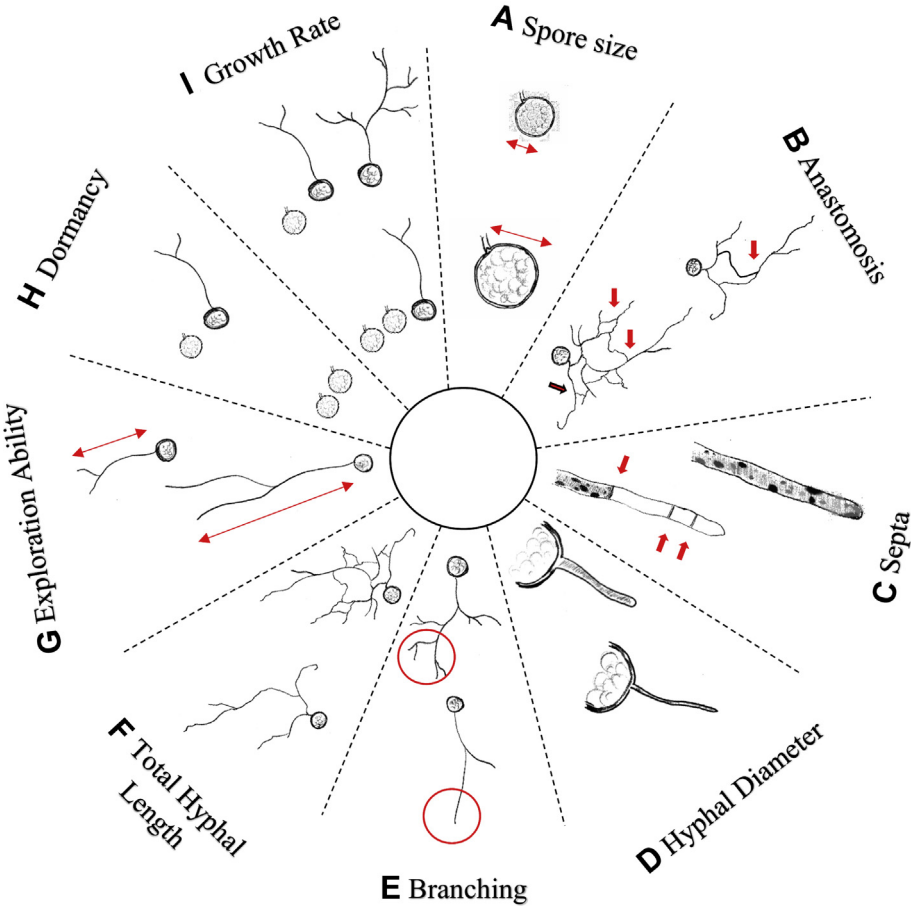

Fig. 1. Predictive framework for the effect of *in vitro* cultivation on asymbiotic AM fungal traits. For each trait, we predict that *in vitro* cultivation will lead to increased or decreased trait expression. Increased distance from the center indicates increasing generations *in vitro* cultivation. (A) *In vitro* propagation may lead to smaller spores (Calvet et al., 2013). (B) Considering the lack of disturbance during *in vitro* propagation, we expect decreased frequency of anastomosis with increased *in vitro* propagation. (C) Due to the stable conditions *in vitro* may lead to decreased formation of septa. (D) We expect hyphal diameter to decrease with increased *in vitro* propagation due to a shift to a more ruderal life history strategy (tradeoff between investment in spores versus hyphae). (E) We expect reduced branching with increased *in vitro propagation* due to the continuous proximity of hosts in the system. (F) We expect that isolates that are highly *in vitro* propagated will have reduced total hyphal length due to fewer resources present in their smaller spores. (G) We expect reduced exploration ability with increased *in vitro* propagation due to less resources in the smaller spores and due to abundant hosts during *in vitro* propagation. (H) We predict that dormancy will not be necessary with increased *in vitro* propagation due to continuous stable environmental conditions. (I) Finally, we expect faster growth rate with increased *in vitro* generations due to a shift to more ruderal behavior, and as a tradeoff to reduced investment in stress/disturbance strategies.

isolates were obtained from GINCO [http://www.mycorrhiza.be/ginco-bel/] except isolate DAOM 197198, obtained from AGTIV product (Premier Tech) and GD50, obtained from Agriculture and Agri-Food Canada, Swift Current Research and Development Centre.

2.2. Spore isolation and sterilization

Spores were extracted from the substrate (sand and vermiculite) by wet sieving and sucrose centrifugation using a modification of Gerdemann and Nicolson (1963). Briefly, approximately 100 g of the substrate was blended at high speed for 5 s, then sieved through a 38 μm mesh. Spores when then transferred to 50 ml falcon tubes, centrifuged twice at 4 °C (at 1200×*g* and 960×*g*), and collected from the supernatant in falcon tubes (50 ml). To obtain spores from monoxenic i*n vitro* cultures with Ri T-DNA-transformed carrot roots (*Daucus carota*), a small piece of the medium containing only spores (not plant tissue from a dual compartment petri dish) was cut and placed in 50 ml falcon tubes with autoclaved RO water followed by vortexing to ensure separation.

All spores, regardless the culture origin, were surface sterilized using the modified procedure of Mertz et al. (1979) as proposed by Bécard and Fortin (1988). Sterile spores were stored in 200 mg/L streptomycin and 100 mg/l gentamycin at 4 °C for 20 days prior the initiation of the experiment in order to exceed the critical time period needed to reduce spore mortality and increase germination rates by breaking the spore dormancy (Juge et al., 2002).

2.3. Experimental design

From each of the 14 isolates, 30 spores were chosen based on morphology. Only healthy-looking spores with intact spore walls and no discoloration were chosen. Spores were imbedded individually in 60 mm × 15 mm petri dishes filled with 1/2 strength MS medium that has been shown not to affect the germination of spores (Mark et al., 1997; St-Arnaud et al., 1996). The medium was solidified with 1 % gellan gum (Alfa Aesar). All spores were first washed with 2 % chloramine T solution then rinsed with autoclaved water before embedding in the center of each petri dish using a pipette. For two isolates (DAOM 240477 and DAOM 240442), only 22 spores per isolate were deemed acceptable. After plating, spores were placed in a growth chamber (Conviron adaptis CMP6010) in a randomized complete block design at 26 °C in dark. To avoid contamination, petri dishes remained sealed with parafilm and were unopened for the duration of the experiment.

2.4. Trait quantification

Only 11 of 14 isolates were followed for the duration of the study as three isolates failed to germinate (isolates DAOM 211734, DAOM 240442 and GD50). These isolates were included only in the analysis of spore size and were excluded from all other analyses. Similarly isolates (DAOM 240440, DAOM 241558, and DAOM 240477) had very low germination success and therefore the replication number did not allow us to include them in any analysis other than spore size and germination.

Over the course of three months (January to March 2018), we measured the growth of 11 asymbiotic traits of germinating spores for 35 days post germination (Table 2). Spores were removed from the growth chamber and photographed individually with a Bioimager BRC-1600 camera on a Nikon SM2 1500 stereoscope. T Capture v 5.1 (© 2011–2018 Tucsen Photonics.) was used to capture photos. Photos were analyzed with Image J v.2.0.0 (Rasband, 2016). Oval selection and brush selection tools were used to calculate spore area (Fig. S1a). The line trace tool was used to measure hyphal reach and hyphal diameter (Fig. S1a, b). Adobe Photoshop CS3 v.10.0.1 was used to concatenate photos when necessary (Fig. S1c).

Hyphal growth over time, hyphal tips, and total hyphal length were determined using the Neuron J plugin (Meijering et al., 2004; Shen et al., 2016) (Fig. S2). Hyphal growth that exceeded the field of view were concatenated with Photoshop (Adobe Photoshop CS3 v 10.0.1) to determine hyphal growth over time (Fig. S2d, e, f). To determine the number of septa (Fig. S3a) and anastomoses (Fig. S3b), a compound microscope was used.

2.5. Statistical analyses

All analyses were performed on Primer v6 with PRIMER + PERMANOVA (Clarke and Gorley, 2006; Anderson et al., 2008) and R studio (Version 1.0.136 – © 2009–2016 RStudio, Inc.).

2.5.1. Do isolates differ in trait values and variation?

We used PERMANOVA (Anderson et al., 2008) to test for difference among isolates for all measured traits. We also examined traits individually for differences among isolates. ANOVA was used when the residuals of models using either raw or log-transformed trait values were normally distributed. When the residuals were not normally distributed, we used Generalized Linear Mixed model (GLM) that does not assume normality (applied only to anastomosis). For traits with significant differences among isolates, we performed *post hoc* comparisons using Tukey HSD, package: Agricolae. We also performed linear regression using the mean for all spores per isolate to determine the relationship between trait

Table 1
R. irregulare isolates used in this experiment. Isolates represent a gradient in the number of generations grown *in vitro*.

Generations *in vitro*	# for this study	Strain	DAOM reference	Habitat of origin	Isolate origin
>80	1		197198	Boreal forest	Canada
>70	2	GC3	234328	–	–
>60	3	CC4	229457	Fallow field	Canada
>50	4	3086R	211734	Sandy shore	Canada
>50	5	9A2	–	Greenhouse	Canada
45	6	4387 Rac10	240440	Fixed dunes	Canada
45	7	4392Srac6	240442	Fixed dunes	Canada
45	8	EC-16	241558	Oil sands	Canada
>40	9	SD2	233751	Greenhouse	Belgium
36	10	JSD-1	240721	Garden soil	Belgium
>30	11	3545	240159	Uncultivated fields	Canada
21	12	Cuba8	–	Cultivated field	Cuba
12	13	Blasz-3	240477	Fixed dunes	Denmark
0	14	GD50	–	Cultivated Field	Canada

Table 2
Asymbiotic traits examined in our study. Single spores were grown aseptically on half strength MS medium (Mark et al., 1997; St-Arnaud et al., 1996) and observed under a stereoscope for the traits below. For some traits, we also used compound microscope (anastomosis, septa). All distances are in micrometers (μm).

Trait	Quantification
Spore area	The circumference of each spore
Germination tubes	The number of germ tubes per spore growing from either the spore or the subtending hypha
Hyphal tips	The number of hyphal tips per spore (not including subtending hyphae)
Hyphal reach	The distance from the center of the spore to the furthest hyphal reach, obtained on the last day that growth was measured.
Hyphal diameter	The diameter of hyphae taken from the base of the germination tube, a random section of the hyphae, and at the hyphal tip.
Anastomoses	Number of anastomoses measured only at the end of the end of the experiment
Time to germination	Number of days before germ tube emergence
Hyphal growth/hyphal tips	Total hyphal growth divided by the number of hyphal tips
Total hyphal length	The summation of the length of germination tubes from a germinating spore
Septa per total hyphal length	The final number of septa divided by the total hyphal length
Hyphal growth speed	Total hyphal length divided by the number of days it took for a spore to stop growing/end of experiment
Days to maturity	Number of days from emergence of germ tube until it growth cessation and/or end of experiment

variation and generations *in vitro*. To assess differences in the amount of trait variation among isolates, we tested for differences in the spread of data around centroids (PermaDISP) (Anderson et al., 2008).

2.5.2. *Is trait variation related to* in vitro *cultivation?*

To determine how i*n vitro* cultivation affected the variation among isolates, we tested the correlation between a distance matrix based on all measured traits and a hypothetical "*in vitro* cultivation" distance matrix whereby distance increased with the *in vitro* generations. We used the Goodness of Fit comparison in RELATE (Spearman rank correlation) (Clarke and Gorley, 2006).

3. Results

3.1. *Do isolates differ in trait values and variation?*

We observed significant variation among isolates for the traits measured (df = 71, Pseudo-F = 2.11, p = 0.0001) (Fig. 2). Isolates also exhibited significant difference in the amount of trait variation within an isolate (dispersion) ($F_{7,64}$ = 7.63, p < 0.001) with isolate DAOM 240721 (36 *in vitro* generations) exhibiting significantly less variation than other isolates (Fig. 3).

3.1.1. *Germination*

Because not all isolates germinated within the time frame of this study, our analysis of traits is limited to the eight isolates having at least three germinating spores. In general, isolates propagated *in vitro* for more generations had higher germination rate than less domesticated isolates (R = 0.64, p < 0.05) (Fig. S4a). The three isolates with most *in vitro* cultivation (60, 70 and 80 generations) had the highest rates of germination (60, 70 and 43 %, respectively). While the isolate with no *in vitro* cultivation did not germinate, this was also true of isolates with 45 and 50 generations *in vitro* (Fig. 4A).

3.1.2. *Spore area*

While spore area differed among isolates ($F_{13,421}$ = 101.5, p < 0.001) (Fig. 4B) there was no evidence of decreasing size with increasing *in vitro* generations (R = 0.11, p = 0.72) (Fig. S4b).

3.1.3. *Number of germ tubes*

Although isolates differed considerably in the number of germ tubes per spore (min = 1, max = 9, median = 2), this difference was not statistically significant ($F_{7,64}$ = 1.6, p = 0.15) (Figs. 4C and 6A, B), and there was no change in germ tube number with *in vitro* cultivation (R = −0.18, p = 0.68) (Fig. S4c).

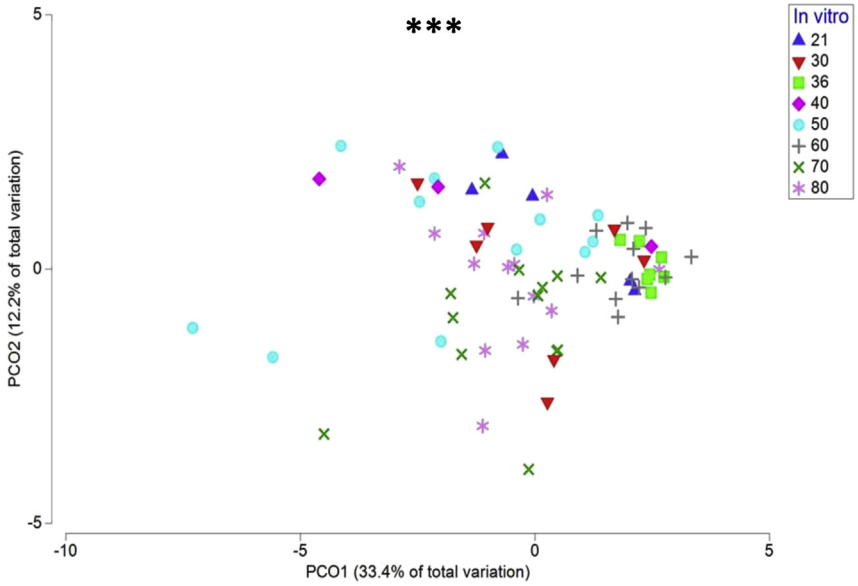

Fig. 2. Principal component ordination plot differentiating germinating isolates based on the traits measured. The x and y axis describe 45.6 % of trait variation between isolates. Different colored symbols reflect the various isolates differing in the number of generations propagated *in vitro*. Stars signify the level of significance (***p < 0.001).

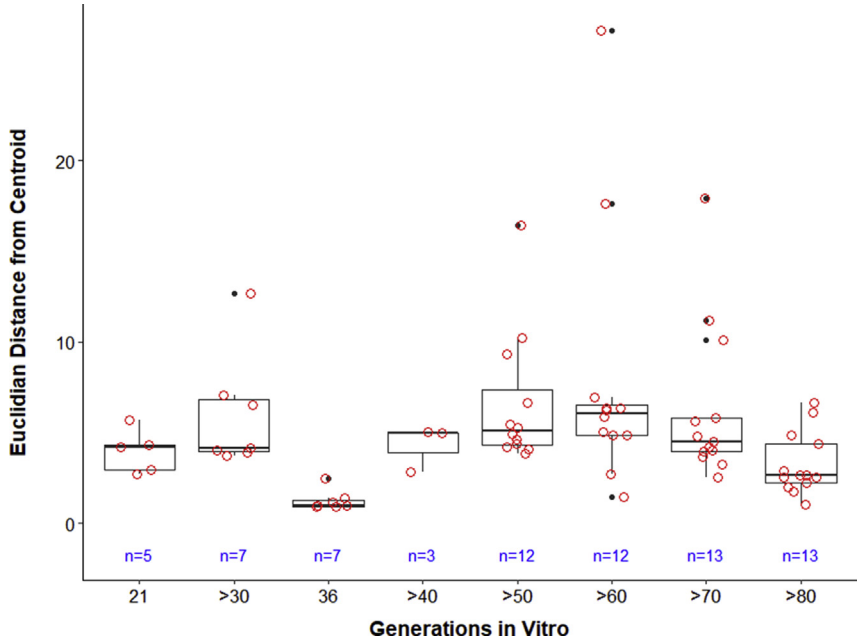

Fig. 3. Variation within isolates, including traits that were not heavily correlated. Hyphal growth speed was excluded because it was heavily correlated to total length ($r^2 = 0.96$, $p < 0.01$). Increased distance from centroid demonstrates increased more trait variation within an isolate. Isolate number represents a gradient of propagation *in vitro*, from 1 to over 80. Red solid circles represent mean values and whiskers represent SE. Open circles represent individual observations. Star signify the level of significance ($^*p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.1.4. Hyphal tips

The number of hyphal tips differed significantly among isolates ($F_{7,64} = 2.1$, $p < 0.05$) (Fig. 4D). There was no evidence that this was related to length of time *in vitro* cultivation ($R = 0.01$, $p = 0.19$) (Fig. S4d).

3.1.5. Hyphal reach

The reach of the germ tube(s) different significantly among isolates ($F_{7,64} = 10.57$, $p < 0.001$) (Fig. 4E). Contrary to our prediction, the isolates with the fewest generations *in vitro* had among the shortest reach, while the most highly cultivated had among the longest. Though significant, this relationship was not strong ($R = 0.26$, $p = 0.54$) (Fig. S4e).

3.1.6. Hyphal diameter

Isolates differed significantly in hyphal diameter ($F_{7,64} = 5.28$, $p < 0.001$) (Fig. 4F), but there was no effect of *in vitro* cultivation ($R = 0.0001$, $p = 0.32$) (Fig. S4f).

3.1.7. Frequency of anastomoses

Isolates differed significantly in the number of anastomoses ($x^2 = 55.76$, $p < 0.001$) (Fig. 5A). There was no effect of *in vitro* cultivation on the number of anastomoses ($R = -0.11$, $p = 0.79$) (Fig. S5a). Isolates DAOM 229457 (>60 *in vitro* generations) and DAOM 240159 (>30 *in vitro* generations) did not form any anastomosis during our experiment.

3.1.8. Days to germination

There was no statistical significance in the number of days required for germination ($F_{7,64} = 1.37$, $p = 0.23$) (Fig. 5B) nor was it related to length of time *in vitro* cultivation ($R = -0.22$, $p = 0.61$) (Fig. S5b).

3.1.9. Total hyphal length

Isolates produced significantly different amounts of hyphae ($F_{7,64} = 7.91$, $p < 0.001$) (Fig. 5C). There was not a significant signal of *in vitro* cultivation ($R = 0.23$, $p = 0.58$) (Fig. S5c). Two growth patterns were observed: intense branching close to the spore versus a long, single tube with limited branching farther from the spore (Fig. 6E and F).

3.1.10. Septa per unit hyphal length

Isolates produced significantly different numbers of septa per unit length of hyphae ($F_{7,64} = 5.11$, $p < 0.001$) (Fig. 5D) (min = 0, max = 0.28, median = 0.002). There was no significant, effect of cultivation on septa formation ($R = -0.44$ $p = 0.28$) (Fig. S5d). While all isolates were able to produce septa DAOM 240159 (>30 *in vitro* generations) had the highest number per unit length of hyphae (min = 0, max = 62, median = 13) and DAOM 229457 (>60 *in vitro* generations) had the lowest ability to form septa (min = 0, max = 137, median = 0).

3.1.11. Growth rate hyphae

Isolates exhibited different rates of growth ($F_{7,64} = 7.56$, $p < 0.001$) (Figs. 5E and 6C, D) ranging from min = 0.93 to max = 4793, median = 492.07 μm per day. There was almost no effect of *in vitro* cultivation on growth rate ($R = 0.3$, $p = 0.47$) (Fig. S5e).

3.1.12. Days to maturity

Isolates differed in the number of days before growing ceased. Some isolates grew continued to grow throughout the entire study, showing no signs of cessation at 30 days, whereas others had completed growth by 5 days. These differences were significant ($F_{7,64} = 3.93$, $p < 0.01$) (Fig. 5F) but were not explained by time *in vitro* ($R = 0.32$, $p = 0.44$) (Fig. S5f).

3.1.13. Spore formation

Interestingly, isolate (strain 9A2) (*In vitro* generation > 50) consistently produce spore-like structures, smaller than its actual spores (Fig. 7). In one case it formed multiple such formation in a raw that initially increased in size but then got stabilized to 23 μm diameter.

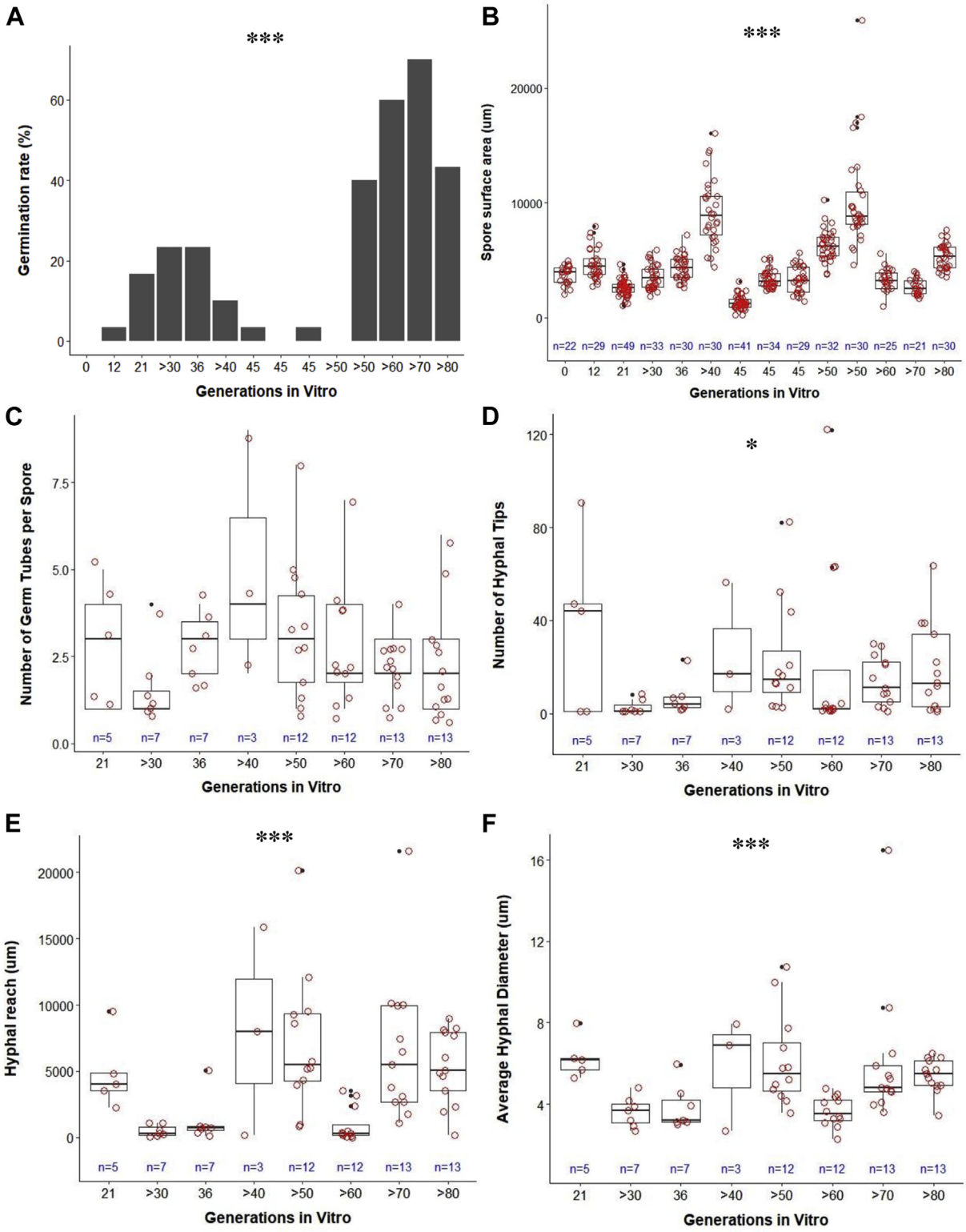

Fig. 4. Trait variation between isolates with at least 3 spores germinating. All measurements (except germination rate) are presented in micrometers (μm). (A) Germination rate (%), (B) spore size, (C) number of germ tubes per spore, (D) number of hyphal tips per spore, (E) hyphal reach, (F) average hyphal diameter. Red solid circles represent mean values and whiskers represent SE. Open circles represent individual observations. Stars signify the general significance of variation between all isolates ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. *Is trait variation related to* in vitro *cultivation?*

Considering the traits as a whole, we found that the number of generations *in vitro* did not correlate with the amount variation within each isolate (Spearman Rank correlation of matrices, $p = 0.379$).

4. Discussion

Asymbiotic traits for isolates of *R. irregulare* were highly variable among isolates. While intraspecific variation among AM fungi has been documented for *R. irregulare* in the symbiotic stage (Ehinger

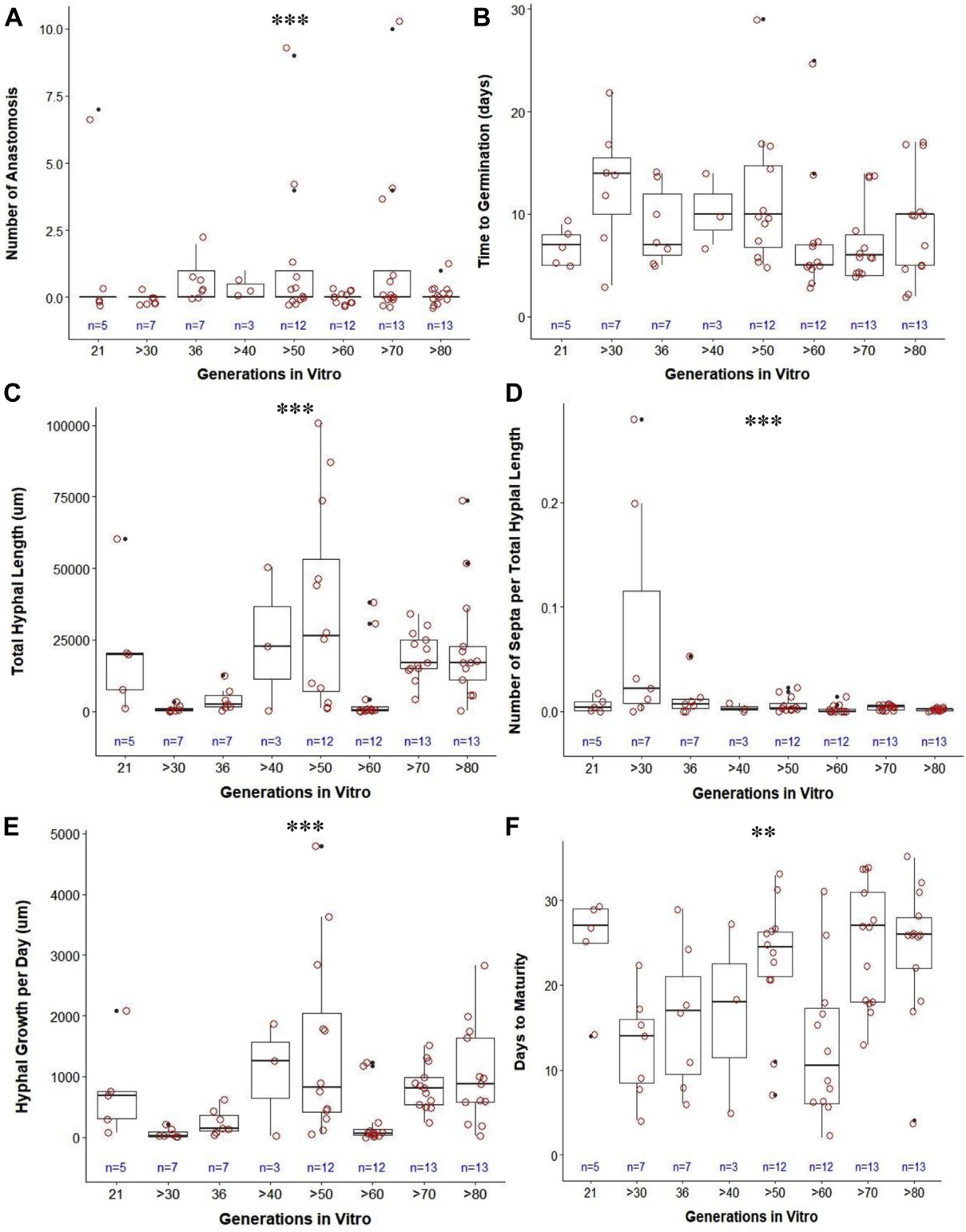

Fig. 5. Trait variation between isolates with at least 3 spores germinating. (A) Number of anastomoses, (B) days to germinate, (C) total hyphal length, (D) number of septa per total hyphal length, (E) hyphal growth per day, (F) days until maturity. Red solid circles represent mean values and whiskers represent SE. Open circles represent individual observations. Stars signify the general significance of variation between all isolates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2009; Hart and Reader, 2002; Munkvold et al., 2004), data on the asymbiotic stage is limited (Juge et al., 2002).

Total hyphal length differed significantly among isolates. Previous research showed that such differences can elicit with different stratification strategies even between spores of the same isolate (Juge et al., 2002). In our study such growth patterns were preserved within isolate. However, isolates producing the most hyphae were not the isolates with the greatest *hyphal reach*. Thus, ability to

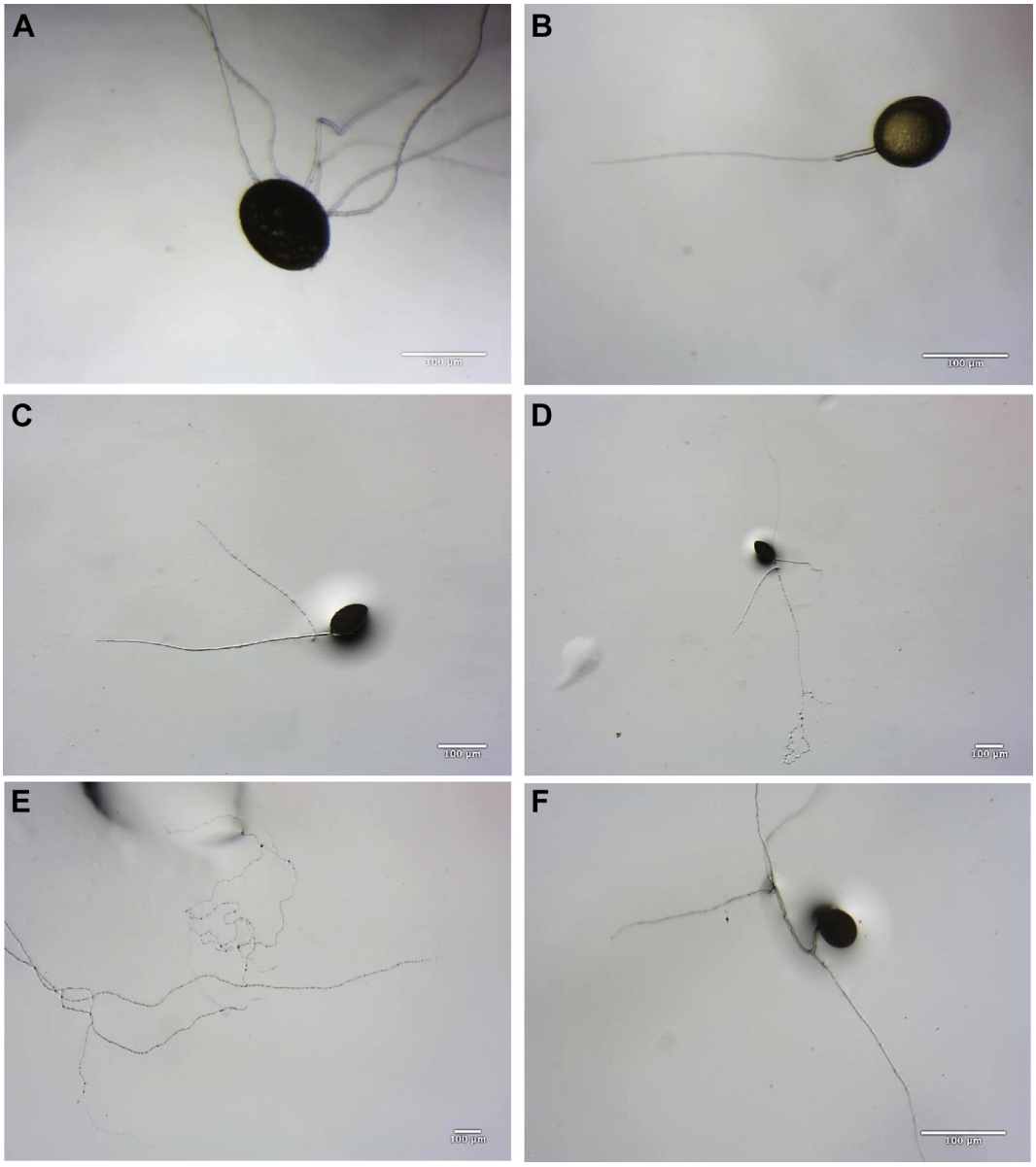

Fig. 6. (A) Multiple germ tubes originating from a single AM fungal spore. (B) Single germ tube originating from a subtending hypha. (C, D) Growth over time from a single AM fungal spore. (E) Complex type of growth with a high number of hyphal tips, branching and increased exploration area. (F) Simple type of growth with hypha spreading without intense branching. Bar = 100 μm.

produce large quantities of hyphae may not be a good predictor of successful host encounters particularly where hosts may be limited, such as disturbed, or early successional sites (Johnson et al., 1991).

As expected, *germination* increased with *in-vitro* propagation. In our study, isolates with the most generations *in vitro* had the highest rates of germination. Differences in germination rate can depend on multiple factors, including dormancy (Tommerup, 1983; Gemma and Koske, 1988), viability (Juge et al., 2002) and sterilization (Bharadwaj et al., 2012). Spore maturation can also affect germination success, especially for DAOM 197198 which needs 30–60 days to reach maturity (Marleau et al., 2011), however all spores in our study were fully mature.

We observed differences in dormancy among isolates. Dormancy is a commonly observed phenomenon in the Glomeromycota (Tommerup, 1983; Gemma and Koske, 1988). Stratification used in this study was sufficient to reduce spore mortality based on Juge et al. (2002), so observed differences in germination are likely due to isolate specific cues, and not spore viability. Regardless of the mechanism, our study shows that taxonomic identity is not a guarantee for successful *in vitro* propagation.

Contrary to our expectations, *in-vitro* propagation did not affect *spore area*, although there were significant differences among the isolates. Although *in vitro* cultivation can lead to smaller propagule in other situations (Calvet et al., 2013), it is not universal. Rather, spore size may be highly conserved, and changes may require more than 80 generations to manifest.

To our knowledge, this is the first study reporting germ tube number variation within *R. irregulare*. While there was no statistical difference in the number of germ tubes among isolates, there was variation observed. Sward (1981) observed variation in the number of germ tubes in a single isolate of *Gigaspora margarita*, with the majority of spores forming a single germ tube but, in some cases,

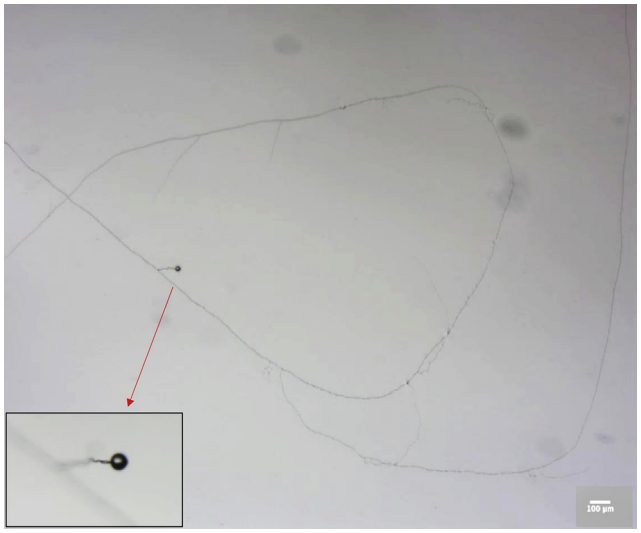

Fig. 7. Spore like formation observed consistently during the asymbiotic growth of *Rhizoglomus irregulare* (strain 9A2). Bar = 100 μm.

multiple germ tubes (up to 12). Juge et al. (2002) reported that one or more germ tubes can emerge from *R. irregulare* but did not report numbers.

To our knowledge, this is the first evidence that AM fungal *germ tubes can produce spores* in the absences of a functional mycorrhiza. In our study, this was restricted to strain 9A2, and was not related to time *in vitro*. Similar structures, characterized as spores, have been described, but only during synchronized growth with the bacterium *Paenibacillus validus* (Hildebrandt et al., 2002). Those spores were later proved to be fertile and able to colonize roots (Hildebrandt et al., 2005). Whether or not the structures we observed function as storage units, or in fungal reproduction or fitness, requires further research.

Isolates differed significantly in the *frequency of anastomoses* but did not depend on the time *in vitro* cultivation. Anastomosis in AMF has been reported only intraspecifically (de la Providencia et al., 2005). Variation in the ability to form anastomoses has been shown before within isolates of *R. irregulare* (Giovannetti et al., 1999), but our study shows that it may be a discrete, rather than continuous trait, even within a species. We know little about the function of anastomosis in AM fungus, and while it can be an important mechanism affecting multiple functions (Chagnon, 2014), it is usually viewed as disturbance resistance mechanism or as a mechanism allowing for genetic exchange within a mycelium (Sanders and Croll, 2010; Young, 2009). Clearly, some of our isolates had lost, or never developed, the ability to anastomose. Our results show that there is wide variation in this trait even among conspecifics, which could result in different ecological outcomes in natural systems.

We found no support for our hypothesis that increased *in vitro* propagation results in reduced septa formation. AM fungi are described as coenocytic, septa are not uncommon in germ tubes, and symbiotic hyphae (Powell, 1976; Mosse, 1988; GIOVANNETTI et al., 1993; Bago et al., 1998; de la Providencia et al., 2005). In our study, septa were always associated with cytoplasm retraction at hyphal tips. Cooke et al. (1987) described a similar phenomenon which he described as a survival strategy, called “clean septa” zones after Bago et al. (1998). Isolates capable of forming septa in our study may be more disturbance tolerant than other isolates, leading to dominance in disturbed scenarios. Alternatively, septation followed by apoptosis could mean a rapid re-distribution of readily available resources for the fungus making that isolate more efficient in low nutrient environment.

In vitro propagation had a small but significant effect on *days to maturity*. Such a difference may result in dramatic fitness disparity among isolates. For example, in stressful conditions a spore that remained viable for 30 days is much more likely to encounter a host compared to an isolate that stops growing after five days. In our study, isolates would have differential success in situations where hosts were infrequently distributed. Such differences may be related to saprobic ability. Germ tubes have limited saprophytic ability (Azcon-Aguilar and Barea, 1985; Giovannetti et al., 1996; Azcón-Aguilar et al., 1999) differences in germ tube lifespan may be an indication of differential saprophytic abilities.

4.1. *Is trait variation related to* in vitro *cultivation?*

Dormancy was strongly affected by *in vitro* propagation. This makes sense as only spores capable of germinating in TRC will be selected for propagation. However, there was little evidence that other traits were affected by the length of time *in vitro* propagation for *R. irregulare*. *In vitro* propagation may represent less of a selective force for *R. irregulare*, as this species has been widely characterized as ruderal – it may already be well adapted to ruderal conditions of TRC, regardless of its degree of cultivation (Declerck et al., 2005; Gerdemann and Nicolson, 1963; Ohsowski et al., 2014). Thus, it may be a chicken or egg scenario: since ruderal traits are desirable qualities for *in vitro* propagation, an isolate will have a higher chance of being used for *in vitro* propagation if it displays a more ruderal life history strategy (Berruti et al., 2016). However, TRC propagation may still represent strong selection pressure on other fungi, especially those that depend exclusively on spores for germination. Fungi in the Glomeraceae (including the isolates in this study) germinate preferentially from hyphal fragments (Hart and Klironomos, 2002), thus effects of *in vitro* growth on spore traits may be under more relaxed selection pressure than for fungi that reproduce exclusively by spores.

Perhaps the most important reason we may have failed to detect a cultivation effect is that our ‘low cultivation’ isolates were limited by dormancy (<3 spores germinated) and were not included in our statistical analysis along with the wild isolate which failed to germinate. Thus, we were not able to test for a full cultivation gradient. Instead, our gradient was limited to isolates which had already been exposed to multiple generations of *in vitro* cultivation. It may be that uncultivable fungi exhibit more stress tolerant, or competitive, traits. But as most fungal traits are difficult to study *in situ*, we do not yet know (Ohsowski et al., 2014). While the fact that the wild isolate or the early AM lineages did not germinate is a limiting factor for our study, nevertheless it is extremely relevant ecologically.

Finally, we looked only at asymbiotic traits, but AM fungi are obligate symbionts, even in the asymbiotic stages, host presence can evoke a suite of responses in a germinating fungus (Becard and Piche, 1989; Giovannetti et al., 1993). A necessary next step is to examine trait variation among isolates in the presence of a host. Whether inter-isolate variation important in other life stages is essential for understanding the consequences of *in vitro* cultivation on AM fungi.

4.2. *Significance of intra-specific asymbiotic trait variation*

In addition to *in vitro* cultivation, our isolates differed in terms of geographic origin and habitat type. Although there is little evidence for endemism among the Glomeromycota, there is evidence that not all taxa are cosmopolitan (Davison et al., 2015), thus there exists potential for locally adapted ecotypes with functional differences

(Antunes et al., 2011). In our study, we cannot completely separate the effect of cultivation from geographic origin. It may only be possible to do so if the effects of cultivation are significantly larger than intra-isolate variation. Future examination of isolate variation by controlling for geographic origin variation is needed to further elaborate differences among isolates.

Recently, Savary et al. (2018) categorized *R. irregulare* as four distinct genetic groups and argued that morphology and function are preserved within genetic groups, regardless of biogeography (even across continents). In our study DAOM 197198 (group 4 after Savary et al., 2018) and DAOM 240721 (also group 4 after Savary et al., 2018) differed significantly in most of the traits examined in this study. Moving forward, it will be important to understand the extent and drivers of intraspecific (and intra-isolate) variation for a complete understanding of AM in natural systems.

5. Conclusions

Our study provides evidence for extreme life history trait variation within an AM fungal species during asymbiotic growth. The observed variation during the asymbiotic growth might reflect important functional and morphological differences during the symbiotic stage. Future research into the extent of locally adapted genotypes of AM fungi is urgently needed to understand the most ecologically relevant level of genetic variation among AM fungi.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.01.005.

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