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

Vasilis Kokkoris*, Miranda M. Hart

Department of Biology, University of British Columbia, Okanagan Campus, 3333 University Way, Kelowna, BC, V1V 1V7, Canada

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In vitro propagation of AM fungi using transformed root cultures (TRC) is commonly used to obtain pure AM fungal propagules for use in research and industry. Early observations indicate that such an artificial environment can alter traits and function of AM fungi over time. We hypothesized that increased *in vitro* cultivation may promote ruderal strategies in fungi by enhancing propagule production and reducing mutualistic quality. To examine the effect of *in vitro* cultivation on the trait and function of AM fungi, we inoculated plants with 11 *Rhizoglomus irregulare* isolates which fell along a cultivation gradient spanning 80 generations. We harvested plants at 10, 20 and 30 d post inoculation to observe differences in fungal and plant traits post infection. *In vitro* cultivation led to increased spore production but reduced plant shoot phosphorus. Our results indicate that *in vitro* propagation may indirectly select for traits that affect symbiotic quality.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are important plant symbionts that interact with the majority of land plants including many economically important crops (Redecker et al., 2000; Smith et al., 2008). Their ability to improve plant growth via enhanced nutrient uptake led to their commercialization and use in agriculture (Berruti et al., 2016; Gianinazzi et al., 2010; Gianinazzi and Vosatka, 2004). In order to have a sustainable industry based on AM fungi, it is necessary to produce large numbers of contaminant free propagules. Because AM fungi are obligate root endophytes, *in vitro* cultivation of AM fungi using transformed root cultures (TRC) is necessary.

TRC propagation allows AM fungi to be cultivated under sterile, laboratory conditions through the use of a genetically modified host (St-Arnaud et al., 1996). Such hosts are called “hairy root cultures” and are modified to grow absorptively, without the need of a shoot (Willmitzer et al., 1982). While high quantities of contaminant free propagules can be produced via *in vitro* cultivation (Rosikiewicz et al., 2017), only limited AM fungal species have been produced in this way, perhaps due to their ability to germinate well after spore surface sterilization. Currently, *Rhizoglomus irregulare* (syn. *Rhizophagus irregularis*) is the most common species in commercial inoculum production (Rosikiewicz et al., 2017).

What effect does TRC have on isolates that are cultivated long term? *In vitro* cultivation represents a highly artificial environment which provides a stable growth environment without stress and disturbance (Fortin et al., 2005). Such a static environment can lead to reduced trait expression range (Wyss et al., 2016) and could also promote a ruderal behavior (Chagnon et al., 2013; Grime, 1977; Hart et al., 2001) as previously proposed (Kokkoris et al., 2019), since ruderal traits such as fast growth rates, early and abundant production of small spores and rapid hyphal turnover seem to align to *in vitro* propagation needs.

There is some evidence that *in vitro* cultivation selects for a more ruderal life history strategy in AM fungi by affecting multiple **fungal traits**. *In vitro* cultivation has been associated with increased germination rates via reduced or eliminated dormancy (Kokkoris et al., 2019) and reduction in reproductive unit size (Pawlowska et al., 1999; Calvet et al., 2013). These traits represent a ruderal strategy for rapid and high reproductive output (Chagnon et al., 2013; Egan et al., 2014). In vitro propagation has also been associated with reduced intraradical colonization (Calvet et al., 2013; Vimard et al., 1999) but despite the scarce presence of arbuscules

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* Corresponding author.
E-mail address: bill.kokkoris@ubc.ca (V. Kokkoris).

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in TRC (Fortin et al., 2002), there is profuse production of extraradical mycelium (ERM) and spores (Fortin et al., 2002) indicating a potential shift to extraradical over intraradical investment.

In vitro cultivation has also been shown to affect the function of the symbiosis. *In vitro* propagation has been associated with reduced nutritional benefit to hosts compared to *in vivo* produced propagules (Calvet et al., 2013). Such changes may be due to preferential resource allocation by AM fungi into propagules. Vesicles (Smith et al., 2008) and spores (Badri et al., 2016) represent a significant investment of resources. A copious propagule production strategy can lead to reduced nutritional benefit to the plant either by fungal sequestration of nutrients for spore production or by inducing carbon stress due to increased flow of lipids from host to the fungus.

Root morphological modifications may also be affected by *in vitro* propagation. Changes to root structure is commonly reported post colonization by AM fungi (Berta et al., 1995, 1993, 1990). Since root morphology is closely linked to overall plant function (Harper et al., 1991), such changes may affect the quality of the symbiosis. While changes in root modification varies between species (Berta et al., 1995), differences between isolates, to our knowledge, have not be reported. If time *in vitro* is causing isolates to become copious spore producers, increased carbon and phosphorus investment extraradically may cause alterations to root similar to alterations caused by nutrient stress, such as, increasing root branching in order to account for P deficiency and root volume and diameter due to carbon stress (Berta et al., 1995; Potters et al., 2007; Zhu and Lynch, 2004).

We predict that fungal trait and AM functional changes are associated with increased *in vitro* cultivation (Fig. 1). We examined the early stages of the symbiosis, using multiple isolates of a single species varying in the time they were propagated *in vitro*. We hypothesized that *in vitro* cultivation is associated to ruderal fungal strategies such as rapid and abundant propagule production and to root alterations that indicate a nutrient deficiency stress reaction on behalf of the plant.

2. Material and methods

To examine the effect of *in vitro* cultivation on the symbiotic traits, trait variation and function of *R. irregulare* (Schenck and Smith) we inoculated *Linum usitatissimum* (Flax, var. Bethune) with isolates of *R. irregularis* that varied in the number of generations grown *in vitro*. The plants were grown in sealed glass tubes in a growth chamber. We harvested at three different time points to observe the development of the symbiosis over time by measuring multiple intraradical and extraradical AM fungal traits as well as plant traits. We used a completely randomized block design (n = 8 per isolate and per harvest time, total 288 experimental units included a non-inoculated control).

2.1. AM fungi

We used 11 isolates of *R. irregulare* (Schenck and Smith), propagated along a chronosequence of *in vitro* propagation (Table 1). *R. irregulare* DAOM 197198 was obtained from AGTIV® FIELD CROPS

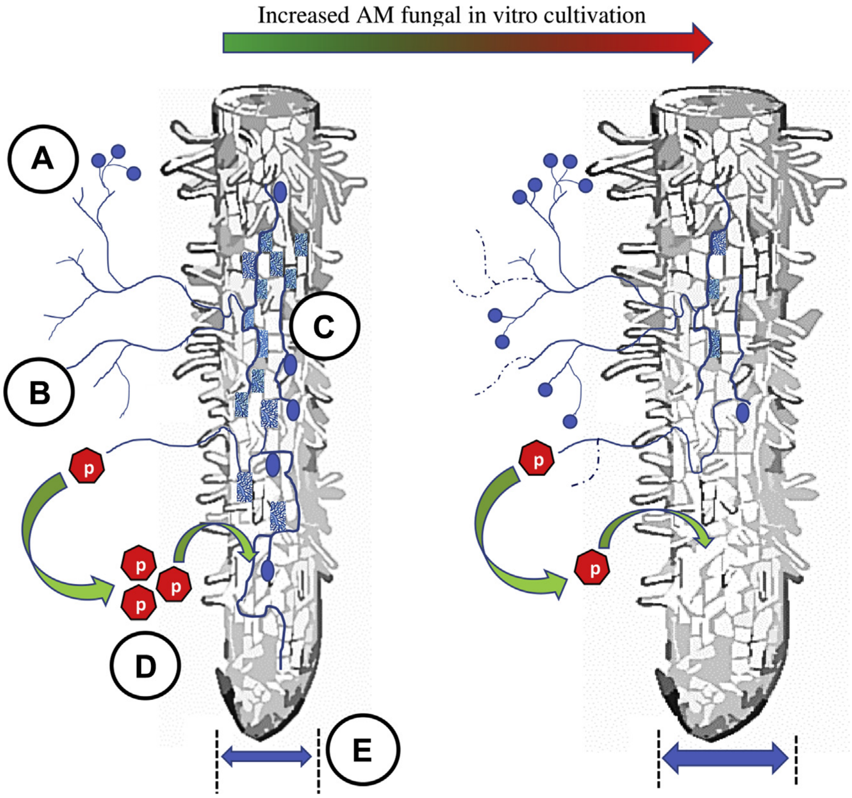

Fig. 1. Theoretical framework of trait and functional changes due to increased propagation *in vitro*. We hypothesized that propagation *in vitro* could lead to ruderal fungal strategies favouring spore production. **(A)** Larger spore numbers are expected with increased *in vitro* cultivation. **(B)** Increase in extraradical mycelium (ERM) is also expected with increased *in vitro* cultivation, due to the expected shift extraradically in order to support increased spore production. **(C)** Consequently, there should be a reduction in the number of intraradical structures with increased *in vitro* propagation (Vimard et al., 1999; Calvet et al., 2013), including arbuscules (Fortin et al., 2002), vesicles and hyphae (Kokkoris et al. unpublished data). **(D)** Reduced plant phosphorus benefit is expected with increased *in vitro* cultivation due to the increased fungal demand with increased sporulation. **(E)** Root alterations related to reduced benefit are expected with increased *in vitro* generations due to nutrient competition and increased carbon flow from the host to the fungus. Specifically, increased branching (increased root tip formation) and increased root volume is expected due to P and carbon limitations (Berta et al., 1995; 1993, 1990).

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

Generations *in vitro*	Species	Strain	DAOM reference	Habitat of origin	Isolate origin
>80	*R. irregulare*	/	197198	Maple forest	Canada
>70	*R. irregulare*	GC3	234328	/	/
>60	*R. irregulare*	CC4	229457	Fallow field	Canada
>50	*R. irregulare*	3086R	211734	Sandy shore	Canada
>50	*R. irregulare*	9A2	/	Greenhouse	Canada
45	*R. irregulare*	EC-16	241558	Oil sands	Canada
>40	*R. irregulare*	SD2	233751	Greenhouse	Belgium
36	*R. irregulare*	JSD-1	240721	Garden soil	Belgium
>30	*R. irregulare*	3545	240159	Uncultivated field	Canada
21	*R. irregulare*	Cuba8	/	Cultivated Field	Cuba
0	*R. irregulare*	GD50	/	Cultivated Field	Canada

(Premier Tech) and *R. irregulare* GD50 was obtained from Dr. Chantal Hamel at Agriculture and Agri-Food Canada, Swift Current Research and Development Centre. The remaining *R. irregulare* isolates were obtained from GINCO [http://www.mycorrhiza.be/ginco-bel/].

2.2. Spore isolation and sterilization

R. irregulare spores for eight out of 11 isolates were extracted from TRC after solubilizing the solidified with 1 % gellan gum (Alfa Aesar) M medium (Becard and Fortin, 1988). The gellan gum was solubilized using sodium citrate (10 mM, pH 6.0) at 35 °C that does not affect the viability of the AM propagules (Doner and Bécard, 1991). DAOM197198 spores were extracted from the commercial product AGTIV® FIELD CROPS (Premier Tech) by wet sieving and sucrose centrifugation using a modification of Gerdemann and Nicolson (1963) as described previously by Kokkoris et al. (2019). GD50 spores were extracted from a substrate (calcined clay) using the same method. Spores were then surfaced sterilized using the modified protocol of Mertz et al. (1979) as presented by Becard and Fortin (1988) and stored at 4 °C in a solution consisted of 200 mg/l streptomycin and 100 mg/l gentamycin for 20 d in order to break their dormant state and prevent spore mortality (Juge et al., 2002).

2.3. Seed sterilization and germination

Flax seeds were surface sterilized with ethanol 70 % for 1 min, followed by a 5 min step in 9.6 % bleach, and rinsed 6 times (1 min each time) in sterile distilled water. Sterile seeds when then placed on wet (with sterile DW water) filter 11 cm papers (VWR®) and kept sealed in petri dishes to germinate in a growth chamber (CONVIRON ADAPTIS CMP6010) at 24 °C (no light) 24-h cycle.

2.4. Experimental set-up

We used a novel method to ensure single AM fungal spore-plant inoculation. The method is described in Fig. 2. The sealed tubes that contained the germinated spore and seedling were kept in a growth chamber (CONVIRON ADAPTIS CMP6010) at 26 °C (daytime 16 h) followed by 22 °C (night 8 h) per 24-h cycle. Tubes were kept sealed during the entire duration of the experiment. Two of our isolates (GD50 and DAOM 211734) failed to germinate in absence of a host, similar to Kokkoris et al. (2019). In order to examine whether the spores inability to germinate was due to a negative effect of spore surface sterilization or due to lack of host signaling we chose the most healthy-looking spores (with clear yellowish color, no visible membrane damage or discoloration and with a dense granulated content) and proceeded with the inoculation as mentioned above.

2.5. Plant sampling and analysis

Plants were harvested 10, 20 and 30 d after the inoculation of the pre-germinated seed with the pre-germinated spore. Shoots were separated from the roots and the wet weight for each part was recorded. The entire root system was gently washed to remove any substrate particles and extraradical mycelium over a 43 μm pore size sieve. Material retained on the sieve were added back to the substrate (in order to avoid loss of extraradical mycelium) and the clean roots were scanned while suspended in water with a versatile large-format scanner (Epson expression 11000XL) using a grayscale at 400 dpi. Number of tips, forks, crossings, root surface area, length per volume and root volume was estimated using WinRHIZO Pro image analysis system (Regent Instruments Inc., Quebec, Canada, 2013). The dry shoots from the plants that were harvested at 30 d were dried at 60 °C for 48 h and then were pulverized and homogenized. % P in above ground tissue for the 30 d grown plants was calculated using acid digestion followed by color development method after Murphy and Riley (1962).

2.6. Root staining

Roots were stained immediately after scanning, to avoid the degradation of arbuscules (Orchard et al., 2017), using a slightly modified ink and vinegar protocol based on Vierheilig et al. (1998). Briefly, roots were cleared in 10 % KOH for 5 min at 90 °C and then rinsed three times with RO water. Cleared roots were stained for 3.5 min in 5 % black Sheaffer ink and vinegar solution at 90 °C. Roots were de-stained for 30 min in RO water with few drops of vinegar, and afterwards were stored in sterile RO water at 4 °C until further processing.

2.7. Quantification of AM fungal colonization

The entire root system of each plant was cut into approximately 2 cm pieces and mounted into multiple slides. Each slide was then examined under a compound microscope at 100× magnification (Bioimager BUM500FL). Each fragment that was identified as mycorrhizal was assigned a number on the slide and then the entire slide containing the fragments was scanned with a versatile large-format scanner (Epson expression 11000XL) using a grayscale at 400 dpi. Each mycorrhizal root fragment surface area and root volume was estimated using WinRHIZO Pro image analysis system (Regent Instruments Inc., Quebec, Canada, 2013). Multiple photos of each colonized root segments were taken using a Bioimager BRC-1600 camera on a Bioimager BUM500FL microscope 100× magnification and T Capture v 5.1 software. The pictures were then combined using a Panoramic Stitching Software (PhotoStitcher 2.0 ©2012–2015 Maxim Gapchenko) (Fig. 3). To calculate the percentage of root colonized by the fungus, each stitched fragment

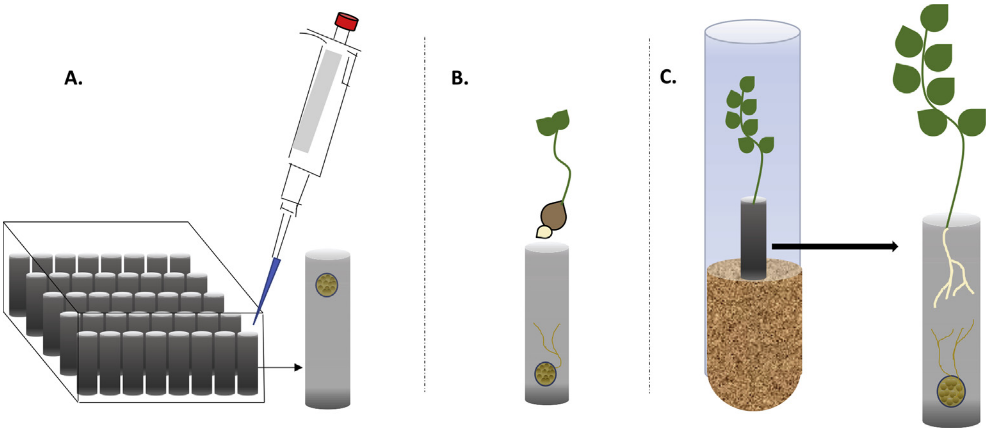

Fig. 2. Single spore inoculation method. **(A)** Magenta boxes (3 × 3 × 4″) were cut in order to fit in the field of view of a Nikon SMZ1500 dissecting microscope. The magenta box was filled with M-medium (Becard and Fortin, 1988) enough to not overcome the length of straws. Straws were cut into 4 cm and sterilized (15 % bleach solution followed by three time washing with autoclaved water for three minutes under aseptic conditions). Eighty one sterile straw pieces were imbedded in the solidified M medium using forceps under aseptic conditions. A surface sterilized single spore was imbedded in the center of each straw. Eighty one spores per isolate in each magenta box were used and a single box was assigned to each isolate. The boxes were closed with their lids and parafilm and stored upside down in a growth chamber (CONVIRON ADAPTIS) at 24 °C (no light). **(B)** After 15 d germination of spores was assessed by viewing the straws under a dissecting scope. 15 d is enough time for the isolates used to fully germinate (Kokkoris et al., 2019). A straw per tube, that contained a germinated spore, was removed from the magenta box and positioned with the spore on the bottom side. A pregerminated seed was added to the top side of the straw. **(C)** Glass tubes 25 × 200 mm were filled with 3 g of sterile vermiculite (45 min at 121 °C) and 15 ml of liquid M medium was added to them. A hole of similar diameter to the straw was opened in the wet vermiculite with a sterile glass rod. Finally, the complex of spore-seedling was then placed in the tube at the pre-opened hole leaving half of the straw exposed above the surface. The glass tubes where then sealed and remained sealed through the duration of the experiment.

photo was analyzed using Pro image color analysis (Regent Instruments Inc., Quebec, Canada, 2013) (Fig. 4). To calculate the root surface area colonized by the AM fungus the relative surface area of the classes that were generated after the color analyses were standardized per fragment, based on the root surface area of each mycorrhizal fragment. Using the total root surface area (projected) and the surface occupied by the fungus, we then calculated the total fungal coverage.

2.8. *Quantification of intraradical AM fungal structures*

All stained arbuscules and vesicles were counted manually via visualization of the stained root fragments at 200× magnification under a compound microscope for the entire root system of each mycorrhizal plant.

2.9. *Quantification of extraradical mycelium (ERM)*

The ERM was extracted and quantified following a slightly modified aqueous membrane filtration (AMFT) method by (Jakobsen et al., 1992). This method was chosen because it has been proved to yield higher hyphal biomass compared to other techniques (Green et al., 1994). Briefly the entire amount of vermiculite (3 g dry weight) was transferred to a blender and 150 ml of dH2O were added. The sample was blended at high speed for 30s. The suspended material was transferred to an Erlenmeyer flask and stirred for 60s using a magnetic stirrer. 30 ml aliquots were removed using a glass pipette and filtered through filter holders (1.2 μm pore size). The water was removed, and the hyphae were stained using 0.05 % trypan blue in lactoglycerol for 5 min. Stained hyphae was quantified under a Nikon SMZ1500 dissecting

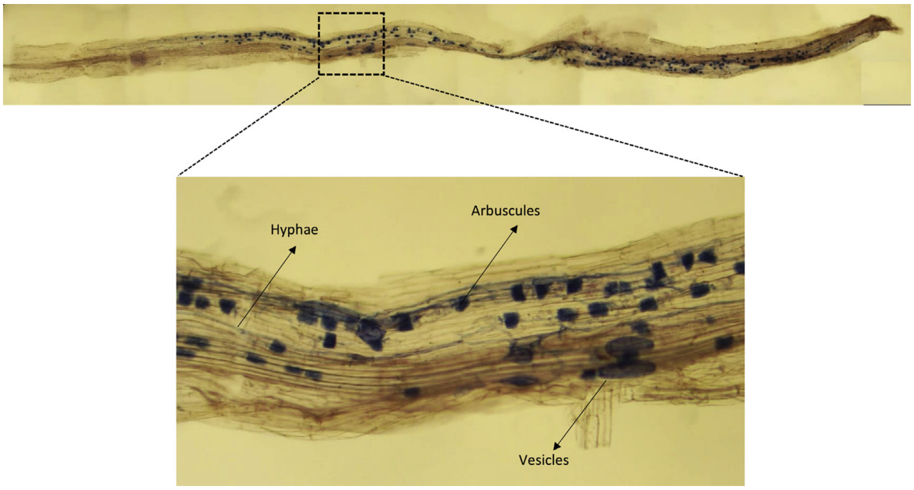

Fig. 3. Fully stitched mycorrhizal root fragment consisted of 27 individual photos. Multiple photos of each colonized root segments were taken at 100× magnification. The pictures were combined using a Panoramic Stitching Software (PhotoStitcher 2.0 ©2012–2015 Maxim Gapchenko). The stitched images were then digitally analyzed in order to calculate the fungal surface area within the roots using microphotography and imaging software.

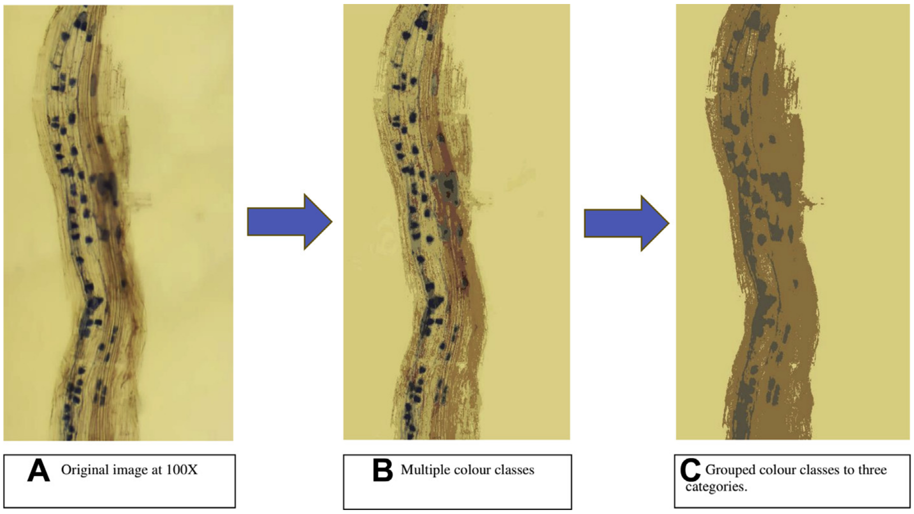

Fig. 4. Intraradical fungal growth determination using image analysis. Pro image color analysis (Regent Instruments Inc., Quebec, Canada, 2013) was used to determine the surface area of the fungus within the roots. Using the original picture **(A)** three categories (background, root and AM fungus) are defined by manually assigning 10 color classes to each category **(B)**. After the classes determination the software analyzes the image and groups the classes to three categories and provides the necessary information (e.g. surface area) for each category. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

microscope using a 3 mm square grid. The grid-line/hyphae intersection counts were converted to hyphal length (mm) using the Tennant (1975) equation, as presented by Juge et al. (2009).

$$[(11/14) \times (\text{intersect counts})] \times 3$$

2.10. Quantification of AM fungal spores

The vermiculite that was used to extract hyphae was not discarded at any time and used to also quantify the AM fungal spores. After extracting hyphae, each sample was returned to the blender and the spores were extracted following a slightly modified protocol from (Gerdemann and Nicolson, 1963) as described previously by (Kokkoris et al., 2019). In addition to spores that were extracted following the sucrose flotation centrifugation technique spores that were extracted alongside the ERM extraction and were visualized in the filters, were added to the final spore number.

2.11. Statistical analyses

For the statistical analysis we used Primer v6 with PRIMER + PERMANOVA (Anderson et al., 2008) and R studio (Version 1.0.136 – ©2009–2016 RStudio, Inc.).

2.11.1. Do isolates vary in traits?

We used PERMANOVA (Anderson et al., 2008) to test for difference among isolates for all measured traits (using 30 d data). We also examined for differences among the isolates looking at traits individually (at 10, 20 and 30 d) by using mixed effect linear model with block as random factor and isolate as fixed factor ("lme4" version 1.1–12, Fitting Linear Mixed-Effects Models) (Bates et al., 2015), when either raw or log-transformed trait values allowed for normal distribution of the residuals, or Generalized Linear Mixed model ("glm2" version 1.2.1, Fitting Generalized Linear Models) (Marschner, 2018) when the residuals of the model were not normally distributed even after transformation of the data (applied to 10 d arbuscule data, 30 d spore data using family "Poisson"). For traits with significant differences among isolates, we performed estimated marginal means (EMM's) analysis (package emmeans version 1.3.0) (Lenth et al., 2018) to evaluate the differences between isolates. We finally examined weather the within isolate variation differed among isolates, by testing for differences in the spread of data around centroids (PermaDISP) (Anderson et al., 2008).

2.11.2. Do isolates differ in function?

We used PERMANOVA (Anderson et al., 2008) to test for difference among plants for all measured traits (using 30 d data) inoculated with the different isolates. Similar to above we also used mixed effect linear model with block random factor and isolate fixed factor (lme4″ version 1.1–12, Fitting Linear Mixed-Effects Models) (Bates et al., 2015) to examine differences in plant phosphorus and biomass individually. For traits with significant differences among isolates, we performed estimated marginal means (EMM's) analysis (package emmeans version 1.3.0) (Lenth et al., 2018) to evaluate the differences between isolates. To assess differences in the amount of isolate function variation, we tested for differences in the spread of data around centroids (PermaDISP) (Anderson et al., 2008).

2.11.3. Is trait and function variation related to *in vitro* cultivation?

We performed linear regression between fungal and plant traits (using 30 d data), and generation *in vitro* (21–80 generations) to determine the relationship between trait variation and the number of generations *in vitro*.

3. Results

3.1. Do isolates differ in trait values along an *in vitro* cultivation gradient?

Isolates differed significantly for the traits measured overall at last harvest (30 d) ($p = 0.001$) (Fig. 5A, for pairwise comparisons see Table S1). Isolates exhibited similar levels of variation within all traits (dispersion) (P (perm) $= 0.123$) (Fig. 6A). Isolates GD50 (no *in vitro* generations) and DAOM 211734 (>50 *in vitro* generations) did not germinate and did not colonize the roots during our experiment and therefore were excluded from all analysis.

a) Spores

Spores were detected only after 30 d at low numbers. We found differences in spore formation at 30 d ($p < 0.001$) (Fig. 8C). Isolates

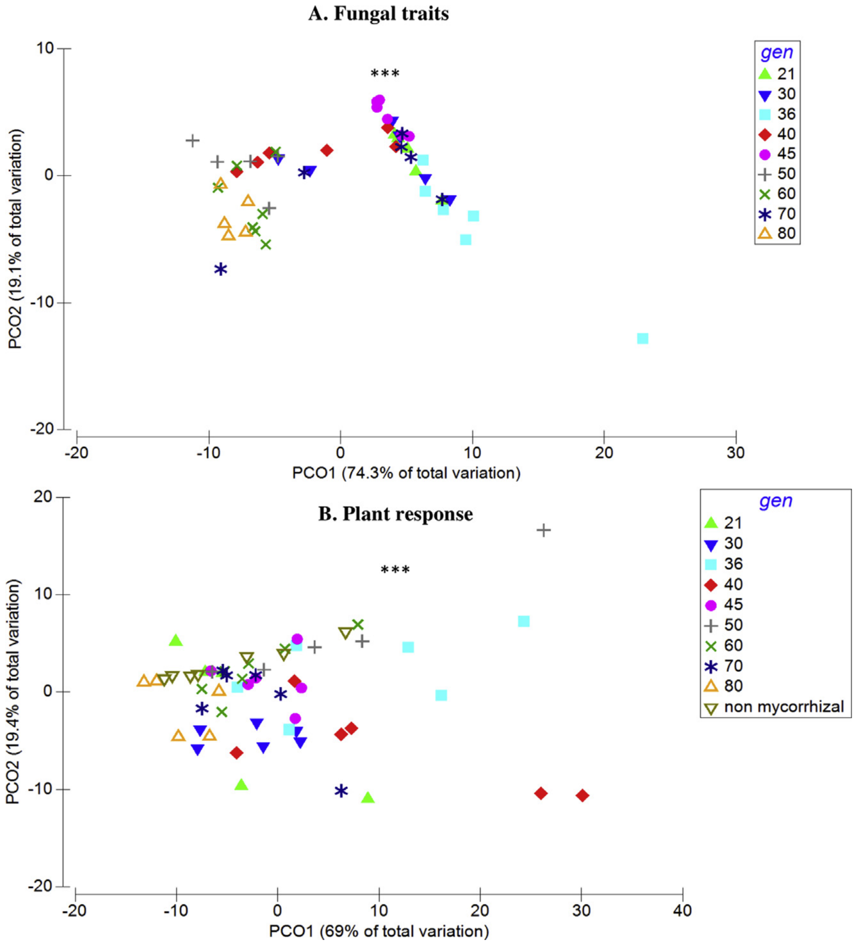

Fig. 5. Principal component ordination plot. PCO differentiating **(A)** AM fungal isolates based on the traits measured, and B) Inoculated plants with different isolates based on the plant traits measured. Different colored symbols reflect the various isolates differing in the number of generations propagated *in vitro*. Three asterisks signify statistical significance at $p < 0.001$.

9A2 (50 generations *in vitro*), DAOM 229457 (60 generations *in vitro*) and DAOM 197198 (80 generations *in vitro*) produced more spores compared to the rest of the isolates. We found evidence of increased spore formation with increased *in vitro* propagation ($R = 0.72$, $p < 0.05$) (Fig. 9A).

b) Intraradical colonization

Isolates exhibited different intraradical growth after 10 d ($p < 0.001$) (Fig. 7A) and continued at 20 ($p < 0.001$) (Fig. 7B) and 30 d ($p < 0.001$) (Fig. 7C). Isolates 9A2 (50 generations *in vitro*) and DAOM 233751 (40 generations *in vitro*) exhibited higher growth compared to other isolates starting at 10 d. Isolate DAOM 240721 (36 generations *in vitro*) grew the least on the course of 30 d. Variation in intraradical colonization was not associated with time in *in vitro* propagation ($R = -0.076$, $p = 0.96$) (Fig. S1A).

c) Arbuscules

We found differences in arbuscule abundance over time starting at 10 d ($p < 0.001$) (Fig. 7D) and continued at 20 ($p < 0.001$) (Fig. 6B) and 30 d ($p < 0.001$) (Fig. 7F). Isolates 9A2 (50 generations *in vitro*) and DAOM 233751 (40 generations *in vitro*) had the most arbuscules at all time points. Isolate DAOM 240721 (36 generations *in vitro*) had the fewer arbuscules after 20 and 30 d. Variation in arbuscule formation was not associated with time in *in vitro* propagation ($R = 0.13$, $p = 0.75$) (Fig. S1B).

d) Vesicles

No vesicles observed at 10 d. We found differences in vesicle formation over time starting at 20 ($p < 0.001$) (Fig. 7G) and continued at 30 d ($p < 0.001$) (Fig. 7H). Isolate DAOM 240721 (36 generations *in vitro*) had the fewer vesicles after 30 d while there were no differences among the rest of the isolates. Variation in vesicle formation was not associated with time in *in vitro* propagation ($R = 0.13$, $p = 0.75$) (Fig. S1C).

e) Extraradical Mycelium

We did not observe ERM at 10 d. We found differences in ERM formation over time starting at 20 ($p < 0.05$) (Fig. 8A) and continued at 30 d ($p < 0.001$) (Fig. 8B). Isolate DAOM 240721 (36 generations *in vitro*) produced the least ERM compared to the rest of the isolates. Variation in ERM was not associated with time in *in vitro* propagation ($R = 0.11$, $p = 0.77$) (Fig. S1D).

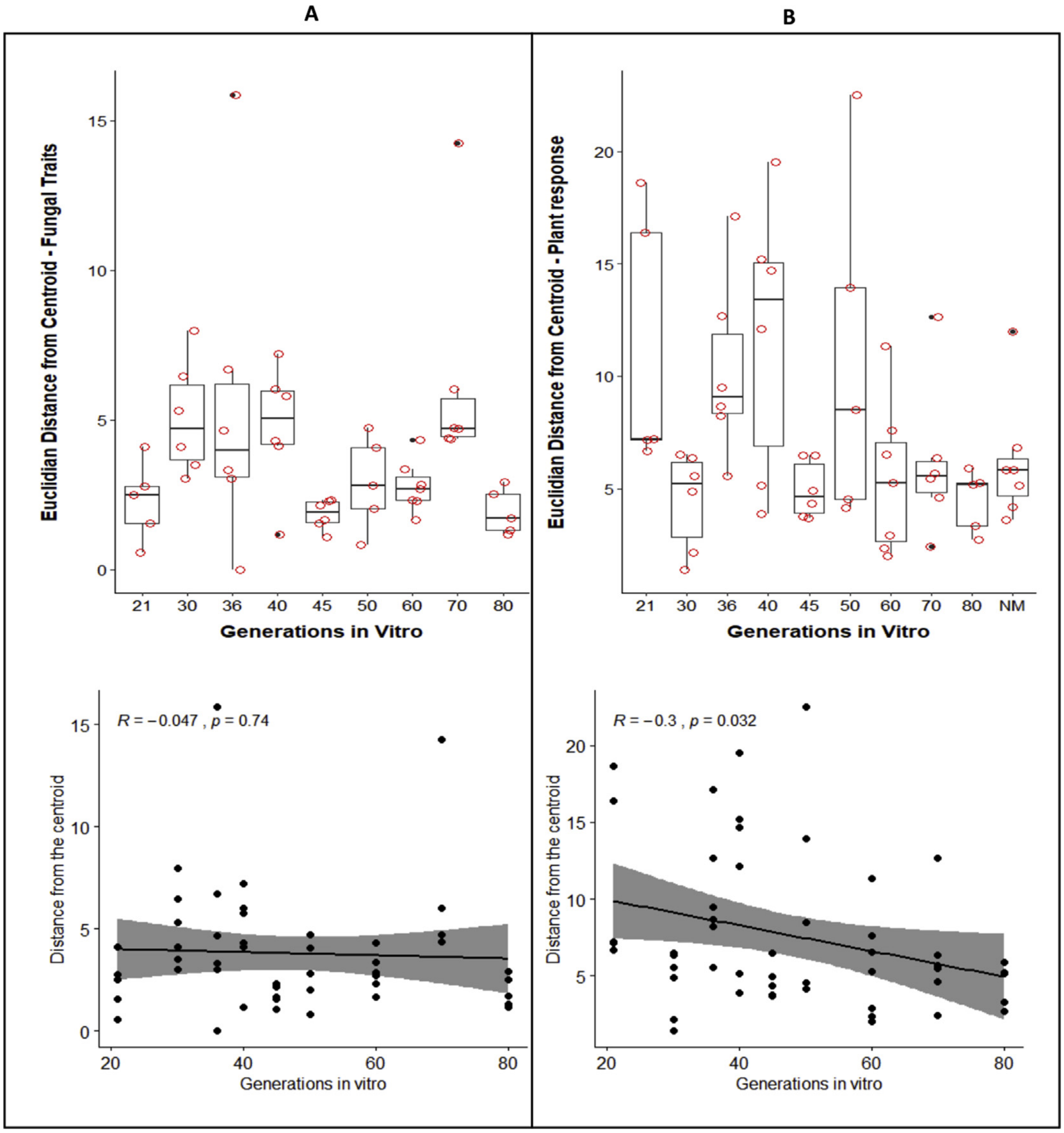

Fig. 6. Relationship between *in vitro* generations and fungal trait, and plant response variation. A) Trait variation within isolates and correlation of that variation to *in vitro* generation. (**B**). Plant response variation to inoculation and correlation of that variation to *in vitro* generation. Increased distance from centroid demonstrates increased trait variation within an isolate. For the boxplots the bold line within the boxplot represent median values and whiskers represent SE. Open circles represent individual observations and black dots represent outliers. For the regression circles indicate individual observations.

3.2. *Do isolates differ functionally along an* in vitro *cultivation gradient?*

There was a significant variation among plant measured traits after 30 d ($p = 0.001$) (Fig. 5B for pairwise comparisons see Table S2). There were significant differences in the plant trait variation after inoculation with different isolates (dispersion) ($p < 0.05$) (Fig. 6B). Isolates cultivated longer *in vitro* led to lower levels of plant trait variation among measured traits ($F = -0.3$, $p < 0.05$). For pairwise comparisons see Table S3.

a) Phosphorus

Shoot phosphorus decreased with increased *in vitro* propagation ($R = -0.28$, $p < 0.05$) (Fig. 9B). Percent shoot phosphorus varied significantly among the inoculated plants ($p < 0.05$) (Fig. 10A). Only isolate DAOM 233751 (40 generations *in vitro*) lead to higher percent shoot phosphorus compared to the control.

b) Plant Biomass

Increased *in vitro* propagation was marginally associated with an increased in host biomass ($R = 0.25$, $p = 0.072$) and shoot biomass ($R = 0.27$, $p = 0.053$) (Fig. S2). Total wet biomass and shoot wet biomass differed significantly among inoculated plants but there was no difference in root wet biomass (Fig. 10B, C, D). Inoculation with the isolate DAOM 197198 (80 generation *in vitro*) resulted in higher total biomass although the differences were significant only for plants inoculated with DAOM 233751 (40 generations *in vitro*) and DAOM 240721 (36 generations *in vitro*). Similarly, inoculation with the isolate DAOM 197198 (80 generation *in vitro*) resulted to higher shoot biomass compared to DAOM

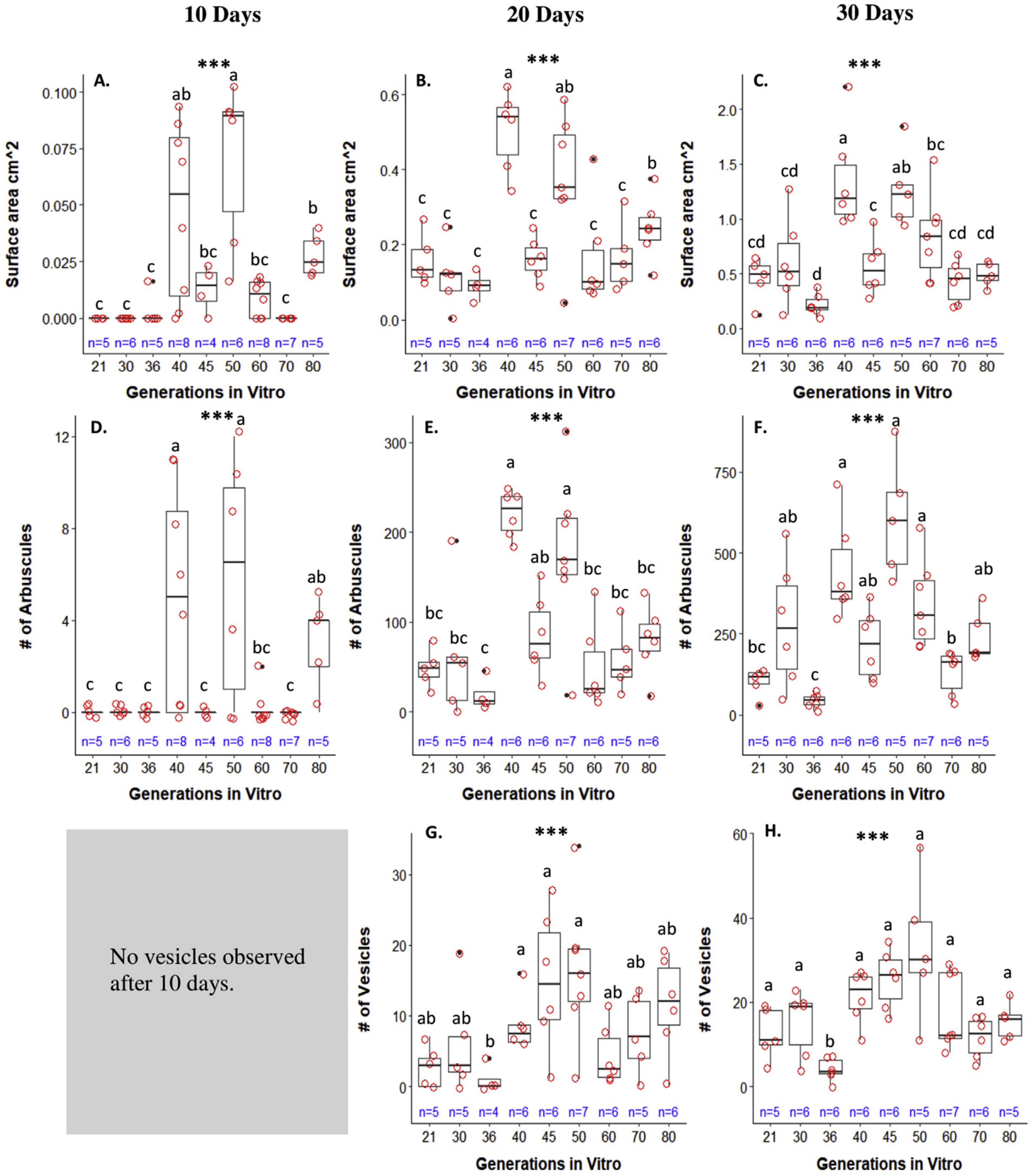

Fig. 7. Intraradical trait variation between isolates after 10, 20 and 30 d. (A, B, C) Intraradical fungal surface area (cm^2) over time, **(D, E, F)** number of arbuscules over time, **(G, H)** number of vesicles over time. Line represents median and whiskers represent SE. Open circles represent individual observations and black dots represent outliers. Stars signify the general significance of variation between all isolates (**$p < 0.01$, ***$p < 0.001$).

233751 (40 generations *in vitro*), DAOM 240721 (36 generations *in vitro*) and DAOM 241558 (45 generations *in vitro*).

c) Root morphology

Root morphology was altered significantly by the generation *in vitro* of the isolates. Specifically, number of root forks ($R = 0.38$, $p < 0.01$), number of root tips ($R = 0.27$, $p < 0.05$), root crossings ($R = 0.42$, $p < 0.01$), root volume ($R = 0.41$, $p < 0.01$) and root length to root volume ($R = 0.4$, $p < 0.01$) (Figs. S2 and S3) were increased with increased generation *in vitro* isolates.

4. Discussion

In vitro propagation of AM fungi led to increased spore production, reduced host response range, targeted root morphological alterations and reduced plant phosphorus benefit. To our knowledge this is the first study that examines multiple traits and

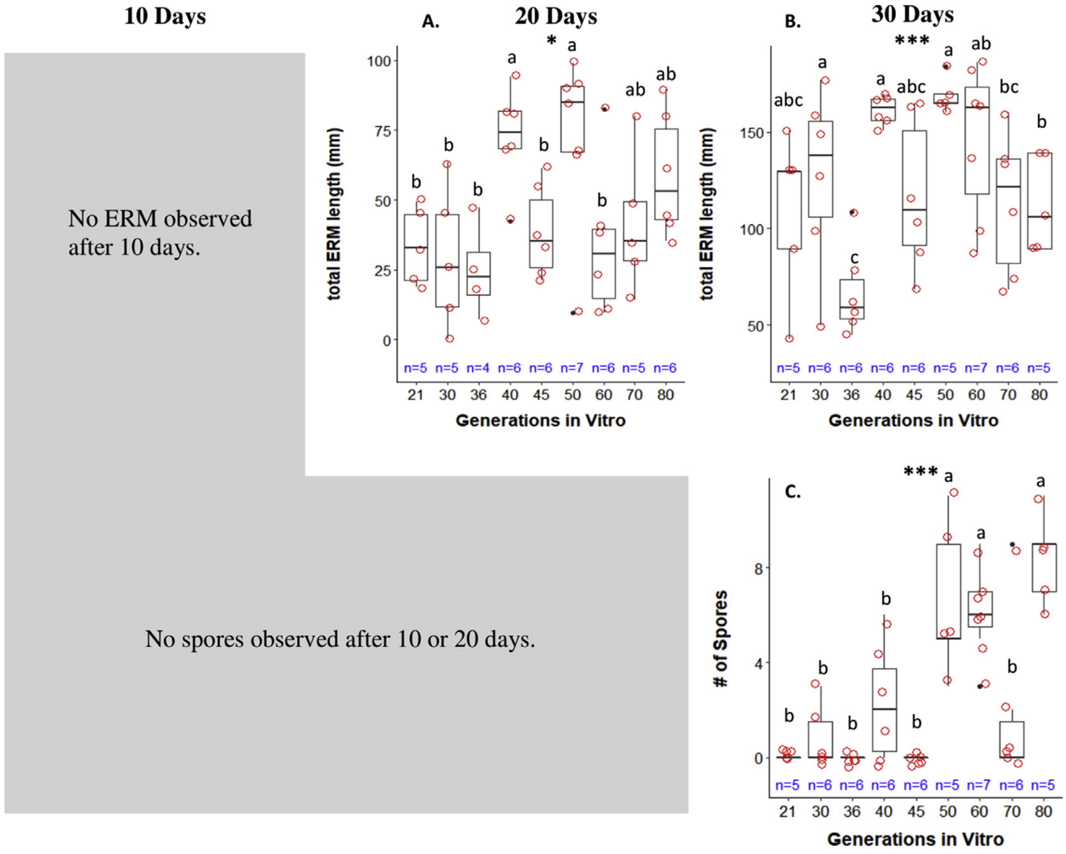

Fig. 8. Extraradical trait variation between isolates after 10, 20 and 30 d. (A, B) ERM quantity (mm) over time, **(C)** number of spores over time. Line represents median and whiskers represent SE. Open circles represent individual observations and black dots represent outliers. Stars signify the general significance of variation between all isolates (**p < 0.01, ***p < 0.001).

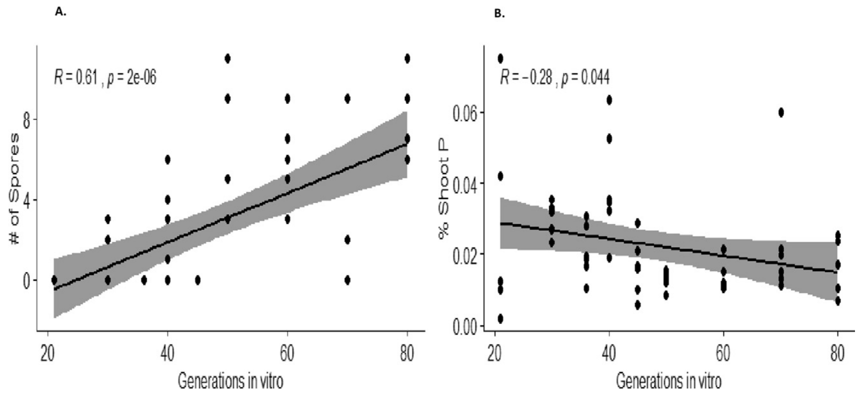

Fig. 9. Relationship between **(A)** spore number, **(B)** % shoot phosphorus content and *in vitro* generations.

functional variation of multiple isolates of *R. irregulare* representing an *in vitro* grown gradient, in a host over developmental stages.

4.1. AM fungal traits

We observed high levels of intraspecific variation among the isolates during the symbiotic stages. Similar levels of intraspecific variation were reported among isolates of *R. irregulare* during the asymbiotic growth (Kokkoris et al., 2019).

To our knowledge this is the first study that reports increased spore production with increased *in vitro* propagation supporting our hypothesis that time *in vitro* will lead to a preferential allocation of resources to spore production. *In vitro* propagation conditions are highly eutrophic (usually using the M medium as proposed by (Becard and Fortin, 1988). It has been shown that a nutrient rich environment with limited P, promotes spore production in AMF (Douds and Schenck, 1990; Douds, 1994) up to 65 000/plate (Douds, 2002). Differences in spore production between isolates have been previously observed (Campagnac and Khasa, 2014; Ehinger et al., 2009; Koch et al., 2004; Lee and Eom, 2015; Munkvold et al., 2004) but they were not examined in relation to *in-vitro* propagation. Calvet et al. (2013) reported reduction in spore size *in vitro* compared to *in vivo* cultivation but did not report spore numbers. Spores are large structures that require a lot

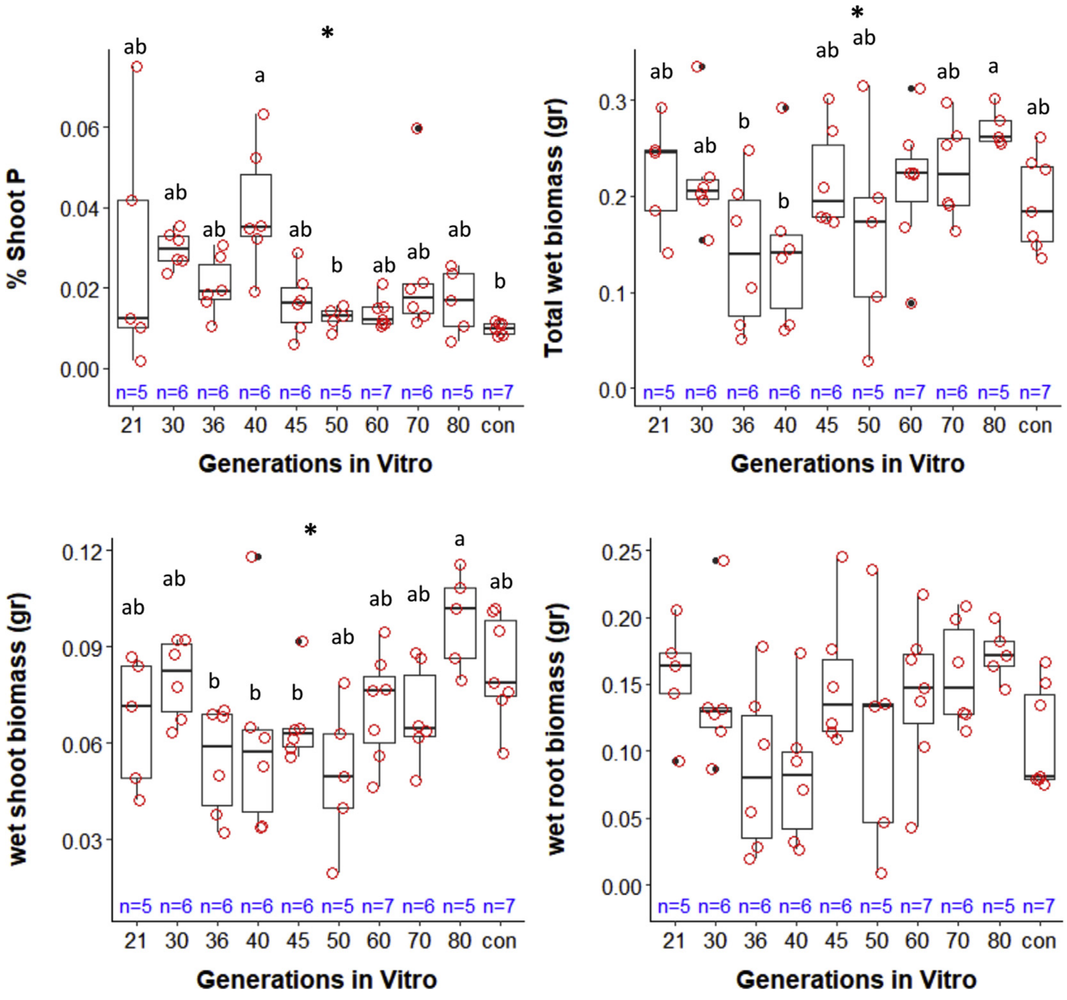

Fig. 10. Response trait variation in plants inoculated with the different isolates 30 d. (A) % shoot phosphorus, **(B)** Total wet biomass (gr), **(C)** Wet shoot biomass (gr), **(D)** Wet root biomass (gr). Line represents median and whiskers represent SE. Open circles represent individual observations and black dots represent outliers. Stars signify the general significance of variation between all isolates (*$p < 0.05$).

of resources to reach maturity. The thick chitinous walls (Bonfante-Fasolo, 1988; Grandmaison et al., 1988), the hundreds of nuclei per spore, that can reach up to 300 for *R. irregulare* (Marleau et al., 2011), the thousands of mitochondria, that can be more than 3000 per spore for that species (Badri et al., 2016) and the large amount of lipids present in the spores which consist up to 14 % of spore volume, bring into attention the costly nature of spores. Taken together with the shift to an increased spore production strategy with increased *in vitro* propagation observed in our study, indicates such isolates might compete for resources with the host leading to less beneficial interactions.

To our knowledge, this is the first attempt to quantify the entire intraradical growth via microphotography. While the majority of studies use observational microscopic approaches that estimate the colonization in a root subsample, e.g. (Trouvelot, 1986; McGonigle et al., 1990) we were able to quantify the stained surface area of the fungus within the entire root. We did not find support for our hypothesis that time *in vitro* cultivation will lead to reduced intraradical growth. Rather, we found significant variation in the intraradical growth between the isolates. Past studies showed that such differences between isolates can elicit important differences in plant response (Hart and Reader, 2002; Munkvold et al., 2004). Because our inoculation approach facilitated physical contact between the host plant and the germinated spore, differences in rate of colonization among isolates are likely due to variable growth rate, appressoria formation ability (Logi et al., 1998) and root penetration speed of the isolates (Brundrett et al., 1985) rather than to spatial stochasticity.

We did not find support for our hypothesis that increased *in vitro* cultivation will cause a reduction in arbuscule number since the number of arbuscules did not change with generation *in vitro*. While we observed differences in the number of arbuscules between the isolates in 10 and 20 d those differences were eliminated after 30 d with the exception of only one isolate (36 generations *in vitro*) that had significantly lower growth in all traits. Our results agree with that of Koch et al. (2017), arbuscule number seem to be a preserved trait between isolates at least at the early establishment of the symbiosis.

Similar to arbuscules, we did not find support for our hypothesis that increased *in vitro* cultivation led to reduced vesicle formation. Vesicles production was largely preserved among the isolates, and this has been previously reported (Koch et al., 2017). In our study, DAOM 240721 (36 generations *in vitro*) formed significantly less vesicles than the rest of isolates. It is possible that we would have observed more variation in vesicle production had we allowed the symbiosis to develop for longer. Vesicle production is highest in older roots (Vukicevich et al., 2019), so differences among isolates in their ability to form vesicles may only manifest in a mature symbiosis. It is also possible that differences may be more apparent under conditions more stressful than our experiment, as stress is associated with vesicle production (Hans-Werner, 1997).

In contrast to our hypothesis, ERM growth did not increase with time *in vitro* propagation, but differed significantly between isolates. Intraspecific variation of ERM has been previously observed (Hart and Reader, 2002; Koch et al., 2004; Munkvold et al., 2004). The ERM values in our study are similar to the values reported by Schubert et al. (1987) (0.06 m/g soil) for *Glomus clarum* when growing with clover (*Trifolium repens*). But ERM length can vary depending on the AM fungal species identity (Pepe et al., 2018) but also on host plant species identity and growing medium (Drew et al., 2006). Compared to other studies that examined the ERM growth of *R. irregulare*, ERM values in our study belong to the low range of reported values. For example, Battini et al. (2017) found that after 30 d' growth with Zea mays, ERM density of R. irregulare (isolate BEG87) was 22.3 m/g, while Pepe et al. (2018) reported 24 m of ERM per Cichorium intybus plant after 30 d. Low values of ERM were expected in our study since the plant–fungus interaction was initiated from a single spore and not from multiple propagules that can simultaneously colonize the host from multiple entry points and therefore produce a larger ERM faster. In our study, isolates with larger ERM were not associated with higher spore production. Additionally, although ERM has been linked to host benefit (Jakobsen et al., 1992) in our study higher ERM did not result in higher benefit either.

4.2. Asymbiotic stage vs symbiotic stage

The isolates that grew the most during the symbiotic stage, were those with higher hyphal growth during the asymbiotic stage (Kokkoris et al., 2019). The isolate DAOM 240721 (36 generations *in vitro*) that had the least growth in the symbiotic stage was among isolates with the smallest hyphal growth during the asymbiotic stage (Kokkoris et al., 2019). While it is known that in the presence of a host the fungal growth is significantly increased even when there is no actual contact due to chemical communication (Giovannetti et al., 1993), it has been suggested that this stimulation is occurring due to activation of mitochondria by strigolactones that may be an important component that enhances lipid catabolism (Besserer et al., 2006). If plant signals boost the genetically prefixed ability of each isolate, examining the fungal growth capacity during the asymbiotic stage or even additional traits such as nutrient uptake ability, could be informative about the symbiotic life stage of the fungus.

4.3. Plant response

We hypothesized that a ruderal strategy favouring spore production due to cultivation *in vitro*, would negatively affect the P transfer to the host and also induce a carbon stress to the host causing differentiated above to below ground biomass allocation and root morphology alterations. To our knowledge we are the first to show a reduction in the **phosphorus** benefit, reduction in plant trait variation and root morphological alteration with increased *in vitro* propagation. While variation in plant response among conspecifics has been previously observed (Munkvold et al., 2004) such changes have not been linked to *in vitro* propagation.

Supporting our hypothesis, we found a reduction in P to the host with increased *in vitro* propagation. Considering that we also observed an increase in spore production with time *in vitro*, such a result is not surprising. Spores harbor hundreds of nuclei and phospholipids (Marleau et al., 2011) which require significant amounts of phosphorus (Raven, 2013). While none of the isolates caused a negative plant response in the shoot phosphorus content in this study, the increased need for nutrients (for the fungus) with increased time *in vitro*, in order to sustain the higher sporulation rates, could lead to negative plant responses after inoculation in a later stage of the symbiosis. Negative responses using commercial inocula have been observed in the past (Christophersen et al., 2009; Facelli et al., 2010; Grace et al., 2009; Poulsen et al., 2005), especially under light limited conditions (Fitter, 1991). Such responses could be explained with the observed shift to a spore producing strategy.

Inoculation with isolates with more generations *in vitro* led to reduced variation among plant traits. If the unique environment of *in vitro* cultivation using TRC has the ability to reduce fungal genetic variation as previously observed (Wyss et al., 2016) then variation in plant response could also be reduced. For example isolate DAOM 197198 which is the most *in vitro* cultivated isolate has been shown to have reduced genetic variation compared to other isolates (Wyss et al., 2016) and is also homokaryotic (Chen et al., 2018). Our result suggests that inoculation with the isolate DAOM 197198 could lead to reduced variation in plant response due to reduction of genetic variation in the fungus (Limpens and Geurts, 2014).

Finally, in alignment with our hypothesis, we observed significant root morphological alterations with increased *in vitro* propagation cycles. Root morphological alterations have been observed after inoculation with different species but to our knowledge there are no reported results of differentiated responses among conspecifics. The changes include, increase in: root tip number, root volume and root length per volume. Important root morphological alterations caused by AM fungi have been reported in the past (Berta et al., 1995; 1993, 1990). *R. irregulare* led to increased root branching, root diameter and volume (Berta et al., 1995). While increased root branching is expected to increase the P uptake by the roots, AM fungal colonization causes reduction of meristematic activity of root tips which can lead to overall reduced P uptake by the plant (Berta et al., 1990). Furthermore, increased root diameter and volume can occur as a stress response (Haase and Rose, 1993) such as carbohydrate starvation (Berta et al., 1995). The marginally significant increase to above ground biomass with time *in vitro* alongside with the reduced phosphorus benefit observed, may further support a plant response to stress strategy. Based on the observed increased sporulation ability with time *in vitro*, an increased need for carbon allocated extraradically is expected. Increased fungal carbon needs, can lead to plant carbon limitations (Fitter, 1991), followed by a preferential resource allocation above ground in order to compensate for the carbon losses to the fungus.

5. Conclusion

In vitro cultivation over multiple generations resulted in reduced symbiont quality for this particular AM fungal species and host. Nevertheless, it is important to consider that the *R. irregulare* isolates used in this study originated from different habitats and geographic locations and therefore they may present a certain level of genetic variability. It is known that there is important genetic variation between isolates (Chen et al., 2018) that can be translated into functional variation partially driven by local adaptation (Antunes et al., 2011). But despite the extensive variation across isolates for many traits we were able to detect a cultivation effect. In order to further examine whether *in vitro* cultivation is primally responsible for the variation observed in our study, additional research that will analyze the level of genetic variation among strains is needed. We found that propagation *in vitro* leads to increased spore production and reduced phosphorus benefit to the host alongside with reduced range of plant responses and significant root system alteration. Our results indicate that *in vitro* propagation may have consequences for mutualism quality, which should be considered in mass production of AM fungi for use in industrial applications. More research is needed in order to identify whether the similar results are consistent among different hosts.

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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.2019.06.009.

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