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Research Article

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Posted Date: June 1st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9717525/v1

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Additional Declarations: No competing interests reported.

Inoculation timing affects inoculant performance and resident arbuscular mycorrhizal community

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Abstract 232 words

Main document 4466 words

Abstract

Arbuscular mycorrhizal fungi (AMF) are widely used as biofertilizers, yet field outcomes remain difficult to predict. One explanation is that AMF isolates differ in their ability to establish, compete with resident communities, and influence host performance. We tested how inoculation timing and inter-isolate variation affected inoculant establishment, host responses, and resident AMF communities using a greenhouse experiment with three conspecific isolates of *Rhizophagus irregularis*. Plants were inoculated either before exposure to field soil containing resident AMF or at transplant. We quantified inoculant abundance in roots and soil, plant growth and phosphorus responses, and resident AMF community composition.

AMF isolate identity was a stronger predictor of inoculant abundance and persistence than inoculation timing. Cuba8 maintained consistently high abundance across inoculation strategies, whereas DAOM197198 responded strongly to pre-inoculation but remained less abundant overall. These findings demonstrate substantial functional variation among conspecific AMF isolates in their responses to competition and colonization history.

Despite successful establishment, inoculation did not improve host biomass beyond the benefits already provided by resident AMF communities. Instead, most inoculation treatments reduced shoot phosphorus mycorrhizal response, suggesting that introduced AMF altered nutrient acquisition dynamics without enhancing host performance. Inoculation timing affected resident AMF communities, primarily through shifts in rare taxa.

Together, our results show that AMF inoculation outcomes can be affected by both inoculation timing and inter-isolate variation, making biofertilizer performance difficult to predict in soils containing established resident AMF communities.

Introduction

Rising demand for food production has led to a growing market for biofertilizer products. (Sudheer et al. 2023). Although biofertilizers have potential to reduce inputs in agriculture, there is no consistent evidence for their efficacy, especially in field conditions (Schütz et al. 2018; Herrmann et al. 2022). Arbuscular mycorrhizal fungi (AMF) are essential for the health and performance of most crops (Hamel 1996) and, as a result, are increasingly used as biofertilizers. However, AMF inoculation has produced mixed outcomes in field settings (Pellegrino et al. 2020; Arcidiacono et al. 2024) with some reporting no benefit to hosts(Rosa et al. 2020; Thomsen et al. 2021)). Large-scale analyses suggest the efficacy of AMF biofertilizers is highly context dependent (Lutz et al. 2023; Rog et al. 2025).

There are many factors that may limit biofertilizer performance, including the widespread availability of low-quality or inviable products (Salomon et al. 2022; Koziol et al. 2024). However, because most AMF developed as biofertilizers are selected for broad host compatibility and environmental tolerance (Kokkoris and Hart 2019a), their performance may be more strongly constrained by competitive interactions with resident AMF (Hart et al. 2018).

Inoculation timing may be critical for successful establishment of AMF biofertilizers (Verbruggen et al. 2013) by promoting inoculant establishment over residents. *Priority effect* describes is a well-documented phenomenon where the first propagule to arrive at an unoccupied root system has an advantage (Kennedy et al. 2009). For AMF, being first to arrive not only reduces competitive interactions with residents, but hosts may also suppress colonization of subsequently arriving fungi (Werner and Kiers 2015). Consequently, plants exposed to AMF biofertilizers before contact with resident AMF may have greater success, regardless of their intrinsic competitive ability. For farmers, the timing of inoculation may be all that is limiting the success of these products across different environmental and cropping contexts.

Several studies suggest that pre-inoculation can enhance AMF establishment in field soils. For example, pre-inoculation has been shown to increase colonization rates (Douds et al. 2016), improve mycorrhizal development and plant nutrient concentrations (Sorensen et al. 2008) and

81 increase crop yields (Snellgrove and Stribley 1986). However, these studies did not directly test
82 inoculation timing because they did not compare pre-inoculation with field inoculation. Thus, it
83 is unclear whether these benefits would have occurred had the inoculants been forced to
84 compete with residents.

85 Among the relatively few studies that have directly compared inoculation timing strategies,
86 results have been mixed. Pre-inoculation has been associated with increased AMF colonization,
87 improved root development, and greater nutrient uptake (Sohn et al. 2003). In contrast, other
88 studies have reported reduced colonization and host performance following pre-inoculation
89 (Koyama et al. 2019) compared to field inoculated hosts. Re-inoculating pre-inoculated
90 seedlings at the time of field planting has also been shown to increase AMF colonization and
91 improve root morphological traits, including root diameter and surface area (Tian et al. 2024).

92 Inoculation timing may influence not only inoculant establishment, but may also impact
93 resident AMF communities. However, even less is known about how inoculation timing affects
94 soil communities (Cornell et al. 2021). While reports of inoculation effects on resident AMF
95 communities are increasing (Basiru and Hijri 2022), no studies have specifically looked at the
96 role of inoculation timing in shaping these effects. Pre-inoculation has been showing to alter
97 resident AMF community composition (Mummey and Rillig 2009) while others report little
98 effect (Antunes et al. 2009) resident AMF. Similarly, coating seeds with AMF spores has been
99 shown to modify resident AMF communities (Ilyas et al. 2024), although this was not explicitly
100 testing inoculation timing.

101 To date, no studies have directly compared the effects of pre-inoculation vs. field application on
102 resident AMF communities. If biofertilizers gain a competitive advantage through priority
103 effects, pre-inoculation may have produce stronger effects on resident AMF communities.
104 Conversely, if inoculants are inherently superior competitors, then inoculation timing may have
105 little influence on the establishment of inoculants and their impact on residents. We
106 hypothesised that pre-inoculation prior to transplant into soils containing resident AMF would
107 increase inoculant abundance in roots and soil, leading to greater host response compared with

hosts inoculated at the time of transplant, and exert greater impacts on the resident AMF communities.

Materials and Methods

Experimental design

To test the effects of inoculation timing on AMF inoculant establishment, host performance, and resident AMF communities, plants were inoculated either before or at the same time as exposure to resident soil AMF. We conducted a randomized complete block design pot experiment (3 inoculation timing treatments × 3 AMF isolate treatments × 2 harvest times × 7 replicate pots = 126 experimental units, plus 14 non-inoculated controls; 140 total experimental units) in the Plant Growth Facility greenhouse at UBC Okanagan, Kelowna, Canada, from September 2022 to January 2023. Independent variables were inoculation timing, AMF inoculant identity, and harvest time. Dependent variables were AMF inoculant abundance in roots and soil, host plant growth response, and resident AMF community composition in soil.

Inoculation timing

Host plants were assigned to four inoculation treatments. In the *pre-inoculation* treatment, AMF spores were applied to a seeds in sterile growth medium (1 sand : 1 vermiculite : 1 Turface©) and grown for 6 weeks in a growth chamber (supplemented with liquid M-medium and grown) at which point visual root colonization was confirmed using ink–vinegar staining (Vierheilig et al. 1998)(Fig S10). Plants were transferred to greenhouse pots containing field soil. In the *co-inoculation* treatment, spores were introduced at the time of seedling transplant into soil containing resident AMF (n = 7). In the *combination treatment*, plants were inoculated at both germination and transplant (n = 7). Control plants received no inoculum (n = 7).

AMF identity

Three conspecifics of *Rhizophagus irregularis* were cultivated *in vitro* using Transformed Root Cultures (TRCs) (DAOM197198, CC4 and Cuba8). All isolates were obtained from the Canadian

Culture of Arbuscular Mycorrhizal Fungi (CCAMF), Agriculture and Agri-food Canada, Ottawa. Briefly, to obtain sterile propagules for inoculation, spores were extracted from plates using citric acid-sodium citrate buffer solution . Healthy spores were selected using microscopy inside a sterile laminar flow hood and placed on non-nutrient agar plugs. Each agar plug contained 100-200 spores and one plug was inoculated into each pot upon planting. Control agar plugs without AMF inoculant were also included (n=10).

Resident AMF community

We collected field soil containing natural AMF populations from an active orchard at the Agriculture and Agri-Food Canada Summerland Research and Development Centre (Summerland, BC). To re-establish the resident AMF community prior to treatment application, a trap culture was initiated using leek (*Allium ampeloprasum*), which was grown for eight weeks. Root systems were subsequently cleared and stained to confirm the presence of an active resident AMF community before inoculation (Vierheilig et al. 1998).

Plant Host

Plantago lanceolata L. seeds were surface-sterilized with 70% ethanol and 9.6% bleach, then stratified at 4 °C for five days before being sown into seedling trays filled with sterile peat. And thinned to a single individual per pot. Plants were maintained at 16 h day light and 20–30 °C. Irrigation was delivered via an individual drip system, with each plant receiving approximately 15 mL of water twice daily. Hosts were harvested at 8 and 16 weeks following exposure to field soil. At each harvest, shoots were cut at the soil surface and dried . Root systems were rinsed with tap water, dried, and stored at −20 °C. Bulk soil not adhering to roots was collected separately and stored at −20 °C for downstream analyses.

AMF quantification

Abundance of inoculated AMF in root and soil samples was quantified using ddPCR with isolate-specific primers and probes (Table S1). DNA was extracted from 0.25 g of root and soil using the DNeasy 96 PowerSoil Pro Kit (Qiagen, Hilden, Germany) and stored at −20 °C. To account for higher variability in root-associated AMF (Hart et al. 2026), two independent DNA extractions

and ddPCR assays were performed per pot. Reactions (24 µL) contained 12 µL ddPCR Supermix for Probes (no dUTP; Bio-Rad), 1.2 µL 20× primer–probe mix (final: 500 nM primers, 250 nM probe; IDT), 2.4 µL template DNA, and 8.4 µL DNase-free water. Droplets were generated using DG8 cartridges with 72 µL droplet oil (Bio-Rad) on a QX100 Droplet Generator, transferred to 96-well plates, and amplified on a C1000 Thermal Cycler under primer-specific conditions (Table S2). Droplets were read on a QX100 Droplet Reader and quantified with QuantaSoft (Bio-Rad). Thresholds were determined using *Define The Rain* (https://definetherain.org.uk/) based on serial dilutions of organismal controls (undiluted to 1000×), selecting the dilution closest to sample concentration. Each plate included ≥3 no-template controls and ≥3 positive controls (DNA obtained from our pure TRC for each isolate).

Plant host response and soil chemistry

To determine plant host response to treatments, mycorrhizal response (MR) was evaluated for total plant biomass, root:shoot ratio, shoot biomass, root biomass, and shoot phosphorus (P)

mycorrhizal response (MR) was evaluated using the following formula:

$$MR = \frac{A}{B}$$

where *A* is the response of a mycorrhizal plant minus the mean response of all non-mycorrhizal plants, and *B* is the mean response of all non-mycorrhizal plants (non-inoculated controls). The MR was determined for total plant biomass, root:shoot ratio, shoot biomass, root biomass, root:shoot ratio, and total shoot phosphorus (P) content.

Fresh plants collected were dried at 60°C for 48 h. Foliar phosphorus (n=5) was measured and analyzed at the Analytical Facility of Frind Estate Winery in West Kelowna, BC following the Bray-1 extraction protocol (Carter 1993). We analyzed ten (10) samples from each treatment at each harvest time for soil pH and total phosphorus (Jones 2017). Samples were processed and analyzed at the Analytical Facility of Frind Estate Winery in West Kelowna, BC.

Resident AMF community

DNA was extreacted from soil collected at 16 weeks and prepared for PCR amplification and Next Generation Sequencing (NGS) at the University of British Columbia Sequencing and Bioinformatics Consortium (SBC), Vancouver, BC. PCR conditions, library preparation, and bioinformatic analysis used are described in Supplemental Methods Appendix 1.

Bioinformatics

All bioinformatics were processed in QIIME 2 (v2024.10). Sequences were standardized to equal sampling effort at minimum sample depth of 4000 AMF sequences per sample (Fig. S3). A detailed description of the workflow can be found in Supplemental Methods Appendix 1

Statistical methods

Inoculant establishment and abundance

We used a hurdle framework to account for zero inflation in ddPCR data. For the first step, establishment (presence/absence) was analyzed using generalized linear models (GLMs) with a binomial error distribution and logit link. Fixed effects included inoculation timing, AMF isolate identity, harvest time, and their interactions. Next, inoculant abundance (copies per 20 µL well) was analyzed using generalized linear models with a Tweedie distribution and log link to accommodate zero-inflated, right-skewed data (Zhang 2013).

Plant mycorrhizal response and soil chemistry

Host responses were quantified as shoot biomass mycorrhizal response (ShootMR), root biomass mycorrhizal response (RootMR), shoot phosphorus mycorrhizal response (ShootPMR), soil phosphorus concentration (SoilP) and soil pH. Model assumptions were evaluated using residual diagnostic plots, Shapiro–Wilk tests of normality, and Levene's tests for homogeneity of variance (Figs. S4-S8)(Tables 9, 10, 14, 15). Each response was analyzed separately using linear models with AMF isolate identity, inoculation timing, harvest time, and their interactions as fixed effects. Type III ANOVA was used to assess main effects and interactions. Tukey-adjusted pairwise comparisons among inoculation timing treatments were conducted within each AMF isolate and harvest time combination when model terms were significant.

Resident AMF Community

We looked at the effects of Inoculation timing, AMF isolate identity and their interaction on resident AMF communities at 16 weeks (Harvest 4). Species richness, Shannon diversity and Pielou evenness using generalized linear models. Species richness was analysed with negative binomial distributions to account for overdispersion, while Shannon and Pielou evenness were analyzed using linear models because residuals were approximately normally distributed (Fig. S7-S9).

Resident AMF community composition was analyzed using amplicon sequence variant (ASV) abundance data. Bray–Curtis and Jaccard dissimilarity were calculated from the rarefied community matrix (4000 sequences/sample)(Fig. S3). The effects of AMF isolate identity, inoculation timing, and their interaction were tested using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. To evaluate whether differences in community composition were driven by heterogeneity in dispersion, homogeneity of multivariate dispersion (PERMDISP) was assessed using *betadisper* (999 permutations). All analyses were conducted in R using the *vegan* (Oksanen et al. 2022).

Principal coordinates analysis (PCoA) and SIMPER (Clarke 1993) were used to visualize changes in resident AMF community composition and identify taxa contributing to dissimilarity among treatments. Inoculation timing effects were visualized as fitted vectors on PCoAs derived from dummy-coded treatment variables, representing directional gradients of community association us with 999 permutations. SIMPER comparisons were restricted to contrasts between each inoculation timing treatment and the uninoculated control. For each comparison, Bray–Curtis dissimilarities were decomposed to quantify the contribution of individual taxa to overall community differences. Taxa were ranked based on their average contribution to dissimilarity across all pairwise sample comparisons, retaining the top 10 contributing taxa. Permutation tests (999 permutations) were used to assess the significance of group differences associated with the underlying dissimilarity structure. All analyses were performed with *vegan* (Oksanen et al. 2022).

To evaluate if differences among our inoculation timing treatments were driven by changes in the abundance rare taxa, we tested for treatment effects on the number or rare taxa (defined as taxa occurring in ≤20% of samples). Differences in rare taxa counts among treatments were evaluated using GLM with a negative binomial distribution and log link to account for overdispersion (Pearson's χ^2 = 2.21, p 0.001). Model significance was assessed using likelihood ratio tests (Type III ANOVA).

Data availability

All statistical analyses and visualizations were performed in R (v4.4.2). All workflows are archived at https://doi.org/10.5281/zenodo.20185822. DNA sequences and project metadata are archived in NCBI Sequence Read Archive PRJNA1464753.

Results

Inoculant establishment

Both roots and soil had identical levels of detection at both harvest times. Pre-inoculation (p = 0.0028) and combination (p = 0.019) increased establishment success of all isolates compared to co-inoculation (Fig. 1, Table 2). For Harvest 2, both CC4 and Cuba8 had 100% detection in root and soil, compared to 197198 which had establishment success for 77-91% of samples for Harvest 2 (Table 3). Because both CC4 and Cuba8 fully colonized at Harvest 2, this resulted in model overparameterization, so Harvest 2 was excluded from the analysis (dAIC = 54) (Table 1). At harvest 4, CC4 and Cuba8 establishment were both still higher than 197198 (p=0.004).

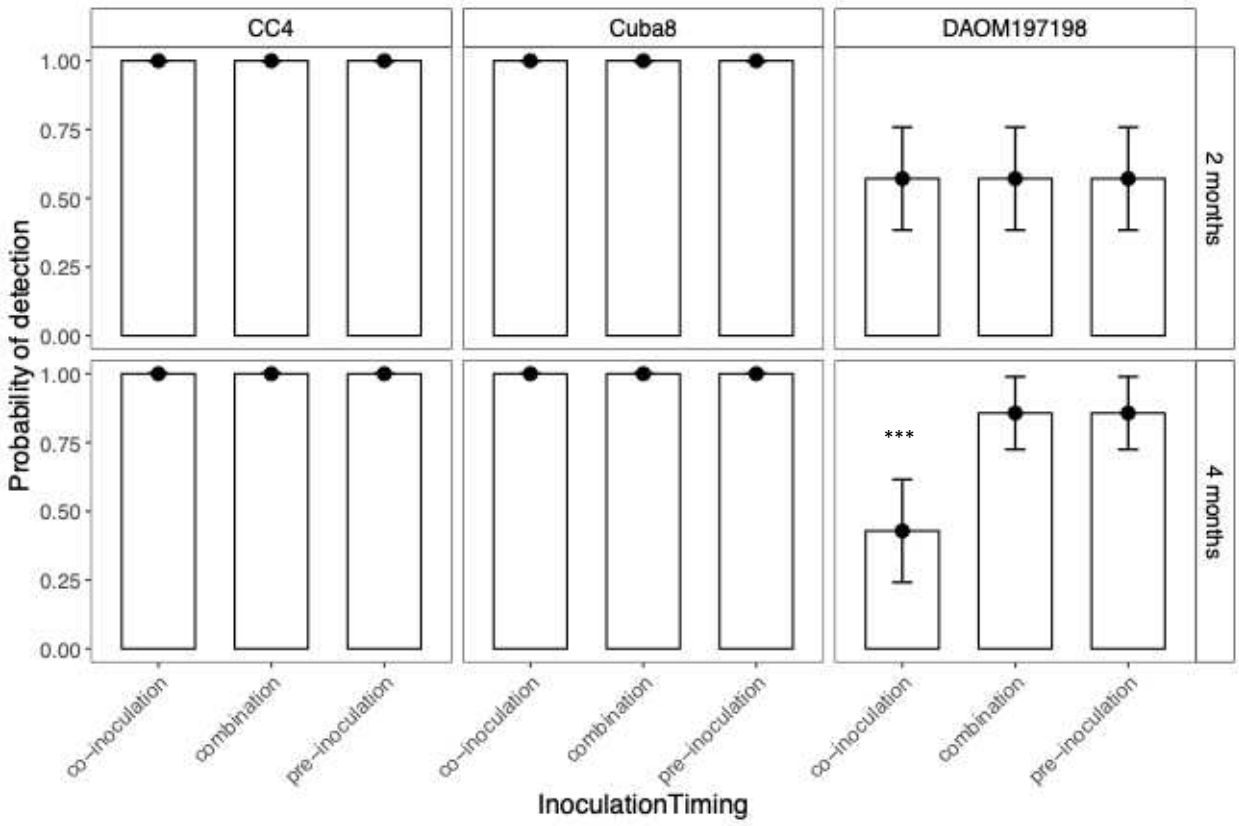


Fig. 1 Inoculant establishment in soil and roots at two months (top) and four months (bottom) post-inoculation for AMF isolates and inoculation strategy. Proportion of samples with successful inoculant establishment (detection > 0). Soils were inoculated with one of three arbuscular mycorrhizal fungal (AMF) (CC4, Cuba8 and DAOM197198) and introduced to hosts before exposure to soil (pre-inoculation), at the same time as exposure to soil (co-inoculation) or both (combination). Bars represent mean (SE) proportions of samples with detectable inoculant. Detection in roots and soil was identical.

a

Inoculant abundance

In roots, inoculation treatment interacted with Harvest time and AMF identity (Fig. 2, Table 4). Pre-inoculation (p = 0.014) and combination (p = 0.05) enhanced root colonization at Harvest 4. Pre-inoculation (p = 0.019) and combination (p = 0.002) enhanced 197198 abundance in roots more than other isolates. This effect disappeared at Harvest 4, with 197198 showing larger reductions in root colonization for both pre-inoculation (p = 0.05) and combination (p = 0.021) .

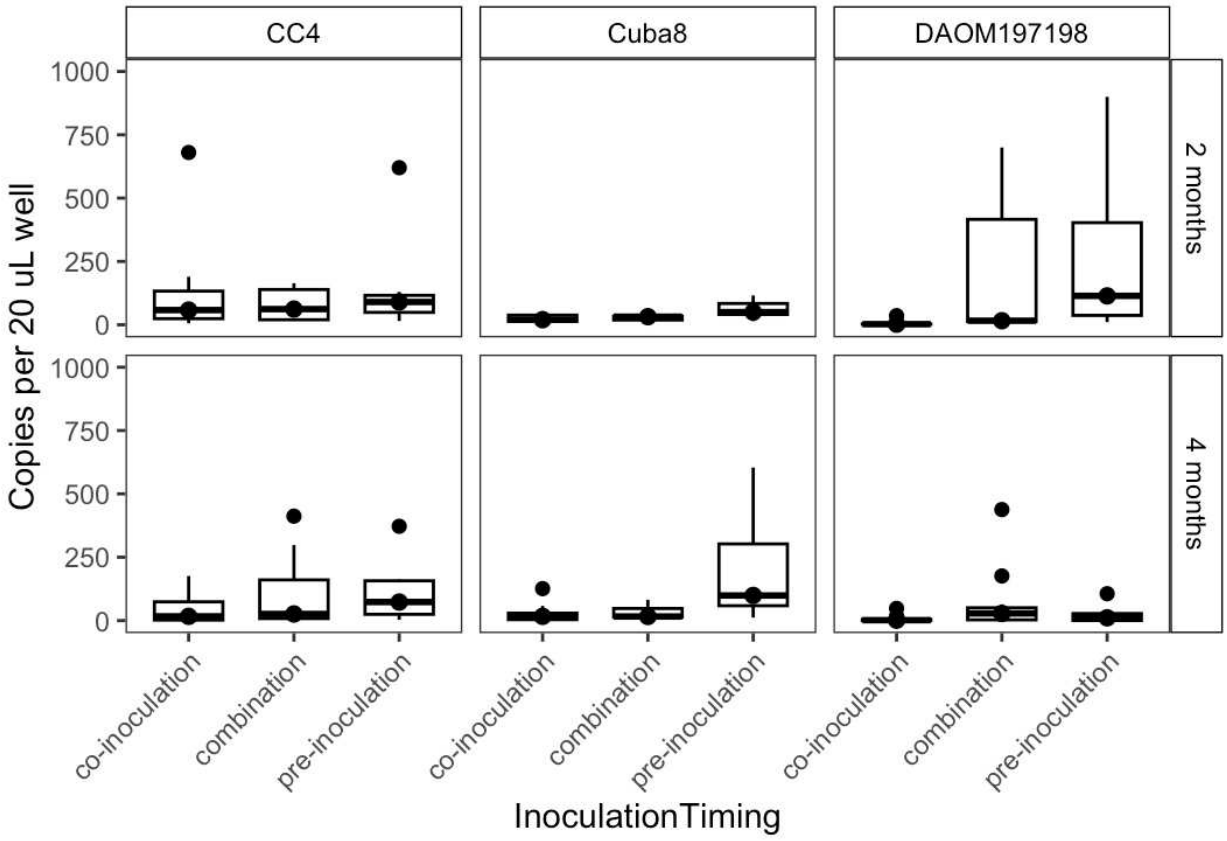


Fig. 2 AMF inoculant concentration in roots at two months (top) and four months (bottom). AMF concentration is the concentration of the target gene (copies μL^{-1}) measured by digital droplet PCR (ddPCR). Soils were inoculated with one of three arbuscular mycorrhizal fungal (AMF) (CC4, Cuba8 and DAOM197198) before host exposure to soil (pre-inoculation), at the same time as soil exposure (co-inoculation) or a combination. Boxplots represent the median and interquartile range (IQR), with whiskers extending to 1.5 × IQR.

AMF abundance in soil differed among AMF isolates, with Cuba8 more abundant and 197198 less abundant in soil compared to CC4 ($p = 0.017$) (Fig. 3, Table 5). Both Cuba8 and 197198 had higher soil colonization associated with pre-inoculation but only 197198 was statistically higher ($p = 0.0004$).

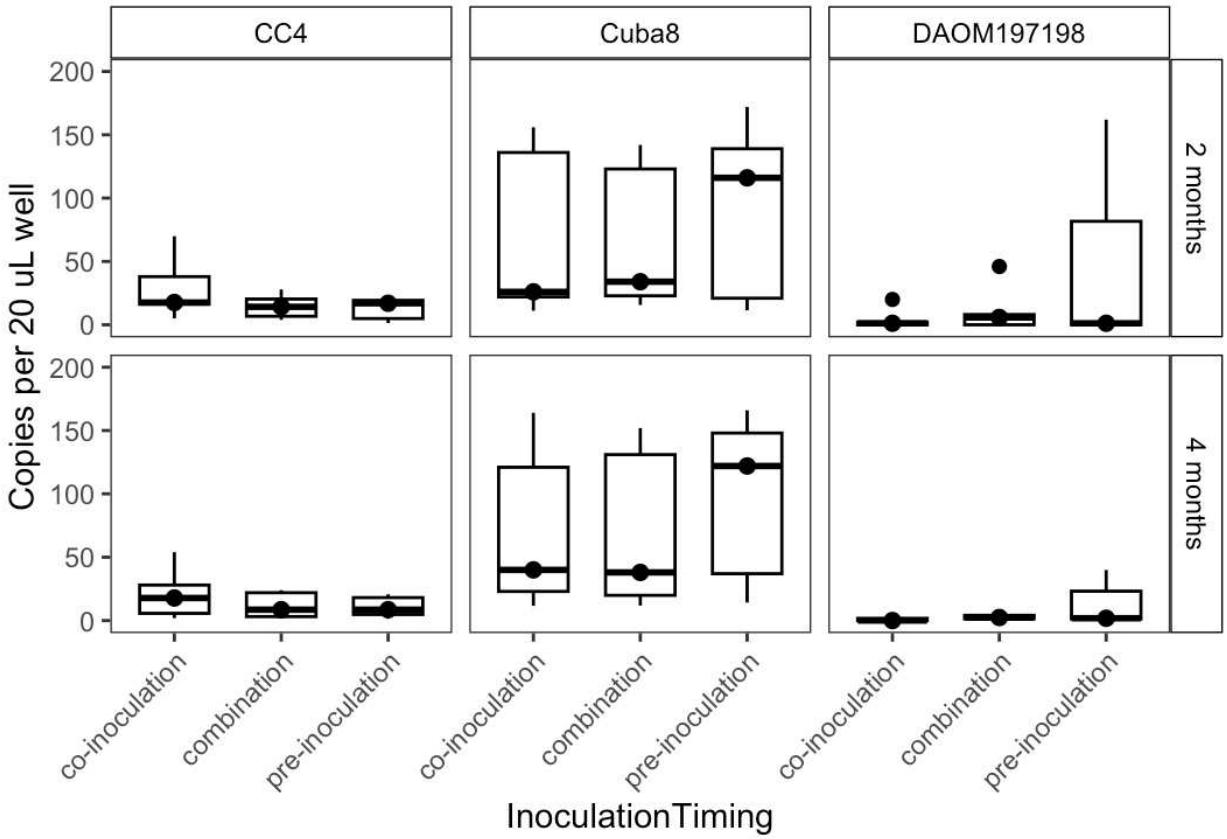


Fig. 3 AMF inoculant concentration in soil at two months (top) and four months (bottom). AMF concentration is the concentration of the target gene (copies μL^{-1}) measured by digital droplet PCR (ddPCR). Soils were inoculated with one of three arbuscular mycorrhizal fungal (AMF) (CC4, Cuba8 and DAOM197198) before host exposure to soil (pre-inoculation), at the same time as soil exposure (co-inoculation) or a combination. Boxplots represent the median and interquartile range (IQR), with whiskers extending to 1.5 × IQR.

Plant and soil responses

Harvest was the most important determinant of host response. **ShootMR** differed only among harvests with Harvest time 4 showing positive MR for all isolates and inoculation treatments ($p = 0.001$, Fig. 4. Table 6). Similarly, **RootMR** was positive for Harvest 4 ($p = 0.001$). However, for RootMR, hosts benefited more from 1971978 at Harvest 4 ($p = 0.06$) especially when 199718 was pre-inoculated ($p= 0.042$, Table 7). **ShootPMR** showed the opposite response, with positive values at Harvest 2 and negative or near-zero values at Harvest 4 ($p = 0.001$) particularly for Pre-inoculated Cuba8 and 197198 Harvest 4 ($p = 0.058$ 197198, $p = 0.029$ Cuba8, Table 8).

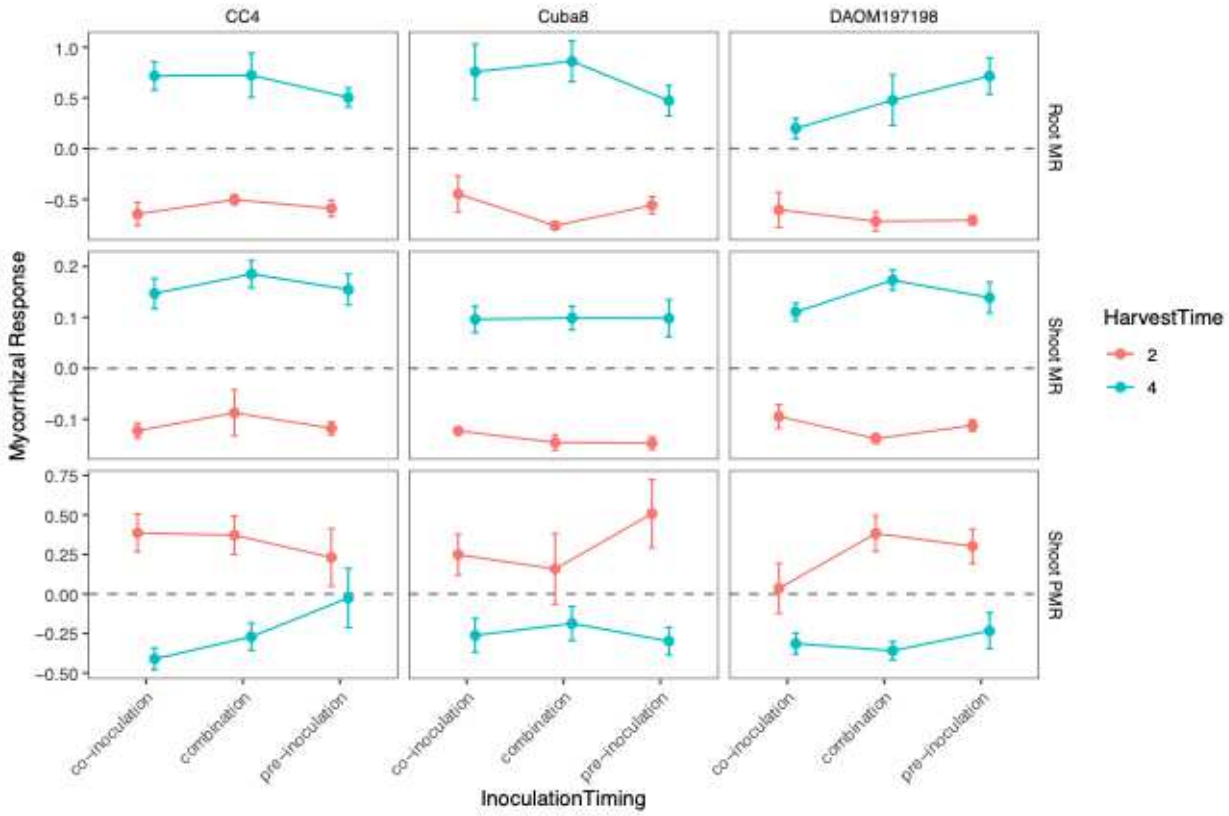


Fig 4. Mycorrhizal responseless for Plantago lanceolata inoculated with one of three arbuscular mycorrhizal fungal (AMF) (CC4, Cuba8 and DAOM197198) before host exposure to soil (pre-inoculation), at the same time as soil exposure (co-inoculation) or a combination at 2 months (red lines) and 4 months (blue lines) post inoculation. Dots represent mean and SE. Values below dotted lines indicate inoculation depressed host performance compared to uninoculated controls. Shoot MR = dry shoot mycorrhizal response, Root MR = dry root mycorrhizal response, Shoot PMR = total shoot phosphorus content mycorrhizal response.

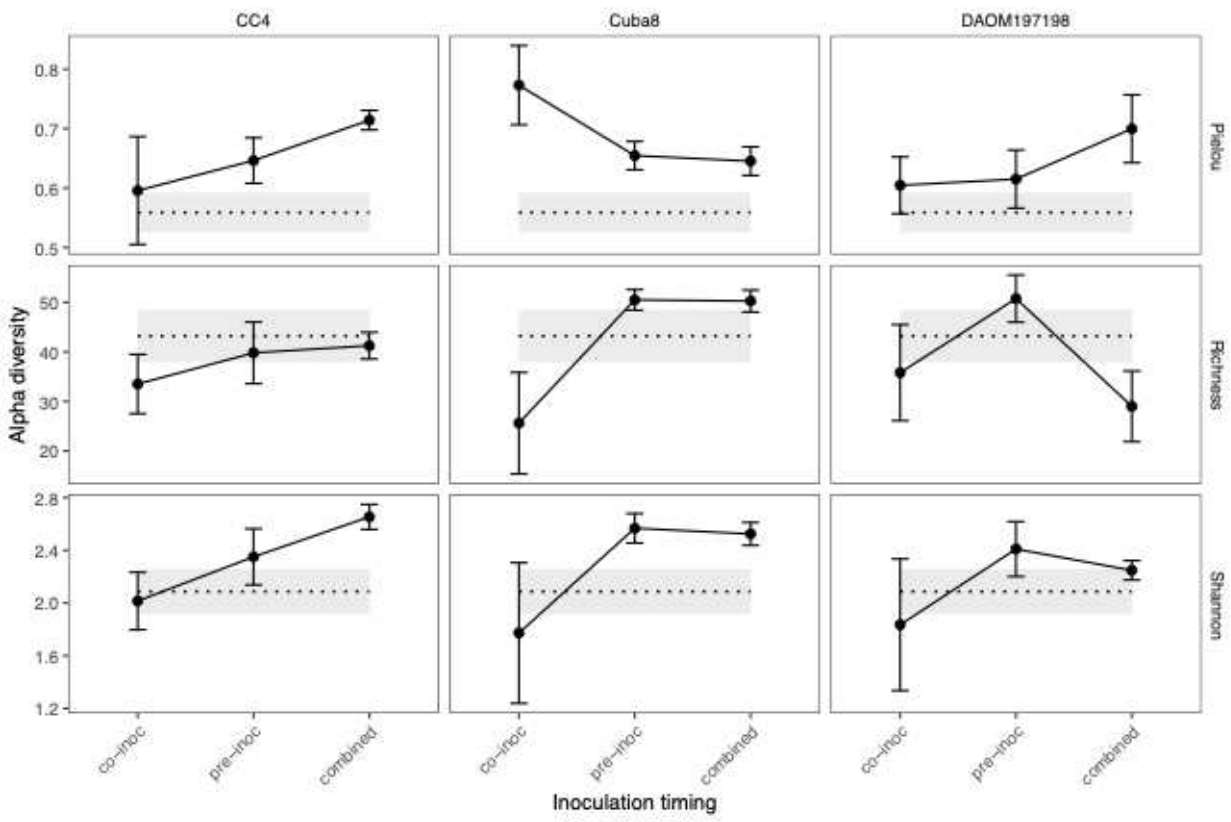
Soil phosphorus was not affected by inoculation timing, AMF isolate or time (Fig. S1, Table 12). A single interaction occurred to reduce soil P between Harvest 4 and pre-inoculation timing, ($p = 0.028$), however, the overall model was not significant ($p = 0.081$). **Soil pH** was more affected by Inoculation strategy , with pre-inoculation ($p = 0.034$) and combination ($p = 0.06$) associated with reduced soil pH at Harvest 2, but higher pH at Harvest 4 for all isolates ($p = 0.031$). For Cuba8, however, both pre- ($p = 0.013$) and combination treatments ($p = 0.049$, Table 13) led to smaller increases in pH in Harvest 4.

Resident AMF communities

316 Amplicon sequencing of the 18S SSU gene retrieved a total of 3,393,326 paired-end raw reads
317 were obtained for all 50 samples (77,866 ± 9298 reads per sample, 95% CI) and after quality
318 trimming of the raw reads, 3,019,083 million paired-end reads (60,381 ± 9097 reads per sample,
319 95% CI) were kept and further processed. Sequence analysis obtained Glomeromycotan
320 sequences belonging to six families and seven genera representing 102 virtual taxa (VT) (Fig.
321 S2). Soil samples were dominated by the genera *Glomus* and *Paraglomus* in all inoculation
322 treatments and across AMF inoculants.

323 Resident AMF diversity

324 Overall, combined inoculated CC4 (p= 0. 031) and 197198 (p = 0.04) were associated with more
325 even AMF communities, but Cuba8 had the highest evenness for co-inoculation (p = 0.037) (Fig.
326 5, Fig. S2, Tables 18, 19, 20). **Species richness** was not significantly affected by AMF isolate
327 identity or inoculation timing alone (Fig. 5, Table 16) but was significantly reduced by the
328 interaction of Cuba8 and co-inoculation timing (p = 0.054). **Shannon diversity** was not
329 significantly affected by AMF isolate identity, inoculation timing, or their interaction and the
330 overall model was not significant (p = 0.417) (Fig. 5, Table 17).



331

332 Fig 5. Alpha diversity (Pielou Evenness (top row), Species Richness (centre row), Shannon's
333 Diversity (bottom row) for soil AMF communities inoculated with one of three arbuscular
334 mycorrhizal fungal (CC4, Cuba8 and DAOM197198) before host exposure to soil (pre-inoc), at
335 the same time as soil exposure (co-inoc) or a combination at 4 months post inoculation. Dots
336 represent mean and SE. Dotted lines indicate mean diversity for uninoculated control soil, with
337 95% CI (shading). Values below dotted lines indicate inoculation depressed host performance
338 compared to uninoculated controls. Shoot MR = dry shoot mycorrhizal response, Root MR = dry
339 root mycorrhizal response, Shoot PMR = total shoot phosphorus content mycorrhizal response.

340

341 Resident AMF community composition

342 Neither inoculation timing ($p = 0.434$), AMF isolate identity ($p = 0.693$), nor their interaction ($p =$
343 0.502) affected AMF community composition when considering shifts in relative abundance
344 (Bray Curtis) (Fig. 6 Table 22). Using Jaccard dissimilarity, however, inoculation timing
345 significantly affected resident community composition ($p = 0.044$) with all groups diverging

from controls (Fig. 6). Neither AMF (p = 0.778) nor their interaction was significant (p = 0.292 , Table 23).

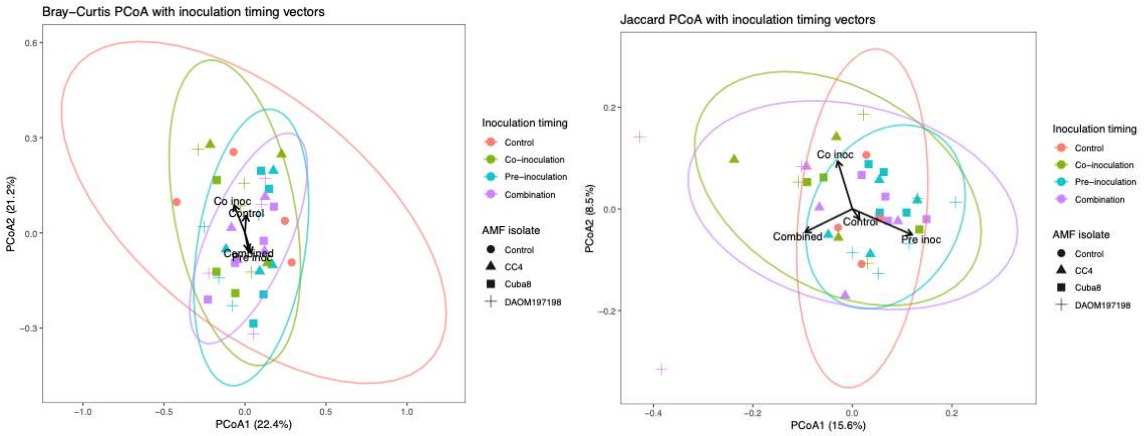


Fig 6 Effect of inoculation timing on AMF community structure based on Bray–Curtis and Jaccard dissimilarities. Principal coordinates analysis (PCoA) ordinations of AMF community composition showing the influence of inoculation timing across samples. The left panel displays Bray–Curtis dissimilarities (rarefied at 4000 sequences per sample), while the right panel shows Jaccard dissimilarities (presence–absence-based). Points represent individual samples, colored by inoculation timing treatment (control, pre-inoculation, co-inoculation, and combined), and shaped by AMF isolate identity. Ellipses indicate group dispersion for inoculation timing treatments. Vector overlays represent fitted environmental variables corresponding to inoculation timing categories, illustrating the direction and relative strength of their association with community structure.

Within group dispersion was similar across inoculation timing treatments for both Bray–Curtis (p = 0.84) and Jaccard distances (p = 0.55)(Fig. 6. Table 24, 25) (Tables 26, 27). Variation among individual AMF taxa were not significantly different among AMF (Table 28) or Timing (Table 29). Changes among pre-inoculation and combined treatments were driven by similar shifts in taxa, generally in the opposite direction of co-inoculation (Fig. 7, Table 30).

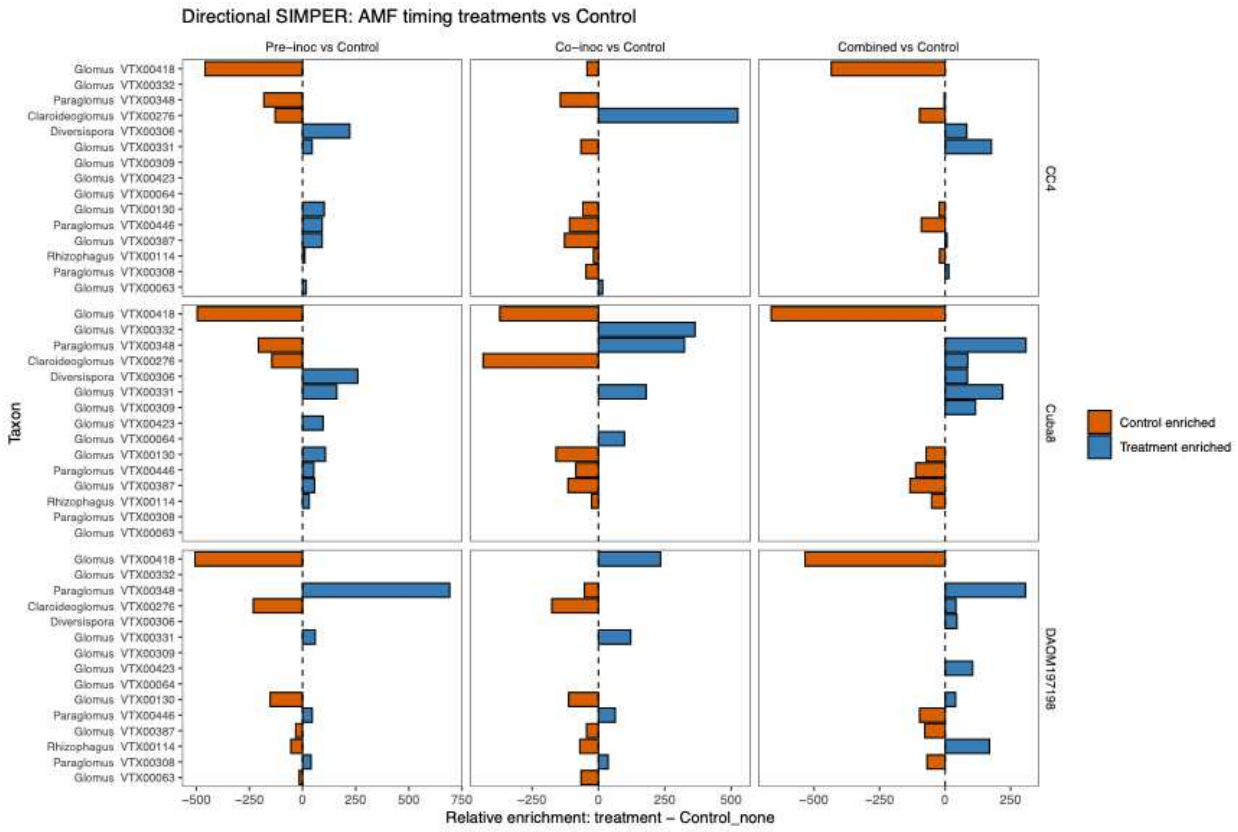


Fig. 7 AMF VTX responsible for 70% of variation between inoculated and uninoculated soil AMF communities (SIMPER). Bars represent the contribution of each taxon (VTX), with negative values indicating greater relative abundance in uninoculated (control) communities and positive values indicating greater relative abundance in inoculated communities.

Relative to the control, several treatments showed reduced numbers of rare taxa ($p < 0.001$, Fig. 8, Table 31, 32). Resident AMF co-inoculated with CC4 ($p = 0.04$) and Cuba8 ($p = 0.078$) had significantly fewer rare taxa compared to the control. All other treatments did not differ significantly from the control ($p > 0.10$)(Table 33).

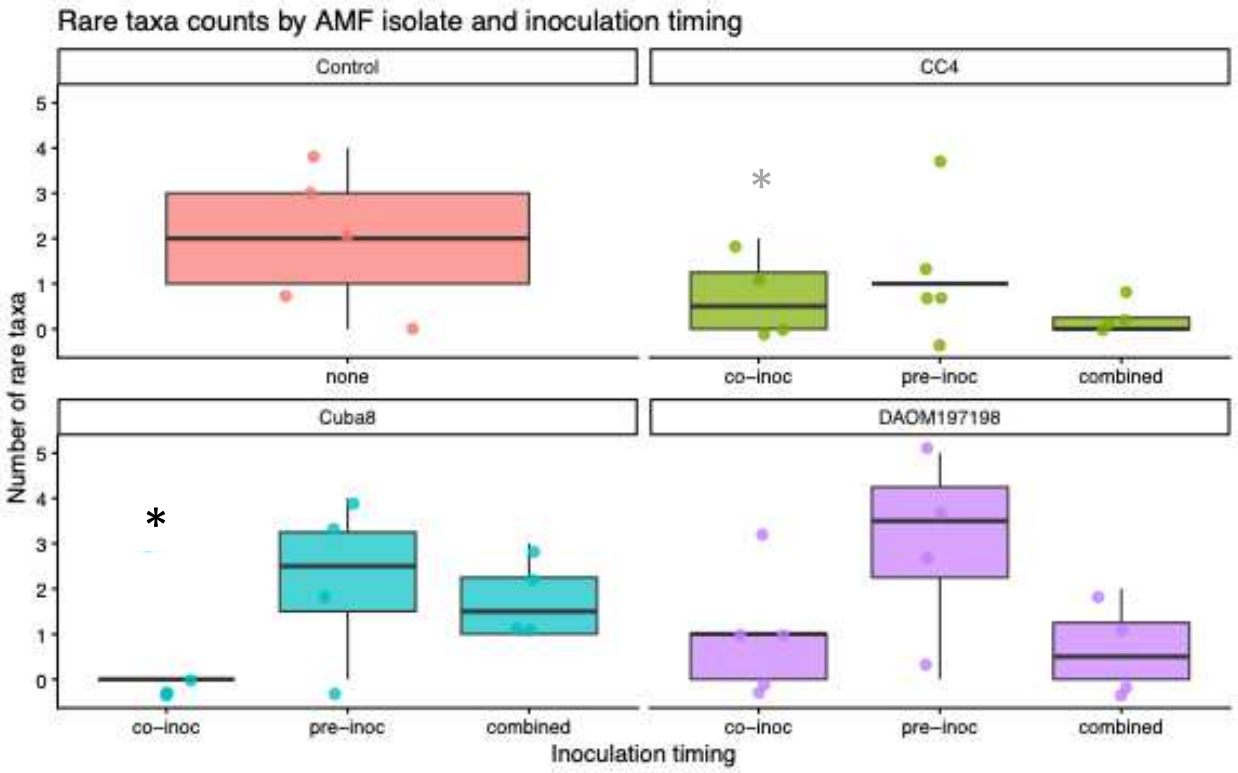


Fig. 8. Rare taxa counts (number of taxa present in ≤ 20% of samples) are shown for each AMF isolate across inoculation timing treatments. Control samples (top left) were not inoculated, while inoculated treatments (CC4, Cuba8, DAOM197198) include co-inoculation, pre-inoculation, and combined inoculation. Points represent individual samples, boxplots show medians and interquartile ranges, and whiskers indicate data spread. Colour of asterisk indicates $p < 0.05$ (black), $p < 0.01$ (grey).

While our approach could not definitively identify the isolates used as inocula, VTX114 is associated with *R. irregularis* sequences and was detected in all samples. Which showed both increases and decreases in VTX114 compared to controls.

Discussion

Inoculant establishment and abundance

We found that both inoculation timing and AMF isolate identity were important determinants of inoculant establishment in roots and soil. But even in our simplistic pot study, using one host and 3 AMF of the same species, context mattered.

All methods of inoculation led to high initial levels of inoculant detection in roots and soil, although 19798 did better when exposed to inoculants before exposure to soil community. This trend was identical for roots and soils. Early arrival has previously been shown to confer competitive advantages in both AMF and ectomycorrhizal systems through preferential access to root space, host-derived carbon, and nutrient exchange interfaces (Kennedy et al. 2009; Werner and Kiers 2015). Such priority effects may arise through mechanisms including spatial pre-emption, resource monopolization, and host-mediated carbon allocation favouring early colonizers (Kiers et al. 2011).

In terms of abundance, however, AMF identity was much more important predictor. In roots 197198 was less abundant overall but was enhanced by pre- and combination inoculation. This suggests that inoculation strategy is most important early and can enhance the successful establishment of an infection unit on the root system. Once established, however, inoculation strategy becomes less important. These results highlight the importance of intra-specific variation in competitive dynamics and persistence within established AMF communities.

Previous studies have shown that while 197198 has the ability to rapidly colonize roots and soil, compared to Cuba8 and CC4, may not be an ideal competitor in pre- and symbiotic growth stages. For example, previous work showed that it had lower germination rate and number of germ tubes, and slower germ tube growth rate compared to Cuba8 and CC4 (Kokkoris et al. 2019). During the symbiotic phase, there is evidence that while 197198 quicker to establish root and soil mycelia, it does not achieve as great size as the other isolates (Hart and Reader 2002; Kokkoris and Hart 2019b). In combination with its fast rate of vesicle development suggest that it may pose a larger carbon drain on hosts, perhaps limiting its growth (Kiers et al. 2011).

Together, these results indicate that both priority effects and isolate identity contribute to inoculant persistence, but that isolates differ in their capacity to capitalize on early establishment opportunities. The consistently high abundance of Cuba8 suggests that some isolates possess traits that enhance competitiveness across both root and soil environments, even among conspecifics.

Plant response

Despite successful establishment, inoculation did not enhance host shoot and root biomass beyond the benefits already provided by the resident AMF community by any inoculation method. This lack of growth response is consistent with studies showing that established resident AMF communities can limit the functional impact of introduced inoculants, particularly in soils already containing active symbionts (Jin et al. 2013; Loján et al. 2017; Elliott et al. 2021)

In contrast, shoot phosphorus mycorrhizal response became negative by Harvest 4 for nearly all isolates and inoculation treatments. Although inoculants have been associated with reduced host functioning in field soils, the mechanism underlying these effects are not clear (Baraza et al. 2024). In our study, inoculation may alter AMF community in ways that reduced phosphorus acquisition efficiency relative to the resident community. AMF taxa are known to differ in phosphorus uptake and transfer efficiency, including differences in external hyphal development and nutrient exchange with hosts (Smith et al. 2011).

The only exception to the negative ShootPMR was CC4 under pre-inoculation, which corresponded to the highest CC4 colonization levels and a concurrent reduction in soil phosphorus. This pattern suggests that CC4 may have been more effective at phosphorus acquisition or transfer to the host once established early within root systems. These findings support evidence that AMF isolates can differ substantially in phosphorus uptake efficiency and host nutrient delivery, even among closely related taxa (Tian et al. 2013; Raphael et al. 2022)

Soil pH also responded to AMF identity and inoculation timing, although patterns were more complex. The increase in we observed soil pH over time suggests that inoculation altered rhizosphere nutrient dynamics, since the pots differed in no other way. AMF can influence rhizosphere chemistry directly through nutrient exchange and proton fluxes (Smith and Read 2010), but also through changes to bacterial and fungal communities (De Gruyter et al. 2022) and leading to broader changes in ecosystem functions (Arcidiacono et al. 2024).

Together, these findings suggest that successful establishment of AMF inoculants does not necessarily translate into improved host function. Competitive interactions between introduced

and resident AMF may instead alter phosphorus acquisition dynamics and rhizosphere conditions without enhancing plant growth.

AMF Resident Communities

We found evidence for small but detectable shifts in resident AMF communities associated with inoculation strategy. Increases in evenness were associated with corresponding increases in diversity, particularly in the CC4 combined inoculation, but were not related to inoculant abundance in roots or soil. This suggests that changes in resident AMF communities were not simply driven by inoculant dominance, but instead may reflect indirect effects on resident taxa, potentially through inter- and intraspecific interactions within the AMF community (Janoušková et al. 2017; Elliott et al. 2021; Islam et al. 2021; Ilyas et al. 2024).

Inoculation with any AMF isolate caused resident AMF communities to diverge from uninoculated controls but these differences were detectable only using Jaccard distance metrics, suggesting they were driven primarily by shifts in rare taxa. Inoculation with both CC4 and Cuba8 reduced the number of rare AMF taxa present in soil communities, consistent with the observed increases in evenness in these treatments. Although the mechanisms underlying these losses remain unclear, rare taxa are generally expected to be more vulnerable to stochastic exclusion or competitive displacement during invasion (van der Putten et al. 2007).

The short duration of this study, and invasion work in general, limits our ability to predict how these compositional changes may develop over longer time scales. Early colonization can generate persistent legacy effects on microbial community assembly, even when inoculants are not dominant (Kong et al. 2025). Inoculation may also have consequences beyond AMF communities. For example, Catford et al. (2003) showed that pre-inoculation with *Rhizobia* or AMF reciprocally inhibited the other. Together, these findings suggest that inoculation timing may influence resident AMF communities not necessarily through inoculant abundance itself, but by altering competitive interactions among residents.

The role of host identity in mediating these responses also remains unclear. AMF isolates are known to differ in their interactions and competitive outcomes when grown with conspecifics (Kokkoris et al. 2021), suggesting that plant community composition may influence how resident communities respond to inoculation.

Conclusion

From an applied perspective, our study shows that AMF biofertilizers are most likely to establish when applied early in plant development, before competition with resident communities. In systems with reduced or functionally limited AMF communities, there may be less of an advantage to pre-inoculate before field planting. Despite pre-inoculation advantages, persistence of inoculants in roots and soil was not closely tied to inoculation strategy. Once the symbiosis is established, the abundance of inoculants may depend more on competitive interactions with residents.

Introduction of all inoculants into a field soil resulted in reduced host phosphorus benefits. These findings suggests that successful inoculant establishment does not necessarily translate into improved host function, and that inoculation may alter AMF community function in ways that are not predictable from colonization alone. The isolates used here may perform more effectively in less diverse or more disturbed soils, as they have previously been categorized as ruderal AMF taxa (Hart & Reader, 2002).

Overall, these results support growing evidence that AMF biofertilizers introduced into soils with established AMF communities can alter resident AMF composition and reduce host performance. We further showed that intraspecific variation among AMF can have functional consequences, making inoculation outcomes difficult to predict for both plant and soil ecosystems.

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Note. Models included AMF isolate identity (CC4 [baseline], Cuba8, DAOM197198), inoculation timing (pre-inoculation. prior to root–soil contact; co-inoculation. at root–soil contact; combined), and harvest time (Harvest Time = 2 and 4), with interaction terms where specified. Binomial models used a logit link (dispersion = 1). PERMANOVA used 999 permutations. Significance codes. * p < 0.05, ** p < 0.01, *** p < 0.001.

Table 1. Binomial GLM results for AMF inoculant presence in roots and soil (Harvest Time = 2 and 4)

Term	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	2.9519	0.6276	4.703	2.56e-06 ***
AMFIsolateCuba8	1.4306	1.1312	1.265	0.205972
AMFIsolateDAOM197198	-2.1716	0.5717	-3.799	0.000145 ***
HarvestTime4	-0.5841	0.4551	-1.284	0.199289
InoculationTiming (combination)	1.5634	0.5652	2.766	0.005670 **
InoculationTiming (pre-inoculation)	1.0143	0.4941	2.053	0.040111 *

Table 2. Binomial GLM Results for AMF Inoculant Presence in roots and soil (Binomial) (Harvest Time = 4 only)

Term	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.8256	0.5535	3.298	0.000972 ***
AMFIsolateCuba8	1.4762	1.1447	1.290	0.197181
AMFIsolateDAOM197198	-1.7951	0.6248	-2.873	0.004063 **
InoculationTiming (combination)	2.4374	0.8158	2.988	0.002812 **
InoculationTiming (pre-inoculation)	1.4107	0.6034	2.338	0.019393 *

Table 3. Predicted probabilities of AMF inoculant presence in roots and soil

AMF Isolate	Harvest Time	Inoculation Timing	Probability
CC4	2	co-inoculation	0.950
CC4	2	combination	0.989
CC4	2	pre-inoculation	0.981
CC4	4	co-inoculation	0.914
CC4	4	combination	0.981
CC4	4	pre-inoculation	0.967
Cuba8	2	co-inoculation	0.988
Cuba8	2	combination	0.997
Cuba8	2	pre-inoculation	0.995
Cuba8	4	co-inoculation	0.978

Cuba8	4	combination	0.995
Cuba8	4	pre-inoculation	0.992
DAOM197198	2	co-inoculation	0.686
DAOM197198	2	combination	0.912
DAOM197198	2	pre-inoculation	0.857
DAOM197198	4	co-inoculation	0.549
DAOM197198	4	combination	0.853
DAOM197198	4	pre-inoculation	0.770

674

675 Table 4. Tweedie GLM results for AMF abundance in roots (copies per 20 µL well).

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	5.0178	0.7136	7.032	<0.001 ***
AMFIsolateCuba8	-1.7263	1.0961	-1.575	0.117
AMFIsolateDAOM197198	-3.0398	1.1763	-2.584	0.0106 *
HarvestTime4	-1.2320	0.9090	-1.355	0.177
InoculationTiming (combination)	-0.6365	1.0390	-0.613	0.541
InoculationTiming (pre-inoculation)	-0.0028	1.0093	-0.003	0.998
DAOM197198 × combination	4.8445	1.5387	3.148	0.00194 **
DAOM197198 × pre-inoculation	3.6308	1.5336	2.368	0.0190 *
HarvestTime4 × combination	2.5073	1.2743	1.968	0.0507 .
HarvestTime4 × pre-inoculation	3.0364	1.2331	2.462	0.0148 *
DAOM197198 × HT4 × combination	-4.4509	1.9111	-2.329	0.0210 *
DAOM197198 × HT4 × pre-inoculation	-3.6451	1.8860	-1.933	0.0549 .

676

677 Table 5. Tweedie GLM results for AMF abundance in soil (copies per 20 µL well).

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	3.3584	0.4906	6.845	<0.001 ***
AMFIsolateCuba8	0.9281	0.6455	1.438	0.153
AMFIsolateDAOM197198	-2.0696	0.8513	-2.431	0.0167 *
HarvestTime4	-0.3555	0.7156	-0.497	0.620
InoculationTiming (combination)	-0.6991	0.7386	-0.947	0.346
InoculationTiming (pre-inoculation)	-0.8179	0.7470	-1.095	0.276

DAOM197198 × pre-inoculation	4.0009	1.0988	3.641	0.000418	***

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679
680 Table 6. Linear model results for shoot mycorrhizal response (ShootMR).

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.123	0.024	-5.13	<0.001
AMFIsolateCuba8	-0.0004	0.0338	-0.01	0.990
AMFIsolateDAOM197198	0.0279	0.0338	0.83	0.410
HarvestTime4	0.269	0.0338	7.98	<0.001**
InoculationTimingcombination	0.0351	0.0338	1.04	0.301
InoculationTimingpre-inoculation	0.0048	0.0338	0.14	0.888
AMFIsolateCuba8:HarvestTime4	-0.0501	0.0478	-1.05	0.296
AMFIsolateDAOM197198:HarvestTime4	-0.0641	0.0478	-1.34	0.182
AMFIsolateCuba8:InoculationTimingcombination	-0.0577	0.0478	-1.21	0.229
AMFIsolateDAOM197198:InoculationTimingcombination	-0.0784	0.0478	-1.64	0.103
AMFIsolateCuba8:InoculationTimingpre-inoculation	-0.0287	0.0478	-0.60	0.549
AMFIsolateDAOM197198:InoculationTimingpre-inoculation	-0.0223	0.0478	-0.47	0.641
HarvestTime4:InoculationTimingcombination	0.0031	0.0478	0.07	0.948
HarvestTime4:InoculationTimingpre-inoculation	0.0031	0.0478	0.07	0.948
AMFIsolateCuba8:HarvestTime4:InoculationTimingcombination	0.0221	0.0676	0.33	0.745
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingcombination	0.103	0.0676	1.52	0.131
AMFIsolateCuba8:HarvestTime4:InoculationTimingpre-inoculation	0.0227	0.0676	0.34	0.737
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingpre-inoculation	0.0426	0.0676	0.63	0.529

681

682 Table 7. Linear model results for root mycorrhizal response (RootMR).

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.644	0.153	-4.22	<0.001
AMFIsolateCuba8	0.197	0.216	0.91	0.363
AMFIsolateDAOM197198	0.0418	0.216	0.19	0.847
HarvestTime4	1.362	0.216	6.31	<0.001**
InoculationTimingcombination	0.141	0.216	0.65	0.516
InoculationTimingpre-inoculation	0.0553	0.216	0.26	0.798
AMFIsolateCuba8:HarvestTime4	-0.157	0.305	-0.52	0.608
AMFIsolateDAOM197198:HarvestTime4	-0.560	0.305	-1.84	0.069*
AMFIsolateCuba8:InoculationTimingcombination	-0.453	0.305	-1.48	0.141
AMFIsolateDAOM197198:InoculationTimingcombination	-0.254	0.305	-0.83	0.407
AMFIsolateCuba8:InoculationTimingpre-inoculation	-0.167	0.305	-0.55	0.586
AMFIsolateDAOM197198:InoculationTimingpre-inoculation	-0.159	0.305	-0.52	0.604
HarvestTime4:InoculationTimingcombination	-0.135	0.305	-0.44	0.660
HarvestTime4:InoculationTimingpre-inoculation	-0.269	0.305	-0.88	0.381
AMFIsolateCuba8:HarvestTime4:InoculationTimingcombination	0.551	0.432	1.27	0.205
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingcombination	0.527	0.432	1.22	0.225
AMFIsolateCuba8:HarvestTime4:InoculationTimingpre-inoculation	0.0956	0.432	0.22	0.825
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingpre-inoculation	0.890	0.432	2.06	0.042*

683 Table 8. Linear model results for total shoot phosphorus mycorrhizal response (ShootPMR).

Term	Estimate	Std. Error	t value	Pr(>|t|)

(Intercept)	0.386	0.134	2.89	0.0047**
AMFIsolateCuba8	-0.137	0.189	-0.73	0.469
AMFIsolateDAOM197198	-0.351	0.189	-1.86	0.066*
HarvestTime4	-0.797	0.189	-4.21	<0.001***
InoculationTimingcombination	-0.0146	0.189	-0.08	0.939
InoculationTimingpre-inoculation	-0.155	0.189	-0.82	0.414
AMFIsolateCuba8:HarvestTime4	0.286	0.268	1.07	0.287
AMFIsolateDAOM197198:HarvestTime4	0.448	0.268	1.67	0.097*
AMFIsolateCuba8:InoculationTimingcombination	-0.0758	0.268	-0.28	0.778
AMFIsolateDAOM197198:InoculationTimingcombination	0.362	0.268	1.35	0.179
AMFIsolateCuba8:InoculationTimingpre-inoculation	0.414	0.268	1.55	0.125
AMFIsolateDAOM197198:InoculationTimingpre-inoculation	0.421	0.268	1.58	0.118
HarvestTime4:InoculationTimingcombination	0.155	0.268	0.58	0.564
HarvestTime4:InoculationTimingpre-inoculation	0.541	0.268	2.02	0.046*
AMFIsolateCuba8:HarvestTime4:InoculationTimingcombination	0.0105	0.378	0.03	0.978
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingcombination	-0.547	0.378	-1.45	0.151
AMFIsolateCuba8:HarvestTime4:InoculationTimingpre-inoculation	-0.836	0.378	-2.21	0.029*
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingpre-inoculation	-0.726	0.378	-1.92	0.058*

Table 9. Residual normality tests for mycorrhizal response models (Shapiro–Wilk).

Response	W statistic	p-value
ShootMR	0.9667	0.00335
RootMR	0.9189	1.24e-06
ShootPMR	0.9643	0.00209

Table 10. Homogeneity of variance tests for mycorrhizal response models (Levene's test).

Response	Df	F value	Pr(>F)
ShootMR	17	1.085	0.377

RootMR	17	1.073	0.389
ShootPMR	17	0.815	0.673

Table 11. Estimated marginal means for mycorrhizal response variables

Response	AMF Isolate	Harvest Time	Inoculation Timing	Mean	95% CI
ShootMR	CC4	2	co-inoc	-0.123	-0.170–-0.075
ShootMR	CC4	4	co-inoc	0.147	0.100–0.194
ShootMR	CC4	2	combination	-0.087	-0.135–-0.040
ShootMR	CC4	4	combination	0.185	0.138–0.232
ShootMR	CC4	2	pre-inoc	-0.118	-0.165–-0.071
ShootMR	CC4	4	pre-inoc	0.155	0.107–0.202
RootMR	CC4	2	co-inoc	-0.644	-0.947–-0.342
RootMR	CC4	4	co-inoc	0.718	0.415–1.020
RootMR	CC4	2	combination	-0.504	-0.806–-0.201
RootMR	CC4	4	combination	0.724	0.421–1.026
RootMR	CC4	2	pre-inoc	-0.589	-0.892–-0.286
RootMR	CC4	4	pre-inoc	0.504	0.201–0.807
ShootPMR	CC4	2	co-inoc	0.386	0.121–0.652
ShootPMR	CC4	4	co-inoc	-0.410	-0.676–-0.145
ShootPMR	CC4	2	combination	0.372	0.107–0.637
ShootPMR	CC4	4	combination	-0.270	-0.535–-0.005
ShootPMR	CC4	2	pre-inoc	0.231	-0.034–0.497
ShootPMR	CC4	4	pre-inoc	-0.025	-0.290–0.240

Table 12. Linear model results for soil phosphorus (SoilP)

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	18.5486	0.9260	20.03	<0.001 ***
AMFIsolateCuba8	-1.0414	1.3095	-0.80	0.428
AMFIsolateDAOM197198	0.3871	1.3095	0.30	0.768
HarvestTime4	0.6657	1.3095	0.51	0.612
InoculationTimingcombination	1.1286	1.3095	0.86	0.391
InoculationTimingpre-inoculation	1.1529	1.3095	0.88	0.381
AMFIsolateCuba8:HarvestTime4	0.1286	1.8520	0.07	0.945
AMFIsolateDAOM197198:HarvestTime4	1.3457	1.8520	0.73	0.469
AMFIsolateCuba8:InoculationTimingcombination	0.7257	1.8520	0.39	0.696
AMFIsolateDAOM197198:InoculationTimingcombination	-0.5557	1.8520	-0.30	0.765
AMFIsolateCuba8:InoculationTimingpre-inoculation	-1.0914	1.8520	-0.59	0.557

AMFIsolateDAOM197198:InoculationTimingpre-inoculation	-0.7357	1.8520	-0.40	0.692
HarvestTime4:InoculationTimingcombination	-1.1557	1.8520	-0.62	0.534
HarvestTime4:InoculationTimingpre-inoculation	-4.1271	1.8520	-2.23	0.0279 *
AMFIsolateCuba8:HarvestTime4:InoculationTimingcombination	1.5957	2.6191	0.61	0.544
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingcombination	-2.9643	2.6191	-1.13	0.260
AMFIsolateCuba8:HarvestTime4:InoculationTimingpre-inoculation	4.7729	2.6191	1.82	0.071
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingpre-inoculation	2.1957	2.6191	0.84	0.404

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693 Table 13. Linear model results for soil pH

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	7.3557	0.1149	64.03	<0.001 ***
AMFIsolateCuba8	-0.1971	0.1625	-1.21	0.228
AMFIsolateDAOM197198	-0.2571	0.1625	-1.58	0.116
HarvestTime4	-0.0957	0.1625	-0.59	0.557
InoculationTimingcombination	-0.3057	0.1625	-1.88	0.063 .
InoculationTimingpre-inoculation	-0.3471	0.1625	-2.14	0.0349 *
AMFIsolateCuba8:HarvestTime4	0.4057	0.2298	1.77	0.080 .
AMFIsolateDAOM197198:HarvestTime4	0.2557	0.2298	1.11	0.268
AMFIsolateCuba8:InoculationTimingcombination	0.4586	0.2298	2.00	0.0485 *
AMFIsolateDAOM197198:InoculationTimingcombination	0.3357	0.2298	1.46	0.147
AMFIsolateCuba8:InoculationTimingpre-inoculation	0.5757	0.2298	2.51	0.0137 *
AMFIsolateDAOM197198:InoculationTimingpre-inoculation	0.1286	0.2298	0.56	0.577
HarvestTime4:InoculationTimingcombination	0.3986	0.2298	1.74	0.086 .
HarvestTime4:InoculationTimingpre-inoculation	0.5029	0.2298	2.19	0.0308 *
AMFIsolateCuba8:HarvestTime4:InoculationTimingcombination	-0.5371	0.3249	-1.65	0.101
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingcombination	-0.4729	0.3249	-1.46	0.149

AMFIsolateCuba8:HarvestTime4:InoculationTimingpre-inoculation	-0.8300	0.3249	-2.56	0.0120 *
AMFIsolateDAOM197198:HarvestTime4:InoculationTimingpre-inoculation	-0.1043	0.3249	-0.32	0.749

694

695 Table 14. Residual normality tests for soil response models (Shapiro–Wilk).

Response	W statistic	p-value
SoilP	0.9911	0.603
Soil pH	0.9558	0.00041

696

697 Table 15. Homogeneity of variance tests for soil response models (Levene's test)..

Response	Df	F value	Pr(>F)
SoilP	17	0.941	0.529
Soil pH	17	0.733	0.763

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699 Table 16. Negative binomial GLM for AMF Species Richness

Term	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.76584	0.21366	17.625	<2e-16
AMF CC4	-0.04619	0.32092	-0.144	0.8856
AMF Cuba8	0.15117	0.31922	0.474	0.6358
AMF DAOM197198	-0.39854	0.32488	-1.227	0.2199
Timing co-inoc	0.21065	0.32635	0.645	0.5186
Timing pre-inoc	0.55962	0.34073	1.642	0.1005
CC4 × co-inoc	-0.41876	0.47178	-0.888	0.3748
Cuba8 × co-inoc	-0.88507	0.45999	-1.924	0.0543*
CC4 × pre-inoc	-0.59540	0.46849	-1.271	0.2038
Cuba8 × pre-inoc	-0.55465	0.47810	-1.160	0.2460

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701 Table 17. Linear Model for AMF Shannon Diversity

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.0872	0.2837	7.356	1.59e-08
AMF CC4	0.5666	0.4256	1.331	0.192
AMF Cuba8	0.4373	0.4256	1.028	0.311
AMF DAOM197198	0.1611	0.4256	0.378	0.707
Timing co-inoc	-0.4131	0.4256	-0.971	0.339
Timing pre-inoc	0.1614	0.4486	0.360	0.721
CC4 × co-inoc	-0.2256	0.6184	-0.365	0.718

Cuba8 × co-inoc	-0.3390	0.6019	-0.563	0.577
CC4 × pre-inoc	-0.4643	0.6184	-0.751	0.458
Cuba8 × pre-inoc	-0.1183	0.6345	-0.186	0.853

Table 18. Linear model for Pielou's Evenness

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.55884	0.04589	12.177	1.49e-13
AMF CC4	0.15540	0.06884	2.257	0.0309*
AMF Cuba8	0.08643	0.06884	1.256	0.2184
AMF DAOM197198	0.14097	0.06884	2.048	0.0489*
Timing co-inoc	-0.09521	0.07256	-1.312	0.1988
Timing pre-inoc	-0.08475	0.07256	-1.168	0.2515
CC4 × co-inoc	-0.02325	0.10262	-0.227	0.8222
Cuba8 × co-inoc	0.22308	0.10262	2.174	0.0372*
CC4 × pre-inoc	0.01684	0.10002	0.168	0.8674
Cuba8 × pre-inoc	0.09412	0.10262	0.917	0.3659

Table 19. Alpha diversity metrics by AMF isolate.

AMF	n	Mean richness	SE richness	Mean Shannon	SE Shannon	Mean Pielou	SE Pielou
Control	5	43.2	5.31	2.087	0.171	0.559	0.034
CC4	13	38.31	3.03	2.341	0.124	0.652	0.032
Cuba8	13	40.85	5.13	2.249	0.223	0.691	0.029
DAOM197198	13	38.31	4.91	2.139	0.202	0.640	0.029

Table 20. Alpha diversity metrics by inoculation timing.

Inoculation timing	n	Mean richness	SE richness	Mean Shannon	SE Shannon	Mean Pielou	SE Pielou
None	5	43.2	5.31	2.087	0.171	0.559	0.034
Co-inoculation	14	31.5	5.08	1.864	0.250	0.658	0.043
Pre-inoculation	13	46.46	3.06	2.436	0.104	0.639	0.021
Combined	12	40.17	3.55	2.475	0.068	0.686	0.021

Table 21. Sample distribution across AMF isolate and inoculation timing treatments before and after rarefaction. Rarefied (4000 sequences per sample) (A) and non-rarefied dataset (B).

A. Rarefied (4000 sequences per sample)

AMF Isolate	Co-inoculation	Combination	None	Pre-inoculation
CC4	3	4	0	4
Control	0	0	4	0
Cuba8	3	4	0	4
DAOM197198	4	4	0	4
Total	10	12	4	12

B. Non-rarefied

AMF Isolate	Co-inoculation	Combination	None	Pre-inoculation
CC4	4	4	0	5
Control	0	0	5	0
Cuba8	5	4	0	4
DAOM197198	5	4	0	4
Total	14	12	5	13

Table 22. PERMANOVA results for AMF community composition (Bray–Curtis distance)(999 permutations).

Term	Df	Sum of Squares	R^2	F	Pr(>F)
AMF	3	0.3005	0.0687	0.8352	0.693
Inoculation timing	2	0.2411	0.0551	1.0051	0.434
AMF × Inoculation timing	4	0.4725	0.1081	0.9850	0.502
Residual	28	3.3578	0.7681		
Total	37	4.3719	1.0000		

Table 23. PERMANOVA results for AMF community composition (Jaccard distance) (presence–absence; 999 permutations).

A. Full model

Term	Df	Sum of Squares	R^2	F	p-value
InoculationTiming	2	0.4718	0.06895	1.5100	0.044 *
AMF	3	0.3932	0.05747	0.8391	0.778
AMF × InoculationTiming	4	0.6656	0.09728	1.0651	0.292
Residual	34	5.3117	0.77630		
Total	43	6.8423	1.0 0000		

B. Pairwise for Inoculation timing

Comparison	Df	Sum of Squares	R^2	F	p-value	p_adj

Control vs CC4	1	0.118	0.0845	1.20	0.198	0.594
Control vs Cuba8	1	0.0531	0.0450	0.612	0.923	0.923
Control vs DAOM197198	1	0.0786	0.0431	0.631	0.920	0.923
CC4 vs Cuba8	1	0.135	0.0705	1.52	0.049	0.294
CC4 vs DAOM197198	1	0.0806	0.0326	0.707	0.852	0.923
Cuba8 vs DAOM197198	1	0.120	0.0508	1.12	0.297	0.594

Table 24. Homogeneity of multivariate dispersion for Inoculation Timing (Bray–Curtis distance).

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Groups	3	0.005265	0.0017549	0.2725	0.8448
Residuals	34	0.218998	0.0064411		

Table 25. Homogeneity of multivariate dispersion Inoculation Timing (Jaccard distance)

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Groups	3	0.01081	0.0036033	0.714	0.5504
Residuals	34	0.17158	0.0050464		

Table 26. Homogeneity of multivariate dispersion for AMF community composition (Bray–Curtis distance).

Term	Df	Sum of Squares	Mean Sq	F	Pr(>F)
Groups	3	0.005265	0.001755	0.2725	0.853
Residuals	34	0.218998	0.006441		

Table 27. Homogeneity of multivariate dispersion for AMF community composition (Jaccard distance)

Term	Df	Sum of Squares	Mean Sq	F	Pr(>F)
Groups	3	0.01081	0.0036033	0.714	0.550
Residuals	34	0.17158	0.0050464		

Table 28. Mean multivariate dispersion (distance to centroid) by AMF isolate by distance metric (Bray Curtis (Bray), Jaccard)

AMF	n	Bray mean	Bray SD	Bray SE	Jaccard mean	Jaccard SD	Jaccard SE
CC4	11	0.316	0.0865	0.0261	0.294	0.0441	0.0133
Control	4	0.335	0.115	0.0576	0.279	0.0411	0.0205
Cuba8	11	0.319	0.0799	0.0241	0.269	0.0402	0.0121
DAOM197198	12	0.311	0.0912	0.0263	0.331	0.110	0.0319

734 Table 29. Mean multivariate dispersion (distance to centroid) by inoculation timing for Bray–
735 Curtis (Bray) and Jaccard distances

Inoculation timing	n	Bray mean	Bray SD	Bray SE	Jaccard mean	Jaccard SD	Jaccard SE
Co-inoculation	10	0.331	0.0911	0.0288	0.299	0.0541	0.0171
Combination	12	0.304	0.0759	0.0219	0.318	0.106	0.0305
None	4	0.335	0.115	0.0576	0.279	0.0411	0.0205
Pre-inoculation	12	0.319	0.0612	0.0177	0.278	0.0394	0.0114

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737 Table 30. SIMPER results for inoculation timing treatments relative to control.

Comparison	Taxon (VTX)	Average contribution	p-value
co-inoc vs none	Glomus VTX00418	0.1046	0.058
co-inoc vs none	Claroideoglomus VTX00276	0.0786	0.283
co-inoc vs none	Paraglomus VTX00348	0.0603	0.548
co-inoc vs none	Paraglomus VTX00446	0.0297	0.394
co-inoc vs none	Glomus VTX00130	0.0291	0.421
co-inoc vs none	Glomus VTX00387	0.0201	0.492
co-inoc vs none	Rhizophagus VTX00114	0.0181	0.450
co-inoc vs none	Glomus VTX00331	0.0177	0.748
co-inoc vs none	Glomus VTX00332	0.0167	0.302
co-inoc vs none	Glomus VTX00063	0.0129	0.158
combined vs none	Glomus VTX00418	0.0934	0.137
combined vs none	Paraglomus VTX00348	0.0720	0.261
combined vs none	Claroideoglomus VTX00276	0.0650	0.556
combined vs none	Glomus VTX00130	0.0286	0.400
combined vs none	Paraglomus VTX00446	0.0252	0.661
combined vs none	Rhizophagus VTX00114	0.0219	0.182
combined vs none	Glomus VTX00331	0.0211	0.514
combined vs none	Glomus VTX00387	0.0198	0.527
combined vs none	Diversispora VTX00306	0.0135	0.787

combined vs none	Paraglomus VTX00308	0.0129	0.221
pre-inoc vs none	Glomus VTX00418	0.0919	0.144
pre-inoc vs none	Paraglomus VTX00348	0.0691	0.345
pre-inoc vs none	Claroideoglomus VTX00276	0.0673	0.534
pre-inoc vs none	Paraglomus VTX00446	0.0360	0.163
pre-inoc vs none	Glomus VTX00130	0.0293	0.377
pre-inoc vs none	Diversispora VTX00306	0.0244	0.275
pre-inoc vs none	Glomus VTX00387	0.0224	0.343
pre-inoc vs none	Rhizophagus VTX00114	0.0185	0.395
pre-inoc vs none	Glomus VTX00331	0.0155	0.887
pre-inoc vs none	Paraglomus VTX00308	0.0116	0.417

738 Table 31. Negative binomial GLM coefficients (log scale) for the count of rare taxa

Term	Estimate	SE	z	p-value
Intercept (Control_none)	1.52606	0.28900	5.280	<0.001
CC4_co-inoc	-1.12059	0.54795	-2.045	0.0408*
Cuba8_co-inoc	-0.83291	0.47283	-1.762	0.0781*
DAOM197198_co-inoc	-0.30228	0.42707	-0.708	0.4791
CC4_pre-inoc	-0.24512	0.42323	-0.579	0.5625
Cuba8_pre-inoc	0.26570	0.41862	0.635	0.5256
DAOM197198_pre-inoc	0.41985	0.41145	1.020	0.3075
CC4_combined	-0.71513	0.49466	-1.446	0.1483
Cuba8_combined	0.03209	0.43152	0.074	0.9407
DAOM197198_combined	-0.83291	0.50851	-1.638	0.1014

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740 Table 32. Estimated marginal means (EMMs) for rare taxa counts

TreatmentGroup	Rate	SE	95% CI (LCL)	95% CI (UCL)
Control_none	4.60	0.959	3.057	6.92
CC4_co-inoc	1.50	0.612	0.674	3.34
Cuba8_co-inoc	2.00	0.632	1.076	3.72
DAOM197198_co-inoc	3.40	0.825	2.114	5.47
CC4_pre-inoc	3.60	0.849	2.268	5.71
Cuba8_pre-inoc	6.00	1.220	4.022	8.95
DAOM197198_pre-inoc	7.00	1.320	4.833	10.14
CC4_combined	2.25	0.750	1.171	4.32

Cuba8_combined	4.75	1.090	3.030	7.45
DAOM197198_combined	2.00	0.707	1.000	4.00

Table 33. Pairwise contrasts vs control (rate ratios) for rare taxa counts

Contrast	Ratio	SE	z	p-value (BH)
CC4_co-inoc / Control_none	0.326	0.149	-2.445	0.1255
Cuba8_co-inoc / Control_none	0.435	0.165	-2.199	0.1255
DAOM197198_co-inoc / Control_none	0.739	0.236	-0.945	0.4661
CC4_pre-inoc / Control_none	0.783	0.246	-0.779	0.4905
Cuba8_pre-inoc / Control_none	1.304	0.381	0.911	0.4661
DAOM197198_pre-inoc / Control_none	1.522	0.428	1.492	0.2443
CC4_combined / Control_none	0.489	0.192	-1.819	0.1551
Cuba8_combined / Control_none	1.033	0.320	0.104	0.9176
DAOM197198_combined / Control_none	0.435	0.178	-2.029	0.1273

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