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A Morpho-biochemical and functional comparison of Arbuscular Mycorrhizal Fungal spores cultured in two distinct conditions

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The present investigation was conducted to compare between Arbuscular Mycorrhizal Fungal spores generated *in vitro* through Root Organ Culture and those obtained *in vivo* through conventional pot culture method. Our study conducts a comprehensive analysis of four AMF species *viz., Rhizophagus irregularis, Rhizophagus clarus, Entrophospora etunicata*, and *Funneliformis mosseae* cultured both in *in vitro* and *in vivo* conditions regarding their morphology, Fatty Acid Methyl Ester (FAME) profiles and infectivity potential. Morphological analysis showed that *in vitro*-produced spores tend to be smaller in size with smooth surface in comparison to their *in vivo* counterparts. The observed spore surface irregularities can be attributed to the sloughing off of the outer layer, the adhesion of Rhizobia, Pseudomonads, and the adherence of soil particles due to the release of Glomalin. FAME profiles showed the presence of marker fatty acids indicative of Glomalean nature in all AMF species. Notably, variations in the fatty acid profiles, especially the presence of Neutral Lipid Fatty Acid (NFLA) C16:ù5c, differentiate *in vitro* and *in vivo* spores, indicating the influence of growth conditions on the biochemical composition of these AMF species. It is also crucial to identify the disparities in colonization percentages between *in vivo* and *in vitro* spores when extrapolating research findings from AMF grown in controlled conditions (*in vitro*) to those cultured in association with plants (*in vivo*). *In vivo* cultivated spores of *R. irregularis, R. clarus,* and *F. mosseae* exhibited higher infectivity potential while *E. etunicata* showed higher infectivity potential among all the *in vitro* cultivated AMF species. These findings suggest interesting opportunities for further exploration for optimizing their use as bioinoculants.

Keywords: AMF, comparative analysis, FAME, infectivity potential *in vitro, in vivo*

INTRODUCTION

Arbuscular Mycorrhizal Fungi (AMF) form symbiotic relationships with approximately 72% of vascular plants, playing crucial roles in both natural and agricultural ecosystems (Brundrett and Tedersoo, 2018).

This symbiosis is increasingly important given global challenges such as a growing population, limited arable land, and excessive chemical fertilizer use (Huang *et al.* 2020; Smith and Read, 2008). Among numerous cultivation techniques, the primary approaches for cultivating these microorganisms include *in vivo* cultivation using pot cultures and *in vitro* cultivation through Root organ cultures. *In vitro* cultivation offers controlled conditions but lacks ecological relevance, reducing host diversity and potentially impacting experimental results (Calvet *et al.* 2013). Morphological plasticity, where AMF spores exhibit variability in response to environmental factors, further complicates species identification (Walker *et al.* 2021). Studies suggest that the choice of AMF production system influences plant growth and experimental outcomes (Calvet *et al.* 2013). *In vitro* cultivation in stable conditions optimizes sporulation but neglects the fluctuating abiotic factors in natural environments (Heinemeyer and Fitter, 2004; Meyer *et al.* 2017). Additionally, *in vitro* lacks biotic interactions, essential for AMF's

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stress resistance and metabolic activity (Engelmoer *et al.* 2014; Gallone *et al.* 2016). *In vitro* cultivation often focuses on a single host, while natural environments involve multiple hosts, influencing AMF gene expression, and symbiotic relationships (Mateus *et al.* 2019; Angelard *et al.* 2010). *In vivo*-cultivated propagules benefit host plants more than *in vitro* generated propagules, leading to increased colonization and plant growth (Calvet *et al.* 2013). Repeated *in vitro* cultivation reduces symbiotic quality, impacting root morphology and phosphorus (P) benefits for plants (Kokkoris and Hart, 2019). Genetic variability among AMF isolates can contribute to functional differences (Pena *et al.* 2020; Kokkoris *et al.* 2019; Chen *et al.* 2018; Mathieu *et al.* 2018; Silvani *et al.* 2014; Sanders, 2010).

The presence and concentration of the Neutral Lipid Fatty Acid (NLFA) 16:1ω5c in AMF serve as an important indicator of the viable biomass of these fungi in both root systems and soil (Lekberg *et al.* 2022). The NFLA 16:1ω5c has been identified as a biomarker for energy storage lipids in AMF (Olsson, 1999). The utilization of Fatty Acid Methyl Ester analysis presents a viable and promising alternative method for the examination of lipids and their constituent fatty acids (Olsson *et al.*, 2003). This study illuminates the complexity of AMF and uncovers their diverse responses to culture conditions, offering insights for optimizing bioinoculants.

MATERIAL AND METHODS

Source of AMF

The AMF species investigated in the study included *Rhizophagus irregularis*, *Rhizophagus clarus*, *Entrophospora etunicata*, and *Funneliformis mosseae*. The AMF spores were obtained from Symbiotic Sciences Pvt Ltd. *In vivo* spores cultivated in a substrate comprising soil, sand, perlite and vermiculite (1:1:1:1 v/v) with *Zea mays* as the host, were isolated from a 90-day-old culture using the wet sieving and decanting method (Gerdemann and Nicolson, 1963). This involved hydrating 100 g of rhizosphere soil in 1000 mL or more distilled water with intermittent stirring. The resulting inoculum was poured onto a nested sieve set with specific mesh sizes (60BSS, 100BSS, and 300BSS), each retaining particular components. *In vitro* spores sourced from a conventional Root Organ Culture (co-cultures) with *Daucus carota* as the host, were isolated from a 90-day-old culture using the chelation solubilization technique (Doner and Bécard, 1991). Both *in vivo* and *in vitro* spores were collected using a micropipette under a stereomicroscope.

Morphological characterization

Optical Microscopy: The spores were observed in a water medium, placed on a glass Petri dish, and examined using an stereomicroscope for taxonomic classification. Extraradical spores ranging from 50-100 were mounted onto slides using PVLG solution (Koske and Tessier, 1983). Additionally, a mixture of PVLG and Melzer's reagent in a 1:1 volume/volume ratio (Brundett *et al.* 1994) was employed for slide preparation. Spore morphology was examined using a compound microscope in accordance with the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM). The spore sizes were determined utilizing software. The definitions of spore morphological and biochemical characteristics, as outlined by Walker (1983, 1986), Morton (1986, 2001), and Spain *et al.* (2006), provide insights into the intricate structures of AMF spores. Murograms visually represent spore wall layer arrangements, offering insights into AMF structural characteristics (Walker, 1983). The morphological analysis enhances our understanding of spore development under different conditions.

Scanning Electron Microscopy (SEM): Around 50 extraradical spores were fixed primarily immersing the samples in a 2.5% glutaraldehyde solution at room temperature (25°C) for 2 hours. Subsequently, a set of five phosphate buffer washes at pH 7.2 (50 mM) were conducted at room temperature, with each wash lasting 10 minutes. For the secondary fixation, a 1% osmium tetraoxide solution was applied at 4°C for 1 hour, followed by an additional six phosphate buffer washes under the same conditions. To facilitate the subsequent steps, a dehydration process was undertaken using chilled 10% ethanol at room temperature for 10 minutes. The spores were

meticulously positioned onto aluminum stubs using carbon double-stick tape, coated with gold and then subjected to imaging using a SEM that operated within the range of 3 to 10 kV (Roesti *et al.* 2005).

Biochemical characterization

In a sealed glass tube, 100 extraradical spores were saponified with 3.75M NaOH in 50% MeOH, then heated for 30 minutes, cooled, and mixed with 3.25M HCl in 45% MeOH, followed by 10 minutes at 80^{0}C. After cooling, Hexane: MTBE solution was added, and the upper layer was collected. A base wash with 0.024M NaOH was performed, and the organic layer was prepared for GC analysis. GC-MS with specific settings were used to identify FAMEs by comparing retention times through the software Mass spectrometry detector to a standard chromatogram as suggested by Olsson and Johansen (2000).

Assessment of AM root colonization

The experimental set-up consisted of 8 treatments in replicates of 3. The treatments were: *in vitro Rhizophagus irregularis* (T1), *in vitro Rhizophagus clarus* (T2), *in vitro Entrophospora etunicata* (T3), and *in vitro Funneliformis mosseae* (T4), *in vivo R. irregularis* (T5), *in vivo R. clarus* (T6), *in vivo E. etunicata* (T7), and *in vivo F. mosseae* (T8). AMF spores were uniformly introduced at a rate of 200 spores per plant using a micropipette. The experiment was conducted in a growth substrate comprising autoclaved sand, soil, vermiculite and perlite in equal proportions (v/v), with *Sorghum bicolor* as the test crop in replicates of three using 250-g pots. *Sorghum* seeds underwent sterilization through ethanol rinsing and treatment with a 4% sodium hypochlorite solution for 1 minute, followed by thorough water rinsing and overnight incubation in darkness for germination. Each pot was planted with three healthy seedlings. Throughout the 8-week growth period, substrate moisture was maintained at 90% field capacity, and half-strength Hoagland solution (Hoagland, 1933) was applied fortnightly. Greenhouse conditions comprised a temperature range of 25°C (77°F), 50% relative humidity, and controlled light conditions (200-400 μmol/m²/s) to support photosynthesis under a 16-8-hour photoperiod. The plants were harvested after 2 months. To assess root colonization by mycorrhizal fungi, the roots were stained using the Phillips and Hayman (1970) method, and the extent of colonization was evaluated employing the McGonigle *et al.* (1990).

Statistical Analysis

Statistical analyses were conducted using R version 4.3.0 (2023-04-21 ucrt) (Copyright © 2023). After conducting a one-way analysis of variance (ANOVA) at a 95% confidence level (alpha = 0.05), a subsequent Tukey HSD test was performed to identify significant differences among the different treatments.

RESULTS

Morphological characterization

A comparison of the spore morphology of *Rhizophagus irregularis*, *Rhizophagus clarus*, *Entrophospora etunicata,* and *Funneliformis mosseae* produced under different conditions was carried out. It was observed that spores developed under *in-vitro* conditions appear smaller than those produced under *in vivo*. A detailed comparative analysis of the spore morphology is presented in Table 1. Fig. 1 presents the spore size distribution of the above-mentioned AMF species grown in different conditions. Across all species, *in vivo* spores consistently exhibited larger diameters and broader ranges than their *in vitro* counterparts. *In vivo* spores of *R. irregularis* exhibited a mean diameter of 161.28μ, significantly larger than the *in vitro* mean of 92.38μ. Similarly, *R. clarus in vivo* spores had a mean diameter of 158.58μ compared to the *in vitro* mean of 141.25μ. In the case of *E. etunicata*, the *in vivo* mean diameter was 177.63μ, notably larger than the *in vitro* mean of 107.43μ. *F. mosseae* showed a larger mean diameter for *in vivo* spores (201.87 μ) compared to *in vitro* spores (185.56μ). The optical microscopic images based on morphological characterization showed no difference between *in vitro* and *in vivo* spores with respect to Melzer's reaction (Fig. 2). Further analysis of morphological structures through SEM micrographs revealed

Table 1 : Morphological analysis of intact spores of different Arbuscular Mycorrhizal Fungi grown in *in vitro* and *in vivo*

Parameters	*R. irregularis*	*R. clarus*	*E. etunicata*	*F. mosseae*
Colour of the spore in water*	Hyaline (juvenile spores 0-0-10-0)* yellow, brown (mature spores 0-10-40-0)	Hyaline juvenile spores 0-10-60-0), white, pale-yellow brown (mature spores 0-10-20-0)	Hyaline (juvenile spores 0-10-90-5), white, yellow, brown (mature spores 0-60-100-0)	Yellow (juvenile spores 0-40-100-10) to brown (mature spores 0-10-60-0)
Colour of the spore in mountant (PVLG)	Hyaline, Yellow, Brown	Hyaline, White, Pale yellow, brown	Hyaline, White, Yellow, Brown	Yellow, B rown
Colour of the spore in mountant (PVLG: Melzer's)	Permanent laminate layer (L3) stains dark-red brown colour	Hyaline mucilaginous layer (L1) stains pink	Hyaline mucilaginous layer (L1) stains pink	Hyaline mucilaginous layer (L1) stains reddish pink
Shape of the spore	Globose, sub globose, ovoid, oblong, irregular appearing knobby	Either borne solitary or in aggregates in variable numbers	Either borne solitary or in aggregates in variable numbers	Globose (mainly), sub globose
Occurrence	Either borne solitary or in aggregates in variable numbers	Either borne solitary or in aggregates in variable numbers	Either borne solitary or in aggregates in variable numbers	Either borne solitary or in aggregates
Diameter of *in vitro* spores (μm)**	34.21 ± 0.40 to 158.50 ± 1.79	19.56 ± 0.29 to 227.71 ± 2.16	57.16 ± 0.53 to 160.70 ± 1.83	25.65 ± 0.16 to 288.36 ± 2.86
Diameter of *in vivo* spores (μm)**	91.43 ± 1.12 to 227.21 ± 3.28	29.32 ± 0.53 to 228.35 ± 4.29	92.83 ± 1.62 to 218.53 ± 4.16	66.28 ± 1.12 to 372.87 ± 4.16
Hyphal wall	Layers 1–3 in both young and most mature spores	Layers 1-3	Layers 1-2	Layers 1-3
Occlusion	Occluded by a curved septum, continuous with the innermost lamina of spore wall layer	No occlusion	Innermost laminae act as septum to the pore	Recurved septum present
Hyphal attachment	Straight, recurvate, flared	Cylindrical to flared	Cylindrical	Flared to funnel shaped
Spores formed within root	Yes	Yesu	Yes	Yes
Sporocarp present	No, spores in loose clusters	No, spores in loose clusters	No, spores in loose clusters	No, spores in loose clusters

*Colour code of AMF spores as recommended by International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM).
**Average range of diameter of spores (min to max), ±=SE

more differences between *in vitro* and *in vivo* spores, with the former exhibiting a smoother surface and the latter a rougher texture (Fig. 3 a-h) Examination of spore wall layers reveals unique spore wall compositions, and corresponding murograms. Both *R. irregularis* and *R. clarus* had two wall layer groups with *R. irregularis* having two layers of temporary hyaline succeeded by one layer of permanent laminate while *R. clarus* had outer wall group comprising of one temporary mucilagenous hyaline layer and permanent hyaline layer followed by one layer of permanent laminate. In contrast, *E. etunicata* displayed a simpler composition with one layer of each. In comparison, *F. mosseae* exhibited a distinct arrangement with one layer of temporary hyaline, an additional layer of temporary hyaline, and one layer of permanent laminate. Our findings were

Table 2: Neutral Lipid Fatty Acid content of different species of Arbuscular Mycorrhizal Fungi grown in i*n vitro* and *in vivo* conditions detected by Fatty Acid Methyl Ester analysis, expressed in terms of percentage

Type of fatty acid	*R. irregularis*	*R. clarus*	*E. etunicata*	*F. mosseae*
In vitro condition				
C12:0	0.44	0.77	0.83	0.29
C14:0		32.06		
C16:ω5c	68.59	25.42	24.85	29.99
C16:1ω9				
C17:1ω10		20.83		
C18:1ω9	18.99		42.23	12.98
C18:2ω9,12			3.11	
C18:3ω9,12,15		12.53		
C20:0		52.74		
C20:1ω11	6.37			
C21:0	3.69		21.83	2.60
C22:0	1.89			54.14
C22:1ω13	0.03		7.15	
In vivo condition				
C12:0	0.99		0.97	0.64
C14:0				
C16:ω5c	60.27	58.64	29.81	51.14
C16:1ω9		21.04		2.64
C17:1ω10		15.03		
C18:1ω9	22.17		37.29	24.72
C18:2ω9,12			12.80	3.19
C18:3ω9,12,15		10.63		
C20:0		7.32		
C20:1ω11	9.46	1.67		
C21:0	5.04		18.21	8.70
C22:0	2.04		0.92	8.97
C22:1ω13	0.02			

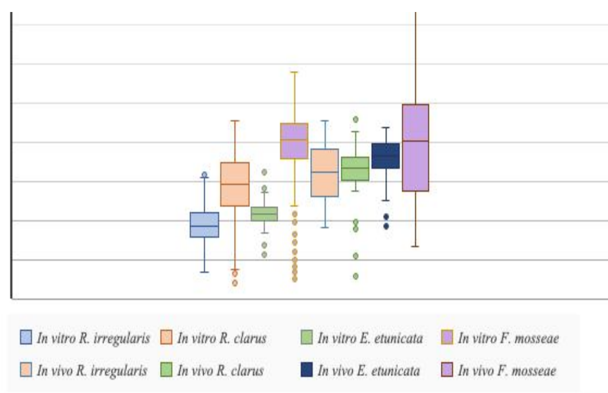

Fig. 1. Spore size distribution of Arbuscular Mycorrhizal Fungi grown in different conditions. Across all species, *in vivo* spores consistently exhibited larger diameters and broader ranges than their *in vitro* counterparts. *In vivo* spores of *R. irregularis* exhibited a mean diameter of 161.28µ, significantly larger than the in vitro mean of 92.38µ. Similarly, *R. clarus in vivo* spores had a mean diameter of 158.58µ compared to the *in vitro* mean of 141.25µ.

consistent with other research, emphasizing differences in the spore sizes, particularly when cultivated under diverse conditions and diverse spore wall structure among these AMF species. The hyaline mucilaginous layer, present in juvenile-stage spores of *R. clarus*, *E. etunicata* and *F. mosseae* cultured across different conditions in all AMFs exhibited a smooth or slightly roughened texture and stained in Melzer's reagent, when present, while it is the permanent laminate layer of *R. irregularis* that stained in Melzer's reagent. The outer layer deteriorated rapidly, sloughing off even in young spores, and was consequently rarely present in the spore wall structure of mature spores. The laminate layer, characterized by a conglomerate of extremely thin sublayers, each typically less than 0.5 µm thick,

appeared as separate groups or innermost sublayers when spores were vigorously crushed (Fig. 3 i-l)

Biochemical characterization

Table 2 and Fig. 4 presents the concentrations of NFLA for *R. irregularis, R. clarus, E. etunicata*, and *F. mosseae*, cultivated both *in vitro* and *in vivo.* Fig. 5 highlights the differences in the retention times of marker fatty acids, both between the species an d among the same species, and the variations in concentrations of these marker fatty acids.

The FAME analysis of *R. irregularis*, *in vivo* conditions, showed significant differences in fatty acid percentages compared to those of *in vitro* conditions. C12:0 (lauric acid) is notably higher at 0.99% *in vivo* versus 0.44% *in vitro*. Other fatty acids, including C18:1ω9 (oleic acid) at 22.17% (*in vivo*) vs. 18.99% (*in vitro*), eicosenoic acid (C20:1ω11) at 9.46% (*in vivo*) vs. 6.37% (*in vitro*), and C21:0 at 5.04% (*in vivo*) vs. 3.69% (*in vitro*). In contrast, C16:1ω5c (palmitoleic acid) was higher *in vitro* at 68.59% compared to 60.27% *in vivo*. Both conditions exhibited low levels of C22:1ω13 (cis-13-Docosanoic acid), with minor differences (0.03% *in vitro* and 0.02% *in vivo*).

The FAME analysis of *R. clarus*, differences in fatty acid composition between *in vitro* and *in vivo* conditions weere evident. In particular, *in vivo* conditions resulted in a substantial increase in the concentration of C14:0 (myristic acid) at 58.64%, compared to 32.06% *in vitro*. Conversely, *in vitro* conditions exhibited a higher percentage of C20:0 (arachidic acid) at 52.74%, whereas *in vivo* conditions showed a lower percentage at 7.32%. Other fatty acids, such as C16:1ω5c (palmitoleic acid), C17:1ω10 (heptadecenoic acid), and C18:3ω 9,12,15c (linolenic acid), displayed lower percentages *in vivo* compared to *in vitro* conditions. C20:1ω11 (eicosenoic acid) was found only in *in vitro* conditions at 1.67%.

The FAME analysis of *E. etunicata,* showed variations in fatty acid composition between *in vitro* and *in vivo* conditions. Specifically, *in vivo* conditions resulted in a slightly higher percentage of C12:0 (lauric acid) at 0.97%, compared to 0.83% *in vitro*. Regarding C16:1ω5c (palmitoleic acid), *in vivo* conditions yielded a higher percentage at 29.81%, while *in vitro* conditions had 24.85%. Similarly, C18:1ω9 (oleic acid) was slightly lower *in vivo* at 37.29%, compared to 42.23% *in vitro*. The most significant difference was observed in C18:2ω9,12 (linoleic acid), which was substantially higher *in vivo* at 12.80% compared to 3.11% *in vitro*. C21:0 (heneicosanoic acid) was also slightly lower *in vivo* at 18.21% compared to 21.83% *in vitro*. Lastly, C22:0 (behenic acid) exhibited a drastic decrease *in vivo* at 0.92% compared to 7.15% *in vitro*.

The FAME analysis of *F. mosseae* showed evident differences in fatty acid composition between *in vitro* and *in vivo* conditions. Under *in vivo* conditions, there was a notable increase in the percentage of C12:0 (lauric acid) at 0.64%, compared to 0.29% *in vitro*. Likewise, C16:1ω5c (palmitoleic acid) displayed a substantial increase *in vivo* at 51.14%, whereas *in vitro* conditions had 29.99%. C16:1ω9 was present only in *in vivo* conditions at 2.64%. C18:1ω9 (oleic acid) also showed a significant increase *in vivo* at 24.72% compared to 12.98% *in vitro*. However, C18:2ω9,12 (linoleic acid) was detected only *in vitro* at 3.19%. C21:0 (heneicosanoic acid) and C22:0 (docosanoic acid) exhibited higher percentages *in vivo* at 8.70% and 8.97%, respectively, compared to lower values *in vitro*.

Infectivity Assay

Fig. 6 showed a statistical difference in the infectivity potential of different AMF spores cultured in distinct conditions at 95% confidence level (Treatments T1 to T8). Fig. 7 showed the root colonization. In the *in vitro* conditions, it can be observed that *R. irregularis* had an infectivity potential of 72.10%, which was lower than that observed in the *in vivo* conditions, where it increased to 82.33%. Similarly, for *R. clarus*, the *in vivo* infectivity potential was notably higher at 51.00% compared to the *in vitro* value of 48.00%. *E. etunicata's* infectivity potential was higher in the *in vitro* conditions (77.72%) compared to the *in vivo* conditions (78.67%). *F. mosseae* exhibited an infectivity potential of 66.55% *in vitro* and 75.33% *in vivo*.

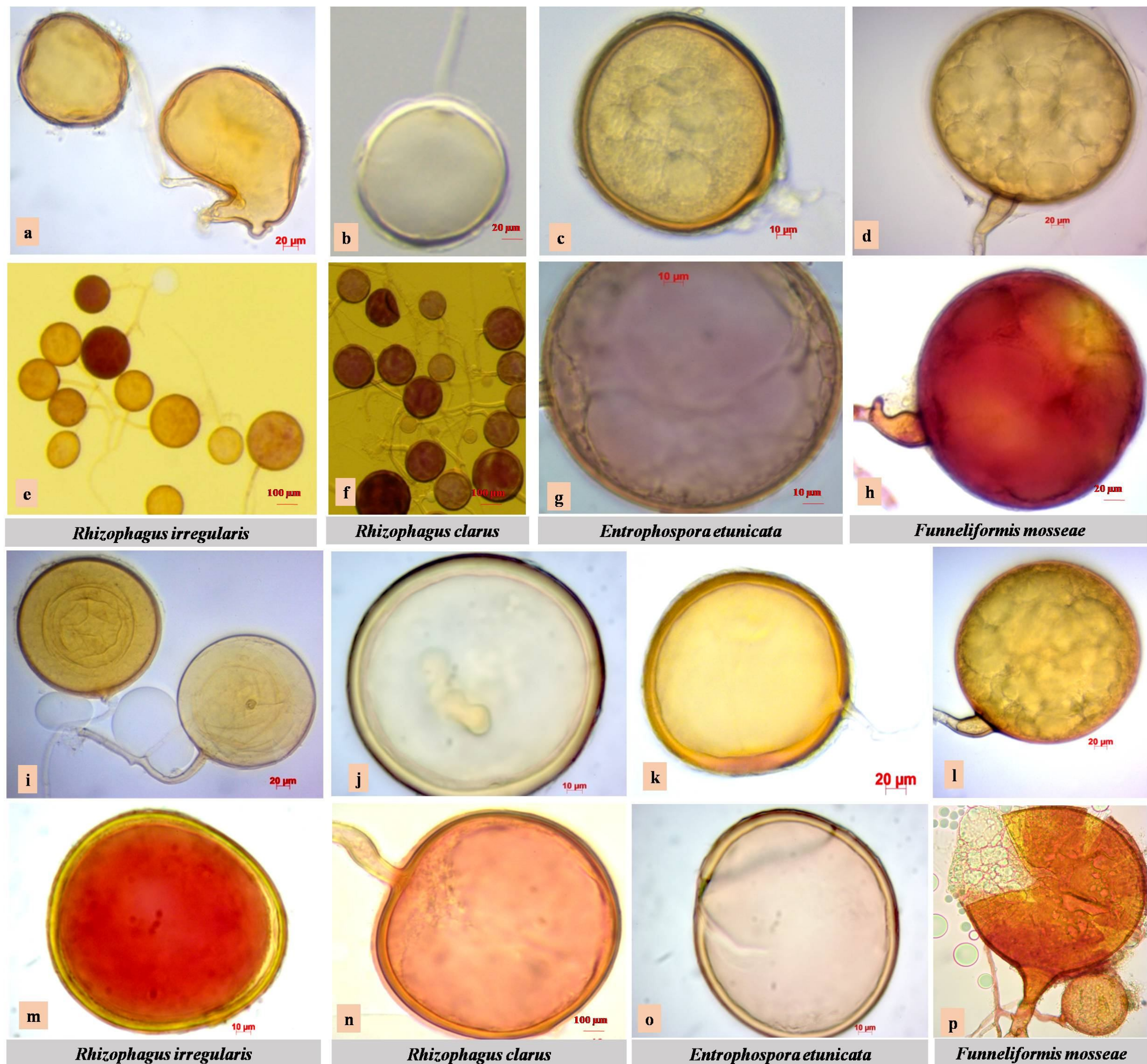

Fig. 2: Microscopic analysis of in vitro (a-h) and in vivo (i-p) spores of Arbuscular Mycorrhizal Fungi. After treating the spores with Melzer's reagent and PVLG. The hyaline mucilaginous layer, present in juvenile-stage spores of *R. clarus*, *E. etunicata* and *F. mosseae* cultured across different conditions in all AMFs exhibited a smooth or slightly roughened texture and stained in Melzer's reagent, when present, while it is the permanent laminate layer of *R. irregularis* that stained in Melzer's reagent.

DISCUSSION

The observation of this study regarding the smaller spore size of *in vitro* differentiated spores than *in vivo* spores align with previous research findings where the spores produced in a laboratory setting tend to be slightly smaller when compared to spores found in natural soil environments (Danesh *et al.* 2016; Calvet *et al.* 2013). This could be due to the specific adaptations of fungus to the growth medium utilized and the depletion of nutrients or carbon within the *in vitro* ROC (Calvet *et al.* 2013). Optical microscopy and SEM have both shown that the spore surfaces cultivated *in vivo* exhibit roughness. This roughness can be likely due to the adherence of soil particles due to the release of glomalin (Li *et al.*, 2022) or sloughing off of outer wall layer or adherence of numerous rhizobia and pseudomonads on the surfaces of spores and hyphae (Roesti *et al.* 2005; Agnolucci *et al.* 2015).

The confirmation of the Glomalean nature of these microorganisms is established by the consistent presence of marker fatty acids, specifically, neutral lipid fatty acid (NLFA) C16:ω5c, found across the

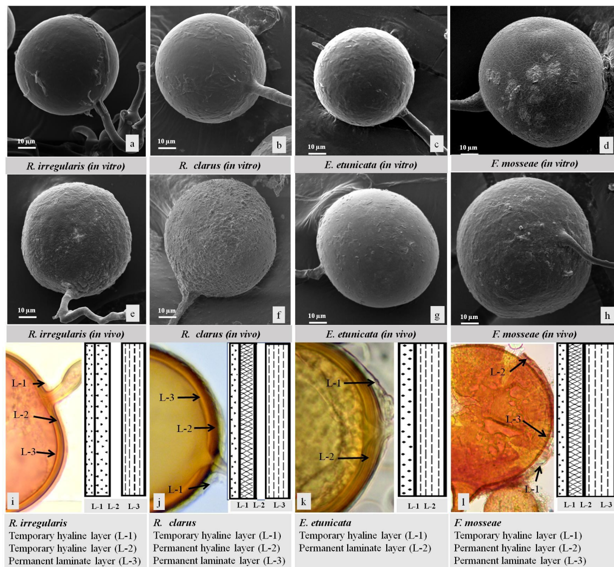

Fig. 3. Scanning Electron Micrograph (SEM) of Arbuscular Mycorrhizal Fungi spores grown in two different cultural conditions (a-h). Spore wall layer analysis of Arbuscular Mycorrhizal Fungi and their corresponding murograms (i-l). Both *R. irregularis* and *R. clarus* had two wall layer groups with *R. irregularis* having two layers of temporary hyaline succeeded by one layer of permanent laminate while *R. clarus* had outer wall group comprising of one temporary mucilagenous hyaline layer and permanent hyaline layer followed by one layer of permanent laminate. In contrast, *E. etunicata* displayed a simpler composition with one layer of each. In comparison, *F. mosseae* exhibited a distinct arrangement with one layer of temporary hyaline, an additional layer of temporary hyaline, and one layer of permanent laminate.

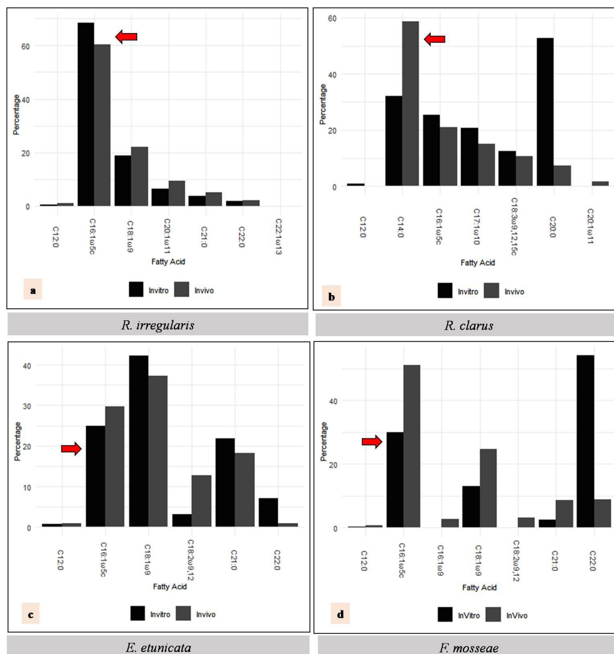

Fig. 4: Comparison C16:ω5c, marker fatty acid percentage observed in *in vitro* and *in vivo* AMF spores. The figure depicts the differences in the retention times of marker fatty acids, both between the species and among the same species, and the variations in concentrations of these marker fatty acids.

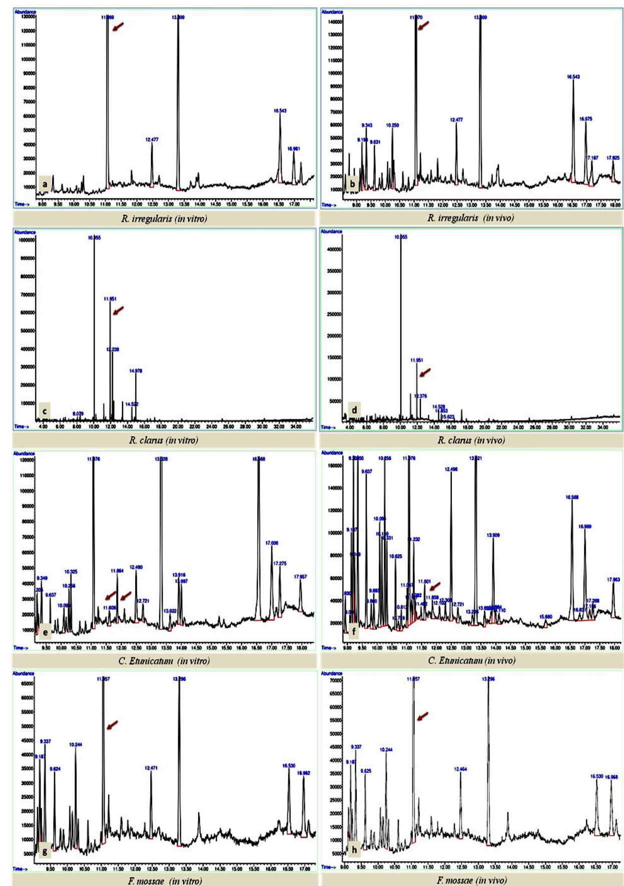

Fig. 5: Fatty Acid Methyl Ester (FAME) chromatograms carried out for comparison of *in vitro* and *in vivo* AMF spores. Peaks indicated by arrow are marker fatty acid Peaks for C16:ù5c, marker fatty acid observed in AMF of both the conditions.

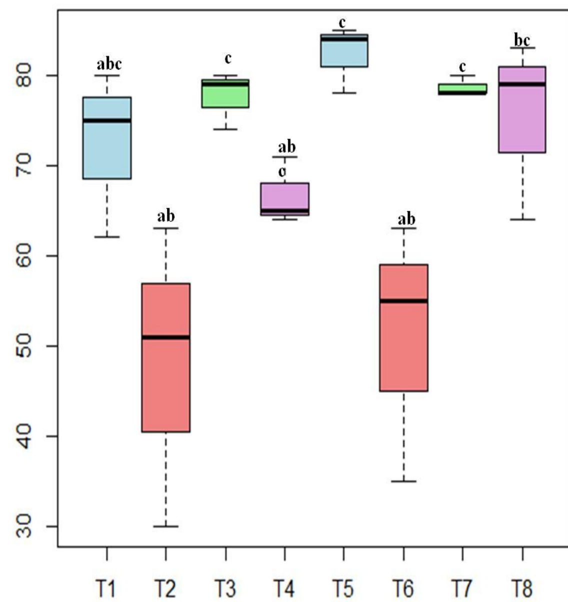

Fig. 6: Mycorrhizal colonization percentage of the Arbuscular Mycorrhizal spores cultured under two distinct cultural conditions. Statistical analysis of the difference in the infectivity potential of different AMF spores cultured in distinct conditions at 95% confidence level (Treatments T1 to T8).

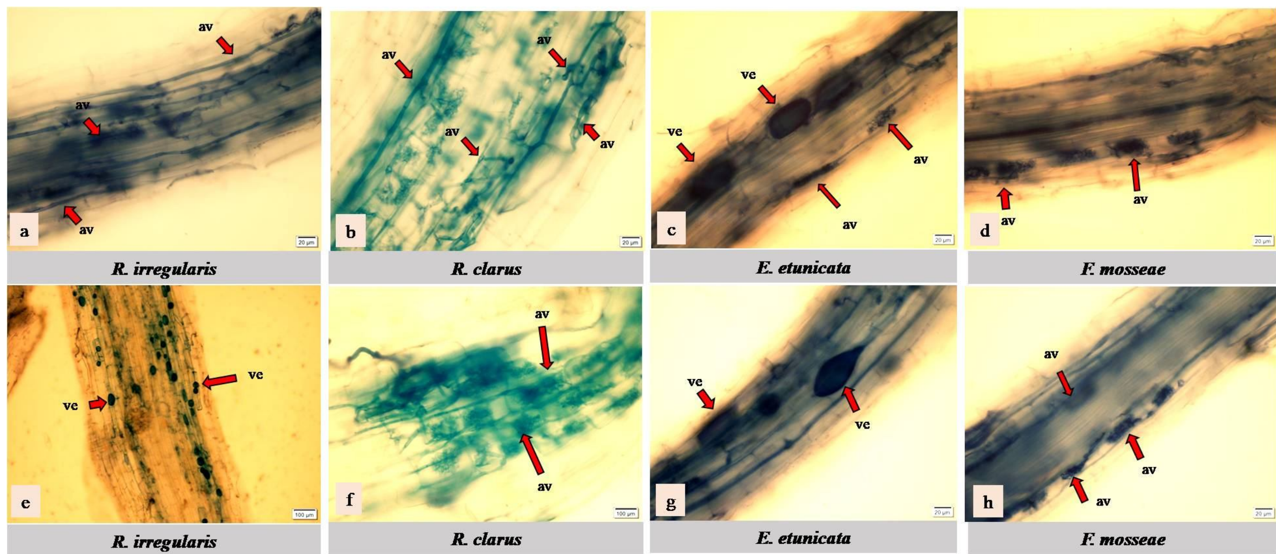

Fig. 7: Infectivity assay by the Arbuscular Mycorrhizal spores cultivated under two distinct cultural conditions. The assay was conducted 60 days post-inoculation with 200 AMF spores per plant. The host for the assay was *Sorghum bicolor*, grown in 250-gram pots filled with a sterile mixture of soil, sand, vermiculite, and perlite in equal proportions (1:1:1:1 v/v).

spores of all AMF species cultured in different conditions. In the study, we observed that the highest levels of C16:ω5c were present in all the spores cultivated *in vivo* whereas only *R. irregularis* cultivated *in vitro* only exhibited its highest concentrations. This accumulation of monounsaturated C16 fatty acids in membrane and storage lipids serves as a protective mechanism for AMF spores and hyphae in the soil, potentially acting as a shield against potential threats posed by bacteria or other fungi. Additionally, the host may perceive it as a symbiotic interaction, potentially aiding in evading the host's immune response (Brands *et al.* 2020). Our findings about the increased presence of fatty acid 18:1ω9c and decreased levels of 16:1ω5c in *E. etunicata*, align with the results reported in the research conducted by Allison and Miller (2004).

Our study shows that the colonization rates were consistently higher in *in vivo* AMF spores compared to those produced *in vitro* spores using Ri T-DNA transformed carrot roots. This disparity could be ascribed to various factors, including the potential presence of beneficial bacteria and absence of carbon availability associated with *in vivo*-produced AMF (Barea *et al.* 2005). Moreover, the absence of coexisting bacteria, the absence of abiotic stimuli, and the lack of host diversity may influence the infectivity of AMF spores raised *in vitro* (Kokkoris and Hart, 2019). The enhanced colonization rate observed in *in vivo* AMF spores could also be hypothetically attributed to their larger spore size as spore size has been shown to have a direct correlation with the number of nuclei that could potentially influence the fungal ability to develop post-germination and establish a symbiotic relationship with plants (Marleau *et al.* 2011). Previous research by Bharadwaj *et al.* (2012) and Lioussanne *et al.* (2010) has established the mutualistic interactions between BLOs and AMF wherein these bacteria play a crucial role in fungal development, affecting hyphal growth and branching, and are commonly found in various AMF species (Lumini *et al.* 2007). Notably, *R. irregularis*, a species forming smaller intraradical spores, experiences changes in the symbiotic process when cultured *in vitro*, potentially linked to the loss of associated bacteria (Calvet *et al.* 2013).

Diop (2003) emphasized the need to investigate the effectiveness of such propagules in natural conditions, and our results reinforce the importance of expanding our understanding of colonization dynamics and interactions between AMF and host plants regarding the origin of inocula. While these observations have led to intriguing hypotheses, our primary objective was to ascertain whether *in vitro*-produced spores of

the same AMF were functionally equivalent or different to those obtained through conventional pot culture systems.

CONCLUSION

This study on morphological and biochemical characterizations of AMF species cultivated in both *in vivo* and *in vitro* conditions has provided valuable insights into the complexity of these symbiotic microorganisms like the difference in spore sizes within the species when cultivated in different environments, the irregularity observed on the surfaces of *in vivo*-cultivated spores, the distinct differences in fatty acid composition between *in vivo* and *in vitro* cultivated spores. The observations suggest that AMF species may exhibit distinct responses to cultivation conditions, potentially influencing their effectiveness as bioinoculants. Significant variability in colonization behaviour is observed among different AMF species, highlighting the importance of understanding the inocula source, dynamic interactions of AMF with biotic and abiotic stimuli like carbon, etc. Our study contributes to the growing knowledge surrounding AMF biology and cultivation, highlighting their intricate relationships with their environment, and the potential for optimizing their use as bioinoculants. Further research in this area holds promise for enhancing agricultural sustainability and ecosystem health.

DECLARATIONS

Conflict of interest : Authors declare no conflict of interest.

REFERENCES

Agnolucci, M., Battini, F., Cristani, C., and Giovannetti M. 2015. Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates. *Biol. Fertil. Soils* **51**: 379–389. 10.1007/s00374-014-0989-5

Allison, V. and Miller, R. 2004. Using fatty acids to quantify arbuscular mycorrhizal fungi. In: *Mycorrhizae: Basic Research and Applications* (Eds. G. Podila, A. Varma) pp.141-161. I.K. International Pvt. Ltd. New Delhi.

Angelard, C., Colard, A., Niculita-Hirzel, H., Croll, D., Sanders, I.R. 2010. Segregation in a mycorrhizal fungus alters Rice growth and symbiosis-specific gene transcription. *Curr. Biol.* 20: 1216–1221. doi: 10.1016/j.cub.2010.05.031

Barea, J., Pozo, M.J., Azcón, R. 2005. Concepción Azcón-Aguilar, Microbial co-operation in the rhizosphere, *J. Exp. Bot.* 56: 1761–1778. 10.1093/jxb/eri197

Bharadwaj, D.P., Alström, S. and Lundquist, PO. 2012, Interactions among *Glomus irregulare*, arbuscular mycorrhizal spore-associated bacteria, and plant pathogens under *in vitro* conditions. *Mycorrhiza* **22**: 437–447. 10.1007/s00572-011-0418-7

Brands, M., Cahoon, E.B., Dörmann, P. 2020. Palmitvaccenic acid (Ä11-cis-hexadecenoic acid) is synthesized by an OLE1-like desaturase in the arbuscular mycorrhiza fungus *Rhizophagus irregularis*. *bioRxiv* (Cold Spring Harbor Laboratory). 10.1101/2020.01.13.901264

Brundrett M., Melville L., Peterson L. 1994. Practical Methods in Mycorrhizal Research. Guelph: *Mycologue Publications*

Brundrett, M.C., Tedersoo, L. 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytol.* **220**:1108–1115. 10.1111/nph.14976

Calvet, C., Camprubí, A., Pérez-Hernández A., and Lovato, P. 2013, Plant Growth Stimulation and Root Colonization Potential of *In vivo* versus *In vitro* Arbuscular Mycorrhizal Inocula. *Hort Sci.* **48**: 897-901. 10.21273/HORTSCI.48.7.897.

Chen, M., Arato, M., Borghi, L., Nouri, E., Reinhardt, D. 2018. Beneficial Services of Arbuscular Mycorrhizal Fungi – From Ecology to Application. *Front. Plant Sci.* **9**:1270. 10.3389/fpls.2018.01270

Danesh, Y.R., Najafi, S., and Demir, S. 2016. Using *In vitro* Culturing Technique for Studying Life Cycle of Arbuscular Mycorrhizal Fungus (AMF) *Glomus intraradices*. *Yyu J. Agr. Sci.* **26**: 161-167.

Diop, T.A. 2003. *In vitro* culture of arbuscular mycorrhizal fungi: Advances and future prospects. *Afr. J. Biotechnol.* **2**: 692-697. 10.5897/AJB2003.000-1128

Engelmoer, D.J.P., Behm, J.E., and Toby Kiers, E. 2014, Intense competition between arbuscular mycorrhizal mutualists in an *in vitro* root microbiome negatively affects total fungal abundance. *Mol. Ecol.* **23**: 1584–1593. 10.1111/mec.12451

Doner L.W., and Bécard, G. 1991, Solubilization of gellan gels by chelation of cations. *Biotechnol. Tech.* **5**: 25–28. 10.1007/bf00152749

Gallone B., Steensels J., Prahl T., Soriaga L., Saels V.,Herrera-Malaver B. 2016. Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. *Cell* **166**: 1397–1410.e16. 10.1016/j.cell.2016.08.020

Heinemeyer, A., Fitter, A.H. 2004. Impact of temperature on the arbuscular mycorrhizal (AM) symbiosis: growth responses of the host plant and its AM fungal partner. *J. Exp. Bot.* 55: 525–534. 10.1093/jxb/erh049

Huang, D., Ma, M., Wang, Q., Zhang, M., Ma, F. 2020, Arbuscular mycorrhizal fungi enhanced drought resistance in apple by regulating genes in the MAPK pathway. *Plant Physiol. Biochem.* **149**: 245–255. 10.1016/j.plaphy.2020.02.020

Identification of Vesicular Arbuscular Mycorrhizal Fungi. (INVAM). University of Florida, Gainesville

Kokkoris, V., Hart, M.M. 2019. *In vitro* Propagation of Arbuscular Mycorrhizal Fungi May Drive Fungal Evolution. *Front. Microbiol.* **10**. 10.3389/fmicb.2019.02420

Kokkoris, V., Hart, M.M. 2019. The role of *in vitro* cultivation on symbiotic trait and function variation in a single species of arbuscular mycorrhizal fungus, *Fun. Biol.* **123**: 732-744. 10.1016/j.funbio.2019.06.009.

Koske, R.E., Tessier, B. 1983. A convenient, permanent slide mounting medium. *Mycologia Soc. Amer. Newsl.* **34**: 59

Lekberg, Y., Bååth, E., Frostegård, Å., Hammer, E.C., Hedlund, K., Jansa, J., Kaiser, C., Ramsey, P.W., Øezanka, T., Rousk, J., Wallander, H., Welc, M.,Olsson, P.A. 2022. Fatty acid 16:1ù5 as a proxy for arbuscular mycorrhizal fungal biomass: current challenges and ways forward. *Biol. Fertil. Soils*, **58**: 835–842. 10.1007/s00374-022-01670-9

Li, Y., Xu, J., Hu, J., Zhang, T., Wu, X.,Yang, Y. 2022. Arbuscular Mycorrhizal Fungi and Glomalin Play a Crucial Role in Soil Aggregate Stability in Pb-Contaminated Soil. *Inter. J. Environ. Res.Public Health* **19**: 5029. 10.3390/ijerph19095029

Li, C., Cano, M., Acosta-Martinez, V., Veum, K.S., Moore-Kucera, J. 2020, A comparison between fatty acid methyl ester profiling methods (PLFA and EL-FAME) as soil health indicators. Li, C., Cano, M., Acosta-Martinez, V., Veum, K.S., Moore-Kucera, J. 2020, A comparison between fatty acid methyl ester profiling methods (PLFA and EL-FAME) as soil health indicators. *Soil Sci. Soc. Amer. J.* 10.1002/saj2.201118

Lioussanne, L. 2010, The role of the arbuscular mycorrhiza-associated rhizobacteria in the biocontrol of soilborne phytopathogens Span. *J. Agr. Res.* **8** S51- S61

Lumini, E., Bianciotto, V., Jargeat, P., Novero, M., Salvioli, A., Becard, G., Bonfante, P. 2007. Presymbiotic growth and sporal morphology are affected in the arbuscular mycorrhizal fungus *Gigaspora margarita* cured of its endobacteria. *Cell. Microbiol.* **9**: 1716-1729. 10.1111/j.1462-5822.2007.00907.x

Madan, R., Pankhurst, C., Hawke, B., Smith, S. 2002. Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil, Soil Biol. Biochem. 34(1), 125-128

Marleau, J., Dalpé, Y., St-Arnaud, M. and Hijri, M. 2011. Spore development and nuclear inheritance in arbuscular mycorrhizal fungi. *BMC Evol. Biol.* **11**: 5. 10.1186/1471-2148-11-51

Mathieu, S., Cusant, L., Roux, C., Corradi, N. 2018. Arbuscular mycorrhizal fungi: intraspecific diversity and pangenomes. *New Phytol.* **220**: 1129–1134. 10.1111/nph.15275

Mateus, I.D., Masclaux, F.G., Aletti, C., Rojas, E.C., Savary, R., Dupuis, C. 2019, Dual RNA-seq reveals large-scale non-conserved genotype×genotype-specific genetic reprograming and molecular crosstalk in the mycorrhizal symbiosis. *ISME J.* **13**: 1226–1238. 10.1038/s41396-018-0342-3

McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L., Swan, J. 1990. A new method which gives an objective measure of colonization of roots by vesicular—arbuscular mycorrhizal fungi. *New Phytol.* 115: 495–501. 10.1111/j.1469-8137.1990.tb00476.x

Meyer, M., Bourras, S., Gervais, J., Labadie, K., Cruaud, C., Balesdent, M.-H. 2017. Impact of biotic and abiotic factors on the expression of fungal effector-encoding genes in axenic growth conditions. *Fung. Genet. Biol.* **99** 1–12. doi: 10.1016/j.fgb.2016.12.008

Morton, J.B. 1986. Three New Species of Acaulospora (Endogonaceae) From High Aluminum, Low Ph Soils in West Virginia, *Mycologia* **78**: 641-648, doi: 10.1080/00275514.1986.12025300

Morton, J. B., Redecker, D. 2001. Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters, *Mycologia* **93**: 181-195. doi: 10.1080/00275514.2001.12063147

Naumann, M., Schübler, A., Bonfante, P. 2010. The obligate endobacteria of arbuscular mycorrhizal fungi are ancient heritable components related to the Mollicutes. *ISME J.* **4**: 862–871. 10.1038/ismej.2010.21

Olsson, P.A. 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. *FEMS Microbiol. Ecol.* **29**: 303–310. 10.1016/s0168-6496(99)00021-5

Olsson, A., Johansen, A. 2000. Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. *Myco Res.* **104**: 429-434.

Olsson, P.A., Larsson, L., Bago, B., Wallander, H., van Aarle, I.M. 2003. Ergosterol and fatty acids for biomass estimation of mycorrhizal fungi. *New Phytol.* **159**:1-10.

Peña, R., Robbins, C., Corella, J.C., Thuita, M., Masso, C., Vanlauwe, B., Signarbieux, C., Rodríguez, A., Sanders, I.R. 2020. Genetically Different Isolates of the Arbuscular Mycorrhizal Fungus *Rhizophagus irregularis* Induce Differential Responses to Stress in Cassava. *Front. Plant Sci.***11**. 10.3389/fpls.2020.596929

Phillips, J.M., Hayman, D.A. 1970. Improved Procedures for Clearing Roots and Staining Parasitic and Vesicular-Arbuscular Mycorrhizal Fungi for Rapid Assessment of Infection. *Trans. Brit. Myc. Soc.* **55**: 158-161. 10.1016/S0007-1536(70)80110-3

Pivato, B., Offre, P., Marchelli, S. *et al.* 2009, Bacterial effects on arbuscular mycorrhizal fungi and mycorrhiza development as influenced by the bacteria, fungi, and host plant. *Mycorrhiza* **19**: 81–90. 10.1007/s00572-008-0205-2

Roesti, D., Ineichen, K., Braissant, O., Redecker, D., Wiemken, A., Aragno, M. 2005. Bacteria associated with spores of the arbuscular mycorrhizal fungi *Glomus geosporum* and *Glomus constrictum*. *Appl. Environ. Microbiol.* **71**: 6673–6679. 10.1128/AEM.71.11.6673-6679.2005

Sanders, I.R. 2010. 'Designer' mycorrhizas?: Using natural genetic variation in AM fungi to increase plant growth. *ISME J.* **4**: 1081–1083. 10.1038/ismej.2010.109

Silvani, V., Bidondo, L.F., Bompadre, J., Colombo, R., Pérgola, M., Bompadre, A., Fracchia, S., Godeas, A.M. 2014. Growth dynamics of geographically different arbuscular mycorrhizal fungal isolates belonging to the '*Rhizophagus* clade' under monoxenic conditions. *Mycologia* **106**: 963–975. https://doi.org/10.3852/13-118

Smith, S.E., Read, D. 2008. *Mycorrhizal symbiosis.* Academic Press. doi: 10.1016/B978-0-12-370526-6.X5001-6

Spain, J. L., Sieverding, E., Oehl, F. 2006. *Appendicispora*: a new genus in the arbuscular mycorrhiza-forming Glomeromycetes, with a discussion of the genus *Archaeospora*. *Mycotaxon,* **97**: 163-182

Walker, C. 1983. Taxonomic concepts in the Endogonaceae: Spore-wall characteristics in species descriptions. *Mycotaxon* **18**: 443-455

Walker, C. 1986. Taxonomic concepts in the Endogonaceae. II. A fifth morphological wall type in endogonaceous spores. *Mycotaxon* **25**: 95-99

Walker, C., Schüßler, A., Vincent, B., Cranenbrouck, S. and Declerck S. 2021, Anchoring the species *Rhizophagus intraradices* (formerly *Glomus intraradices*). *Fungal Syst. Evol.* **8**:179-197. 10.3114/fuse.2021.08.14