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Molecular identification and *in vitro* propagation of arbuscular mycorrhiza from tea plant rhizosphere

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Abstract

The purpose of this research was to identify arbuscular mycorrhiza spores colonizing the rhizosphere of tea plants and attempt to propagate those spores *in vitro*. Soil samples from the tea plant rhizosphere were sieved, and arbuscular mycorrhizal spores were isolated and identified based on a morphological and molecular approach. Five species of arbuscular mycorrhiza were identified: *Acaulospora mellea*, *Acaulospora spinosa*, *Glomus ambisporum*, *Glomus multicaule*, and *Scutellospora cerradensis*. Eleven arbuscular mycorrhizal sequences were obtained, the phylogenetic analysis grouped the 11 sequences into five clades, belonging to the *Acaulosporaceae*, *Gigasporaceae*, and *Glomeraceae* genera. Among the five species identified in this study, only two were able to germinate during co-cultivation with an *Arabidopsis* root organ culture host. *Scutellospora cerradensis* and *Glomus clarum* germinated after three and seven days of incubation, respectively. However, the germ tube did not develop any further and was thus unable to infect the *Arabidopsis* root. Nevertheless, these results provided an overview of arbuscular mycorrhizal fungal species colonizing the tea rhizosphere as well as challenges for arbuscular mycorrhiza *in vitro* propagation using *Arabidopsis* root culture.

Keywords – axenic culture – *Glomeromycota* – nested PCR – root organ culture – single spore sequencing – SSU rDNA

Introduction

Arbuscular mycorrhizal (AM) fungi play an important role in the process of crop production, due to their symbiosis with host plants, which can improve crop productivity. In the case of tea plants (*Camellia sinensis* (L.) O. Kuntze), it has been reported that inoculation with the AM fungi *Glomus mosseae* under different nitrogen levels promoted the growth of tea plants, including plant height, aboveground and underground biomass, as well as improved the tea quality (Qing-Hua et al. 2014). Also, AM inoculation has been shown to enhance tea resistance against root diseases (Bhutia 2013). Despite the positive effect of AM on tea plants, there have only been a few studies on the interactions of AM fungi with tea plants (Li-sha et al. 2015, Sharma et al. 2013, Karthikeyan et al. 2005), and almost no molecular information exists about the genetic diversity of AM within the rhizosphere of tea in Indonesia. Therefore, a molecular analysis of AM colonizing the tea

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rhizosphere will provide a reference for further studies of the symbiotic relationships between AM fungi and tea plants.

Continuous interaction between tea roots and rhizoflora during plant development results in the establishment of a specific microbial community (Pandey & Palni 2004, 1997, 1996, Pandey et al. 2001, 1997) including AM fungi (Singh et al. 2008) within and around the root zone of established tea bushes. Symbiotic relationships between plants and AM in the division Glomeromycota are widespread in most terrestrial ecosystems (Koide & Mosse 2004). Analysis of AM diversity is important for determining the soil quality in an ecosystem. Currently, molecular species determination for AM fungi is mostly based on ribosomal DNA (rDNA) sequences such as the small subunit (SSU), internal transcribed spacer (ITS) region, or the large subunit (LSU). A specific PCR primer sets (AML1 and AML2) targeting the SSU rDNA of all subgroups of AM has been developed by Lee et al. (2008) for AM species identification. These primer sets have been used in the recent study on AM fungi community in the various ecosystem (Berruti et al. 2018, Li et al. 2018, Varela-Cervero et al. 2016).

The recent advances in molecular genetics have clearly made an analysis of AM fungi communities more reliable. However, with the high genetic variation within clonal AM fungi species, it is necessary to establish monoxenic cultures of the identified clonal AM fungal spores to ensure the identity and facilitate further study on the spore development, as well as biological characteristics of the isolated spore. *In vitro* establishment of AM fungi in root organ culture was first successfully performed by Mosse & Hepper (1975), using the dual culture system for growing *Glomus moseae* in clover root organ culture. As of today, the Ri T-DNA transformed carrot roots has been the mainstream medium for the production of *in vitro* AM culture (Zhang et al. 2019, Rosikiewicz et al. 2017, Bidondo et al. 2012). According to Fortin et al. (2002), 25 species of fungi have been successfully grown with monoxenic cultures using various root organ culture. This research describes the isolation and molecular identification of AM fungi in the soil sample taken from tea rhizospheres and the challenges during the *in vitro* culture of the isolated AM spores using the root organ of *Arabidopsis*, that has been widely used as a model plant. With this study, we hope to give the first overview of molecular information about AM fungi from tea rhizospheres in Indonesia that can be used as a reference for further studies on the symbiotic relationship between AM fungi and tea plants.

Materials & Methods

Morphological identification

Soil samples for this experiment were taken from the tea plant rhizosphere located in the district of Kulonprogo, Yogyakarta province. 300 g of soil from six different sampling sites were collected using a spiral tipped general purpose soil auger and mixed. The AM fungal spores were extracted by wet sieving and decanting followed with a sucrose gradient centrifugation (Sieverding 1991, Daniels & Skipper 1982). After centrifugation, the supernatant was poured through a 38-µm mesh and quickly rinsed with tap water. Spores were grouped under a dissecting microscope according to their morphological characteristics. Afterwards, these results of AM spore characterizations were then compared to the AM spore reference culture collection of the international culture collection of (vesicular) arbuscular mycorrhizal fungi (INVAM; West Virginia University, WV, USA) as well as to previous reports of AM fungi morphology which shows similarity to our isolates (Smith & Schenck 2007, Gerdemann & Bakshi 2009, Spain 1996, Morton 1986, Walker & Trappe 1981).

Molecular identification, sequence alignment, and phylogenetic analyses.

A single morphologically identified spore was separated and used for molecular identification. The spore was washed with distilled water three times, afterward AM fungal DNA was extracted by crushing a single spore with a needle inside a 0.2 mL PCR tube and mixed with 1 µL of sterilized water. Partial 18S rDNA fragments of AM fungi were amplified by nested PCR

(Van Tuinen et al. 1998). The first PCR was performed using universal primers NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') and NS4 (5'-TTC CAT CAA TTC CTT TAA G-3') for 32 cycles (one cycle at 95°C for 3 min, 30 cycles at 95°C for 30 s, at 40°C for 1 min, at 72°C for 1 min, and one cycle at 72°C for 5 min). The first PCR product was used as a template for the second amplification with AM specific primers: AML1 (5'-ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GG TTT CC-3') for 32 cycles (one cycle at 95°C for 3 min; 30 cycles at 95°C for 30 s, at 50°C for 1 min, at 72°C for 1 min, and one cycle at 72°C for 5 min) (White et al. 1990, Lee et al. 2003).

After a 10× dilution with TE buffer, the secondary PCR products were used as DNA templates for sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). At least two overlapping sequence data from each sample were collected and then recalculated with matching forward and reverse reading procedures. Reverse sequences were flipped and aligned with forward sequences. All sequences were subjected to BLAST analysis at the National Center for Biotechnology Information (NCBI; Maryland, USA) and aligned with reference sequences of AM fungi using multiple sequence comparison by a log-expectation (MUSCLE) algorithm in the molecular evolutionary genetic analysis version X software (Kumar et al. 2018). The neighbor-joining method was used for phylogenetic analysis (Saitou & Nei 1987). Isolates used in this study and GenBank accessions are listed in Table 1. Phylogenetic trees and DNA sequence alignments were deposited in TreeBASE with submission number 24076.

Table 1 Isolates used in the phylogenetic analysis, specimens from the current study are in bold.

Species	**Isolate/ Voucher no.**	**GenBank Accession Number**
Acaulospora laevis	W3107	Y17633
Acaulospora mellea	**AM17**	**MF196923**
Acaulospora mellea	Wuy104	FJ009670
Acaulospora spinosa	**AM24**	**MF196926**
Acaulospora spinosa	**AM48**	**MF196929**
Acaulospora spinosa	**AM49**	**MF196930**
Acaulospora spinosa	W3574	NG062381
Acaulospora spinosa	WV860	Z14004
Diversispora epigaea	W3221	AJ276088
Diversispora spurca	W3239	AJ276077
Entrophospora colombiana	WV877	Z14006
Gigaspora albida	FL927	Z14009
Gigaspora rosea	DAOM_194757	X58726
Glomus ambisporum	**AM4**	**MF196920**
Glomus ambisporum	**AM13**	**MF196921**
Glomus ambisporum	**AM15**	**MF196922**
Glomus ambisporum	**AM19**	**MF196924**
Glomus claroideum	pKL2_9a	AJ276075
Glomus clarum	BEG125	AJ505619
Glomus clarum	UFPE08	AJ852597
Glomus fasciculatum	BEG53	Y17640
Glomus lamellosum	pWD100_2_6	AJ276087
Glomus multicaule	**AM40**	**MF196928**
Glomus caledonium	BEG20	Y17635
Glomus etunicatum	pWD106_3_2	Y17639
Glomus geosporum	pKL11_1a	AJ245637
Glomus manihotis	pWD113_4_1	Y17648
Glomus mosseae	BEG69	U96141
Mortierella polycephala	NBRC6335	AB476414
Pacispora scintillans	pWD273_3_1	AJ619947
Pacispora scintillans	pWD273_3_5	AJ619946
Rhizophagus irregularis	pWD164_1_5	AJ301859
Scutellospora cerradensis	**AM23**	**MF196925**
Scutellospora cerradensis	**AM31**	**MF196927**
Scutellospora cerradensis	SC21	AB041344

Surface sterilization of spores

Spore sterilization was performed using the filter-paper sandwich method (Mosse 1959, 1962). Spores were transferred by micropipette to a 45 mm diameter filter paper disc (Whatman no. 1), which was then covered by an identical disc (Fig. 1A). The sandwich was placed in a plastic filter holder (SX0004700; MilliporeSigma, MA, USA) (Fig. 1B). Both paper discs and filter holder were autoclaved (121°C, 20 min). A sterilant, 2% (w/v) chloramine T, plus 0.02% (w/v) streptomycin sulfate, plus a trace of Tween 20, was injected with a syringe through a 0.22-µm sterile syringe filter (SLGL0250S; MilliporeSigma, MA, USA) (Fig. 1C) into the filter-paper sandwich until excess liquid emerged. A total of 20 mL of sterilant was used for 50 spores; the contact time was 20 min, and the sterilant in the filter holder was replenished from the syringe three times during the sterilization process. The sterilant was removed from the spores by passing 200 mL of sterile distilled water through the filter paper sandwich. The sterilized spores were stored between the damp filter papers in Petri dishes at 4°C.

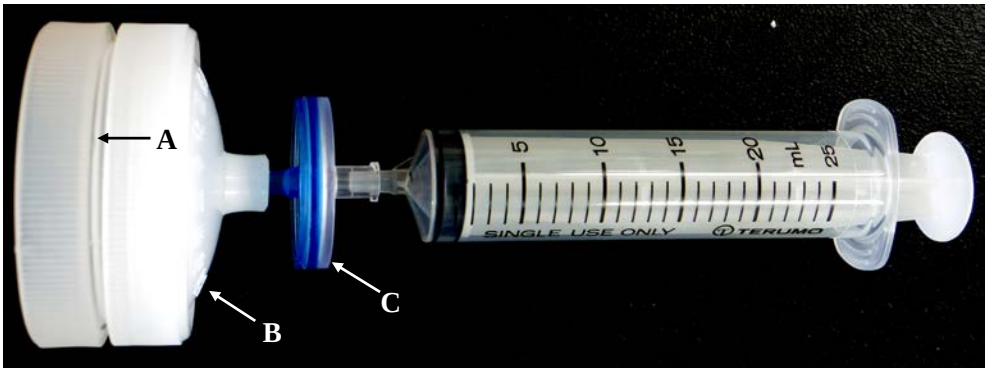

Fig. 1 – Syringe-filter arrangement for spore sterilization: A–45 mm diameter filter paper disc (inside filter holder), B–47 mm filter holder, and C–0.22 µm sterile syringe filter.

***In vitro* root organ culture and AM spore propagation**

In vitro root organ culture was established using *Arabidopsis* roots. To obtain the root organ culture, *Arabidopsis* seed was surface-sterilized with 0.5 µL of 10% Tween 20 and 1 mL of 2% Plant Preservative Mixture (PPM^{TM}; Plant Cell Technology, Inc., WA, USA) then washed in sterile distilled water before being placed on Murashige and Skoog (MS) agar. The plates were stored at 24°C in dark conditions. After seven days post-inoculation (DPI), the leaves and stems were removed, and the roots were transferred onto modified Strullu Romand (MSR) medium. After 30 DPI, the roots were fully developed and ready for AM spore inoculation. The surface sterilized AM spores were placed directly on an agar plate containing the root culture using a micropipette under aseptic conditions and incubated at 24°C in the dark.

Results

Morphological and molecular Identification

The isolated spores were separated based on morphological characters, including spore shape, color, and wall structures, as well as their reactions with Melzer's reagent (Fig. 2). AM spore isolates AM4, AM13, AM15, and AM19 had similar characteristics; they were non-Melzer's-reactive ellipsoid sporocarps with subtending hyphae-bearing walls that were continuous with the outer layer of the spore wall (Fig. 2A). The subtending hyphae could also be observed in the AM40 isolate; however, AM40 had a distinguishable subglobose sporocarp with a very thick outer wall (Fig. 2B), which was also not reactive with Melzer's reagent. On the other hand, the AM17 isolate showed sub globoid sporocarps with germinal walls that stained dark red purple in Melzer's reagent (Fig. 2C). The AM24, AM48, and AM49 isolates had a similar sub globoid sporocarps; however, their germinal walls stained purplish pink (Fig. 2D). The AM23 and AM31 isolates had ellipsoid

sporocarps containing very dark red germinal wall stains in Melzer's (Fig. 2E).

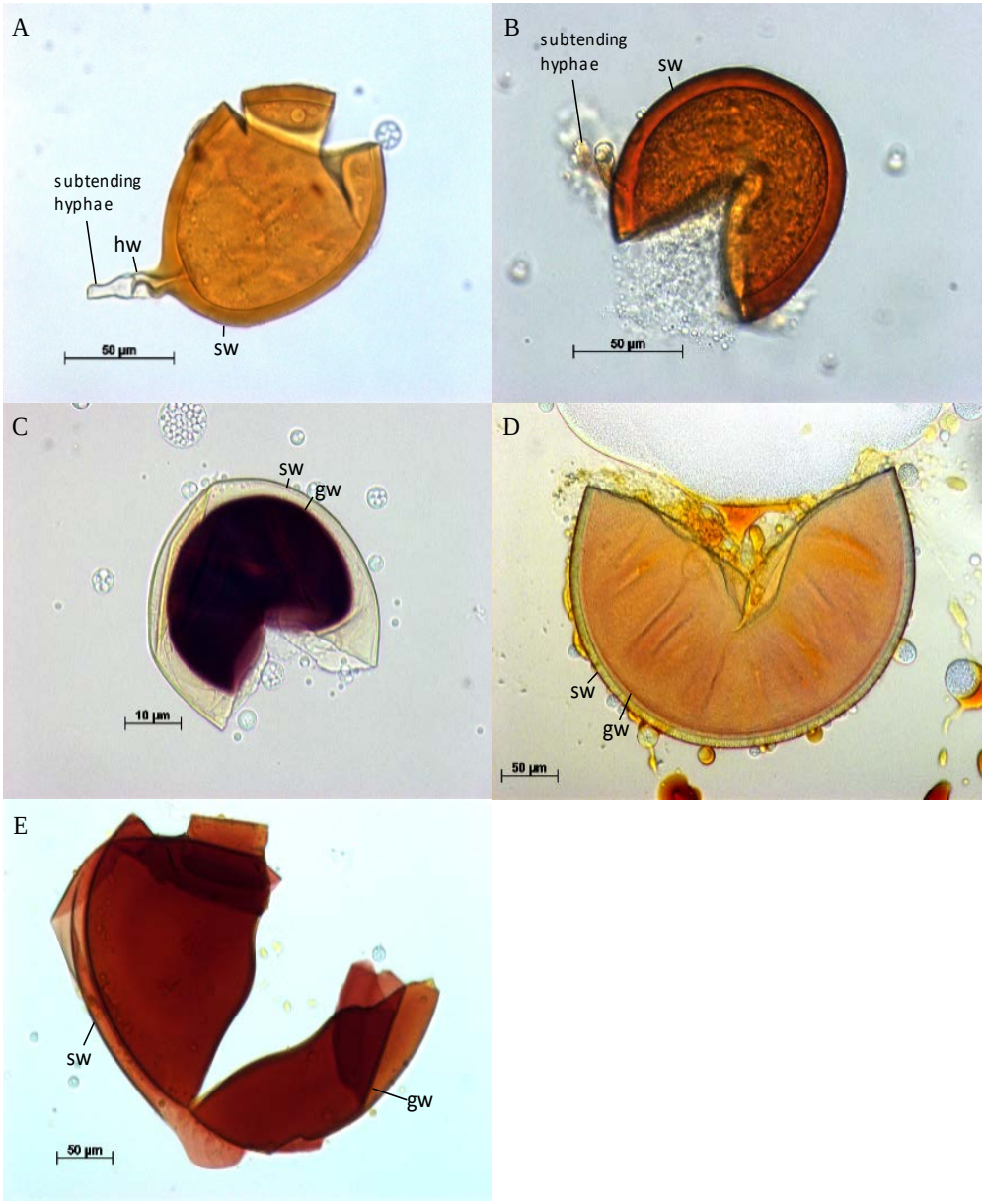

Fig. 2 – Spores of AM fungi isolated from the rhizosphere of tea. A *Glomus ambisporum*. B *Glomus multicaule*. C *Acaulospora mellea*. D *Acaulospora spinosa*. E *Scutellospora cerradensis*. Spores were separated and identified using morphological characters, including spore shape, color, wall structures, and reactions with Melzer's reagent; gw–germinal wall, hw–hyphae wall, and sw–spore wall.

For further identification and to facilitate phylogenetic tree construction, the partial 18S rDNA of the spores were sequenced. A primer pair AML1/AML2 specific to AM fungi was used and successfully amplified DNA from all the species used in this study (Fig. 3), suggesting that the primers were reliable for the identification of AM fungal spores. Eleven sequences were obtained and compared with the GenBank database for molecular identification. The most similar sequences obtained using BLAST analysis and the collected AM fungal sequences were grouped into four species in three genera (Table 1). The neighbor-joining (NJ) analyses of these 11 sequences from AM fungal spores were performed with 24 other sequences from NCBI data. The phylogram obtained from the analysis grouped the isolated 11 AM spores SSU sequences into five clades (Fig. 4).

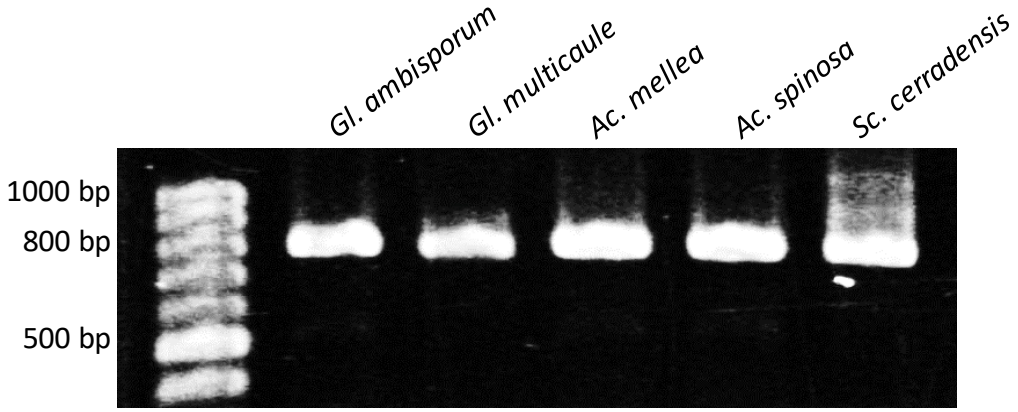

Fig. 3 – The amplification of SSU rDNA genes by using AML1-AML2 primers. SSU rRNA genes were amplified using nested PCR with NS1-NS4 universal fungal primers (1100 bp), and AML1-AML2 AM specific primers (800 bp), respectively.

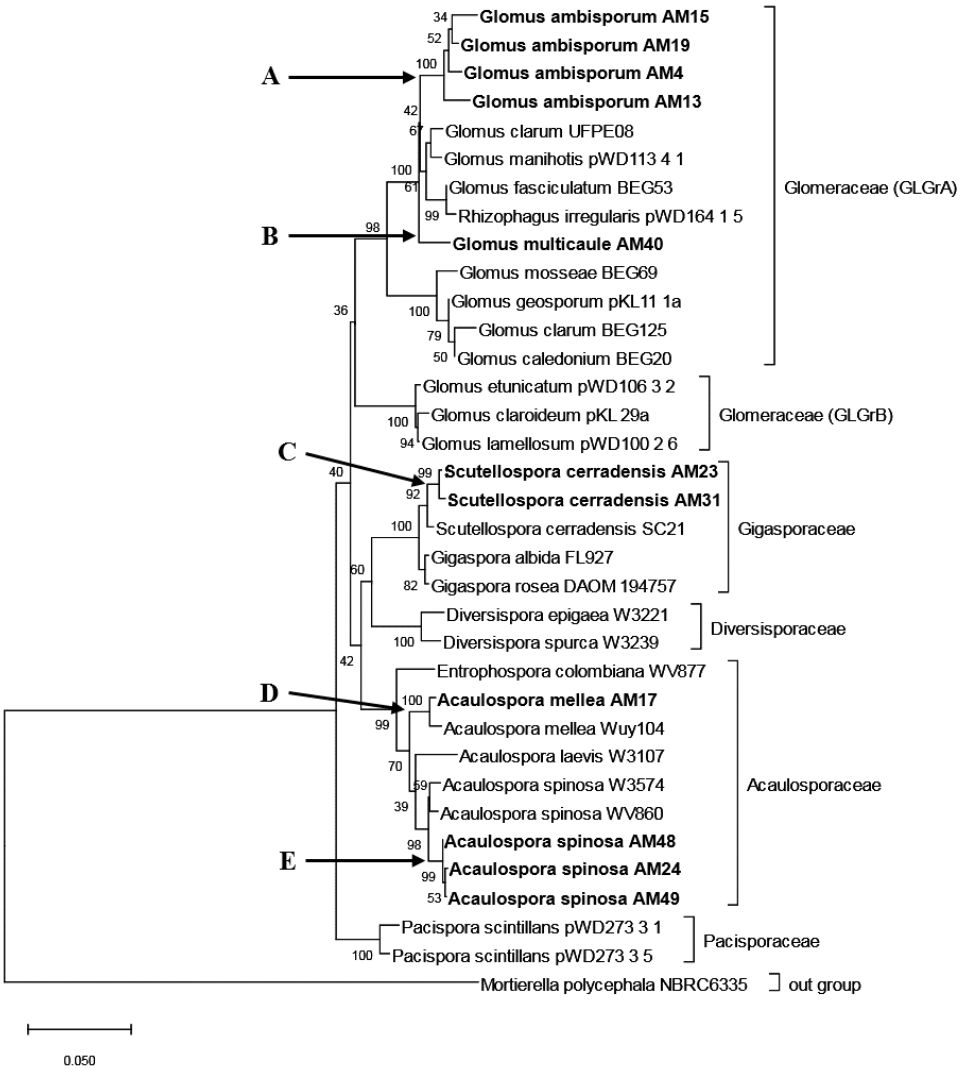

Fig. 4 – Neighbor-joining consensus phylogram for partial 18S rDNA sequence of AM fungal spores. Bootstrapping, 1000 replicates. A *Glomus* sp. A. B *Glomus* sp. B. C *Scutellospora* sp. A. D *Acaulospora* sp. A. E *Acaulospora* sp. B.

***In vitro* root organ culture and AM spore propagation**

Arabidopsis roots were grown *in vitro* on modified Strullu Romand (MSR) agar. After 42 d post-inoculation, the root organs were fully distributed on the agar plate (Fig. 5A). The root organ culture was then used as the host for AM fungi. Surface sterilization by filter-paper sandwich methods yielded 40 spores (80%) that can be successfully sterilized, and ten spores (20%) were contaminated, following incubation on the MSR agar plate. Two species of AM fungi were able to germinate on *Arabidopsis* root organ cultures; *Scutellospora cerradensis* germinated after three days of incubation (Fig. 5B, C), and *Glomus clarum* germinated after seven days of incubation (Fig. 5D, E). However, the germ tube stopped developing after germination and was thus unable to connect with the *Arabidopsis* root.

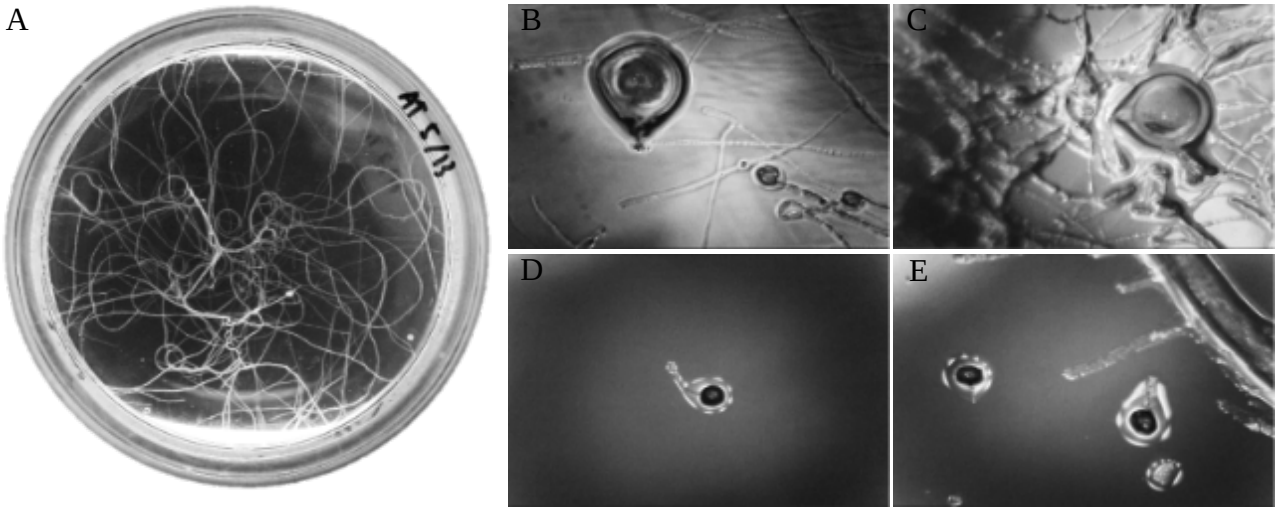

Fig. 5 – Germination of surface-sterilized spores on *Arabidopsis* root culture. A *Arabidopsis* root development after 42 d on MSR agar. B, C *Scutellospora cerradensis* germination after three days post inoculation. D, E *Glomus ambisporum* germination after seven days post inoculation. After cold stratification for seven days, surface sterilized spores were inoculated on *Arabidopsis* root cultures grown on MSR agar and incubated at 25°C in dark conditions.

Discussion

Morphological and molecular Identification

The morphological characters of AM4, AM13, AM15, and AM19 corresponded to the specifications for *Glomus ambisporum*. Smith & Schenck. (2007) described this species as having a spore wall that consisted of two layers, neither of which was reactive in Melzer's reagent. Also, they showed that the subtending hypha at the point of attachment was 5–10 µm wide and consisted of two wall layers that were a continuation of the spore wall. The very thick outer wall of the AM40 isolate, which was non-Melzer's-reactive, was a distinctive character for *Glomus multicaule* (Gerdemann & Bakshi 2009). The AM17 dark red-purple germinal wall corresponded to *Acaulospora mellea* (Morton 1986), while the purplish-pink germinal walls of AM24, AM48, and AM49 corresponded to *Acaulospora Spinosa* (Walker & Trappe 1981). The very dark red germinal walls of AM23 and AM31, as well as their large spore sizes (250–275µm), indicated characteristics similar to *Scutellospora cerradensis*, which belongs to the Gigasporaceae family (Spain 1996).

The *Glomus* isolates (AM4, AM13, AM15, AM19, and AM40) were placed on a subclade of their own "*Glomus* sp. A" (Fig. 4A) and formed a clade together with Glomeraceae group A (GLGrA) reference isolates. Although the most similar sequence to AM4, AM13, AM15, AM19, and AM40 spores was *Glomus clarum*, these sequences were positioned on a quit distance clade in the phylogram. In addition, the AM40 clade "*Glomus* sp. B" (Fig. 4B) was separated into an AM4, AM13, AM15, and AM19 clade, hence indicating a species difference. This was confirmed with the differences in the morphological characteristics that identified AM4, AM13, AM15, and AM19

spores as *Glomus ambisporum*, and the AM40 spores as *Glomus multicaule*. Moreover, *Glomus* isolates were randomly distributed. Stockinger et al. (2010) claimed that only longer (approx. 1500 bp) nuclear rDNA fragments (spanning SSU, ITS region, and LSU) could be successfully applied to species delineation within the GlGrAa and GlGrAb. The two *Scutellospora* isolates (AM23 and AM21) were clustered together in the *Scutellospora cerradensis* clade "*Scutellospora* sp. A" (Fig. 4C). The *Acaulospora* isolate AM17 clearly fell into the *Ac. mellea* clade "*Acaulospora* sp. A" (Fig. 4D), while AM24, AM48, and AM49 fell into the *Acaulospora spinosa* clade "*Acaulospora* sp. B" (Fig. 4E). All of the clades in the phylogram were consistent with morphological identifications; "*Glomus* sp. A", "*Glomus* sp. B", "*Scutellospora* sp. A", "*Acaulospora* sp. A", and "*Acaulospora* sp. B" were morphologically identified as *Glomus ambisporum*, *Glomus multicaule* *Scutellospora cerradensis*, *Acaulospora mellea*, and *Acaulospora spinosa*, respectively (Table 2).

Table 2 The consensus species from the morphological and molecular identifications of AM fungal spores.

Isolates	The most similar sequences in GenBank database			Clade	Consensus species (molecular & morphological)
	Species	SSU	Sequence similarity		
Glomeraceae					
AM4	*Glomus clarum*	AJ852597	813/837 (97%)	*Glomus* sp. A	*Glomus ambisporum*
AM13	*Glomus clarum*	AJ852597	741/769 (96%)	*Glomus* sp. A	*Glomus ambisporum*
AM15	*Glomus clarum*	AJ852597	683/698 (98%)	*Glomus* sp. A	*Glomus ambisporum*
AM19	*Glomus clarum*	AJ852597	690/707 (98%	*Glomus* sp. A	*Glomus ambisporum*
AM40	*Glomus clarum*	AJ852597	704/724 (97%)	*Gllomus* sp. B	*Glomus multicaule*
Acaulosporaceae					
AM17	*Acaulospora mellea*	FJ009670	707/716 (99%)	*Acaulospora* sp. A	*Acaulospora mellea*
AM24	*Acaulospora spinosa*	Z14004	717/723 (99%)	*Acaulospora* sp. B	*Acaulospora spinosa*
AM48	*Acaulospora spinosa*	Z14004	689/694 (99%)	*Acaulospora* sp. B	*Acaulospora spinosa*
AM49	*Acaulospora spinosa*	Z14004	628/635 (99%)	*Acaulospora* sp. B	*Acaulospora spinosa*
Gigasporaceae					
AM23	*Scutellospora cerradensis*	AB041344	717/725 (99%)	*Scutellospora* sp. A	*Scutellospora cerradensis*
AM31	*Scutellospora cerradensis*	AB041345	703/711 (99%)	*Scutellospora* sp. A	*Scutellospora cerradensis*

The molecular identifications of AM spores belonging to the *Glomus* species were not consistent with morphological identifications. This was mainly due to the limited number of AM fungal sequence data in the database that were similar to the sequences from this study. Furthermore, to further validate the phylogenetic tree, several genes, as well as other characteristics, such as morphological and biochemical characters could be used. Nevertheless, the sequence data of AM fungal spores identified in this study could be used as a new reference for the identification of AM fungi colonizing roots, as reference sequences of some species such as *Glomus ambisporum* and *Glomus multicaule* were limited.

In vitro root organ culture and AM spore propagation

Macdonald (1981) reported successful AM culture *in vitro* by prior spore germination on the spore germination medium, followed by transferring the germinated spore to a carrot root organ culture using the agar plug technique. However, that method seemed not to be compatible with the *Arabidopsis* root; the spore development still stopped at the germination stage. It is unclear whether it was host incompatibility or culture conditions that caused the spore development to cease. Notwithstanding, further investigation is required to determine the cause of failure. In the previous report, Mosse & Hepper (1975) experiencing a difficulty when establishing *in vitro* AM culture on red clover and tomato root organ. They observed no AM infection were developed in the media which had been sterilized in a large steam injection autoclave, although root growth was normal.

However, when the media used for the establishment of *in vitro* culture was sterilized in a domestic pressure cooker which has a faster cool down time, AM infection was developed. Also, the optimization of culture conditions needs to be performed to culture the AM fungi using *Arabidopsis* roots as a partner. Fortin et al. (2005) reported that pH and temperature have a significant role in spore germination and development. Optimal temperatures seem to vary between AM fungi. *Glomus mosseae* and *Acaulospora leavis* were able to germinate between 10–18 and 30°C with an optimum germination condition of 20–25°C, while *Gigaspora* germinated at 10–30°C with an optimum germination condition of 20–30°C (Safir 1986). In this research, AM fungal spores were cultivated only at 25°C. Thus, further temperature optimization should be considered.

The axenic culture of AM fungi remains a major challenge because it is far more than just a routine technique. Strict protocols must be followed to achieve it. To develop an *in vitro* culture system for newly isolated AM fungi, a great deal of optimization needs to be performed, such as the selection of appropriate culture systems and symbiotic host root partners, as well as the determination of appropriate culture conditions.

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