BIODIVERSITAS
Volume 24, Number 11, November 2023
Pages: 6271-6278

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d241150

Identification and molecular detection of Arbuscular Mycorrhizal Fungi colonized naturally in citrus roots in nurseries

YENNY SARIASIH[1,2], SITI SUBANDIYAH[3,♥], SRI WIDYANINGSIH[4], TAHIR KHURSHID[5], JIANHUA MO[5]

[1]Doctoral Program in Agricultural Science, Universitas Gadjah Mada. Jl. Flora No. 1, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia
[2]Departement Plant Protection, Faculty of Agriculture, Universitas Bengkulu. Jl.WR. Supratman, Kota Bengkulu 38371, Bengkulu, Indonesia
[3]Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora No. 1, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia. ♥email:sitisubandiyah@ugm.ac.id
[4]Research Center for Horticultural and Estate Crops, National Research and Innovation Agency. Jl. Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, West Java, Indonesia
[5]NSW Department of Primary Industries. 21888 Kamilaroi Highway, Narrabri NSW 2390, Australia

Manuscript received: 26 August 2023. Revision accepted: 28 November 2023.

Abstract. *Sariasih Y, Subandiyah S, Widyaningsih S, Khurshid T, Mo J. 2023. Identification and molecular detection of Arbuscular Mycorrhizal Fungi colonized naturally in citrus roots in nurseries. Biodiversitas 24: 6271-6278.* Citrus plants heavily depend on arbuscular mycorrhizal (AMF), a diverse collection of fungi. The objective of this study was to investigate the root colonization, spore density, spore morphology, and molecular identification of AMF colonizing four citrus root species: Japansche Citroen (*Citrus limonia* Osbeck.), Cleopatra mandarin (*Citrus reshni*), Salam (*Fortunella japonica* cv. "Salam"), and Rough Lemon (*Citrus jambhiri*). AMF was identified by observing spore morphology and molecular PCR methods using the group-specific primers of Glomeromycota, AML1/AML2. Based on the morphological observation, *C. limonia* was found with the highest root colonization (50%), followed by *F. japonica* cv Salam (44%), *C. reshni* (38%), and *C. jambhiri* (36%). The highest number of AMF spores was found on *C. jambhiri* rhizosphere soil, which had 1,379 spores/100 g of soil with the lowest percentage of root colonization. Three mycorrhizal genera were identified, with *Funneliformis (syn. Glomus)* being the most dominant genus, followed by *Gigaspora* and *Acaulospora.* The sequence of PCR products confirmed the highest similarity range from 83.7-99.0% to that of *Funneliformis*, followed by *Acaulospora* at a similarity range from 83.3-99.0% and found on all of the citrus species. The similarity sequence of the PCR products to that of *Gigaspora* ranged between 83.3-95.0%, which was only found on *C. reshni* and *C. jambhiri*.

Keywords: AMF, citrus plants, colonization, *Funneliformis*, molecular detection

INTRODUCTION

Citrus is the most extensively produced tree crop and ranks as the world's top five most favorable fruit. Citrus crops rely heavily on arbuscular mycorrhizal fungi (AMF) (Wu et al. 2017; Yang et al. 2021). Arbuscular Mycorrhizal Fungi (AMF) is the essential symbiotic microorganism for the roots of terrestrial crops. There are several types of AMF, mainly endo and ectomycorrhizae. Arbuscular mycorrhizae (endomycorrhizal) is the most widespread crop-root symbiosis and nearly 90% of terrestrial plant species, including citrus crop (Sudová et al. 2020; Paz et al. 2021). Endomycorrhizae, specifically AMF, has a wide host range, approximately 150 species (Willis et al. 2013; Jacott et al. 2017). The major AMF spore in the citrus rhizosphere is the genus of *Funneliformis*, a synonym of *Glomus*, despite the presence of *Sclerocystis*, *Gigaspora*, and *Acaulospora* species (Toh et al. 2018; Gusmiaty et al. 2019; Cheng et al. 2022).

Instead of having a single species of AMF, citrus orchard soil has numerous communities of AMF. Since citrus trees have short root hairs, they need AMF colonization to uptake sufficient nutrients and water (Morgan et al. 2016). The host plant has been shown to affect the interaction of AMF. More fungal activities in AMF hyphae have been demonstrated to affect citrus growth and nutrient uptake. More than one AMF may colonize the citrus plant's root at once, indicating that the soil in citrus orchards is rich in diversity of AMF, which enhances a citrus plant's ability to absorb nutrients and flourish. Therefore, to promote optimum growth, it is important to understand the nutrients citrus plants need while growing. Utilizing plant rhizosphere mechanisms, such as mycorrhizal inoculation, to promote growth is beneficial because fertilizers are scarce, expensive, and have an unknowable long-term environmental impact. Inoculating seedlings with AMF earlier is advantageous since citrus plants depend on mycorrhizae colonization.

Arbuscular mycorrhizal fungi (AMF) can only be grown in the presence of their host plant since they are obligate biotrophs (Sun et al. 2022). Identifying different AMF species is challenging because they frequently share the same roots and exhibit little change in hyphal morphology in soil or plants. However, even within an AMF species, there are significant variations in spore form in nature (Redecker et al. 2013). Moreover, many AMFs might only proliferate vegetatively without creating spores (Gehlot and Singh 2015; Paz et al. 2021). It has the potential to identify actively growing fungus in field root samples without spore morphological criteria; therefore, molecular analysis offers a solution to this problem. However, comprehensive studies utilizing morphological

and molecular methods can help identify the biodiversity of AMF in citrus roots.

The aim of this paper is to detect AMF's existence and identify the species of AMF that colonized the roots of four citrus seedlings in the nursery before transplanting/treatment. In this paper, we report our study of AMF colonization and identification in four species of citrus seedling roots through spore morphological characterization and molecular detection. The species of citrus rootstocks used in this study were Japansche Citroen (*Citrus limonia* Osbeck.), Cleopatra mandarin (*Citrus reshni*), Salam (*Fortunella japonica* cv. "Salam"), and Rough Lemon (*Citrus jambhiri*). It is crucial to determine whether AMF is present at the roots of the four types of citrus that are frequently used as rootstocks in the propagation of citrus seedlings to make sure that the roots of these citrus seedlings are healthy and strong enough to be transplanted and grafted with other varieties of citrus.

MATERIALS AND METHODS

The plant materials used in this study were four species of citrus seedlings, namely: Japansche Citroen (*Citrus limonia* Osbeck.), Cleopatra (*Citrus reshni*), Salam (*Fortunella japonica* cv. "Salam"), and Rough Lemon (*Citrus jambhiri).* The seedlings were taken from the *Indonesian Citrus and Subtropical Fruits Research Institute* (ICSFRI), which produces disease-free planting materials through a standard procedure for indexing seedling health and purity of varieties.

Mycorrhizal colonization

Mycorrhizal colonization was observed on the root system of citrus. The root tip samples were taken from each species, kept into a plastic clip, and labeled. AMF colonization was observed microscopically by the root staining technique. Firstly, the root samples were washed thoroughly, cut into ± 1 cm pieces, and kept in a plastic film bottle. The root samples were added with 10% KOH solution, boiled at 100°C for 10 minutes, and then stored for ±12 hours at room temperature. The 10% KOH solution was removed, and the root samples were washed in tap water until the water became clear.

Next, 3% new hydrogen peroxide (H_2O_2) solution was added to the samples, and the samples were left at room temperature for about 12 hours. Then, H_2O_2 solution was discarded, and the samples were washed with tap water. The 1% Hydrochloric acid (HCl) solution was added and then stored for ± 12 hours at room temperature. The 1% HCl was removed, methylene blue solution was added, then boiled at 100°C for 5 minutes and stored for ±12 hours at room temperature. For microscopic examination of the mycorrhizal structures, root samples were stacked in rows of 16 root pieces on each glass slide, covered with cover glass, and sealed with clear nail varnish. The colonization percentages were calculated based on the presence of spores, hyphae, or vesicles in the citrus root cells.

Mycorrhizal spore density and identification

Fresh soil from the rhizosphere was used to observe AMF spores density, spores morphology, and the physical-chemical characteristics of the rhizosphere soil. The spores were extracted from 100 g soil around the rhizosphere of citrus seedlings at the end of the experiment using the wet sieving method. 100 g soil sample was added with 900 mL of water to obtain 1 L final volume, then stirred for ± 10 minutes to get homogeneous soil suspension. The suspension was allowed to stand for ± 5 minutes until the large particles settled down. A multilayer filter with 75 and 54 m particle sizes collected the supernatant liquid.

The residue of each filter was rinsed with tap water to ensure that all tiny particles were flushed away. The filter residues of 75 µ and 54 µ pores were poured into a 100 mL measuring cup by water spraying bottle to reach a volume of 20 mL. The suspension was poured at a volume of 5 mL into a 5 cm diameter petri dish for AMF spore observation under the Optilab microscope. Spore density was observed by counting all spores in the suspension. The morphological properties of the spore (color, cell wall, shape, and addition structure) were used to identify them. The diameter of spores was measured by Image Raster 3.0 and Optilab microscope.

DNA extraction

The citrus root samples were taken in plastic clips and stored in the freezer at -20°C. The amount of 0.1 g of frozen root samples of each species was frozen with nitrogen and extracted using a Geneaid Genome Kit for Plant Protocol to obtain the purified DNA elution.

PCR amplification

The DNA samples were amplified by PCR using the universal primer-set of *Glomeromycota*, AML1 (5'ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'GAA CCC AAA CAC TTT GGT TTC C-3') (Lee et al. 2008). The reaction mixture consisted of 5 µL My Taq buffer (Bioline), 1 µL AML1 primer Forward, 1 µL AML1 primer Reverse, 1 µL DDH_2O, and 2 µL DNA sample. The cycling steps were 3 minutes of initial denaturation at 94°C, 1 minute of denaturation at 94°C, 1 minute of annealing at 50°C, 1 minute extension at 72°C and 10 minutes of final extension at 72°C for 30 cycles. with the reaction was run using a PCR machine (Biorad T100, Singapore) and the amplicons were visualized by UV Transilluminator E3000, USA) after electrophoretic migration on 1,5% agarose gels in the Biorad Electrophoresis machine (Type T, Singapore) which 4 µL volumes for each sample.

Nucleotide sequencing and analysis

The amplicons were sequenced by 1st BASE DNA Sequencing Services to determine AMF in the citrus roots. Sequencing results were analyzed using Bioedit software. Sequence similarities to NCBI Gen-Bank assessments were performed using BLAST software. The sample GenBank's 12 AMF gene sequence dataset was used to align all of the study's sequences. Using MEGA11, the phylogenetic tree was created using the Kimura 2-parameter model with 1000 bootstraps.

Physical and chemical properties of soil

The rhizospheric soil of each citrus species was taken and analyzed for chemical properties including pH, C-Organic, and P available. C-Organic was measured using the Walkley and Black technique, phosphate was measured using the Olsen method, and pH was assessed using a pH meter.

Data analysis

All quantitative data (percentage of AMF spore density and colonization) were tabulated and analyzed statistically by SPSS statistic 26. The significance level was tested using the F test, and Duncan posts hoc 5% was used to test the treatment differences.

RESULTS AND DISCUSSION

Results

There were various patterns of AMF hyphae penetration in colonized root cells. In the first pattern, hyphae adhered to the root's surface perpendicularly, developed perpendicular branches at the bottom, and then waded inside the root to form intracellular hyphae (Figure 1.A). In the second pattern, the hyphae entered the cell by breaking through the root surface (Figure 1.B). In the third pattern, both hyphal ends enter the cell once and multiply there (Figure 1.C).

Additionally, there were various ways to produce AMF vesicles. Some AMF were found to form many vesicles in a single root cell, while others produced few vesicles in a single root cell. The shape of vesicles also varied as well, including oval, imperfectly spherical with thickened borders, and completely round with smooth edges (Figure 2). The information on spore density and colonization percentages is presented in Table 1. In this study, the native AMF invaded every citrus root, forming vesicles and intracellular hyphae, which are characteristic AMF features (Figures 1 and 2).

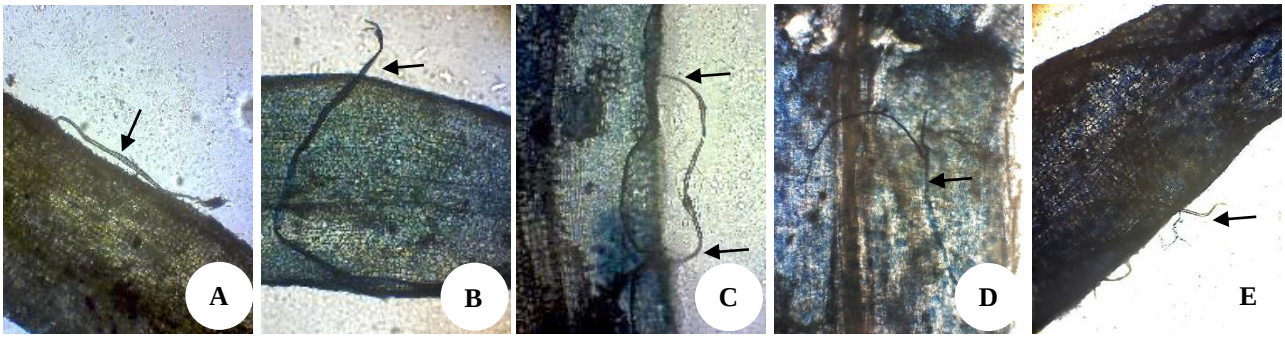

Figure 1. Penetration type of AMF hyphae on citrus roots (A-E): A. hyphae adhered to the root's surface perpendicularly, B. hyphae entered the cell by breaking through the root surface, C. both hyphal ends enter the cell once and multiply there, D. intracellular hyphae, E. extraradical hyphae

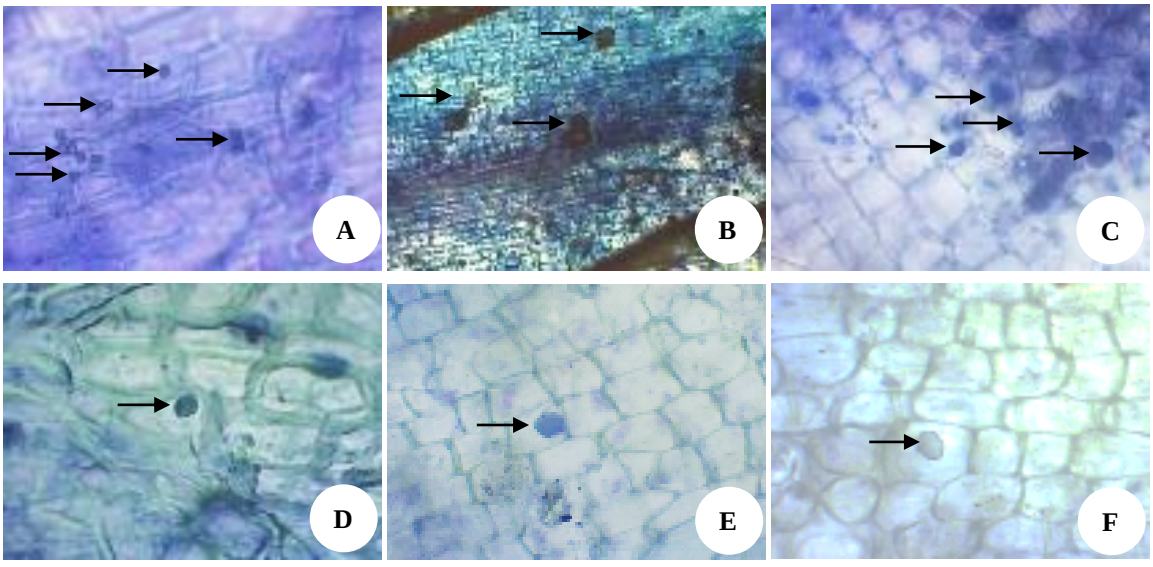

Figure 2. Vesicles type of AMF on citrus roots cell. Observation using the Optilab microscope with the camera on the ocular lens at 400 X magnification power

Table 1. Percentage of AMF spore density and colonization

Citrus species	Spore density (spore)	AMF colonization (%)
Citrus limonia	282±21.8[a]	50±32.9[a]
Fortunella japonica cv. "Salam"	355±4.1[b]	44±32.4 [a]
Citrus reshni	376±4.1[c]	38±28.9 [a]
Citrus jambhiri	1379±0.5[d]	36±20.7 [a]

Note: Data was presented as mean±Std.deviation from 10 sample replicates. Different superscripted letters within a column indicate significance from each other at Duncan posts hoc test 5%

The morphological observation of stained root and spore shows that *C. limonia* had the highest root colonization (50%), followed by *F. japonica* cv. "Salam" (44%), *C. reshni* (38%), and *C. jambhiri* (36%). The highest spores were found in *C. jambhiri* rhizosphere soil (1,379 spores/100 g), followed by *C. reshni* (376 spores/100 g of soil), *F. japonica* cv. "Salam" (355 spores/100 g of soil), and *C. limonia* (282 spores/100 g of soil). AMF colonization data were not statistically different, while spore density data were found to be differ compared to other types. *Citrus jambhiri* had the highest spore density. It was observed that the shape and color of AMF spores varied with species. The AMF spores of *Glomus* (*Funneliformis)* are represented in Figure 3, AMF spores of *Gigaspora* (Figure 4), and AMF spores of *Acaulospora* (Figure 5) were found in the rhizosphere soil.

The spores of *Funneliformis* (*Glomus*) were light yellow, and hyphae were attached to the spores. In this study, *Funneliformis* spores were small in size, less than 100 µm. The spore of *Funneliformis* were globose, ovoid, and ellipsoid. The distinctive characteristic of *Funneliformis* spores was that the spore wall was visible, and hyphae tips were attached to the spore surface or subtending hyphae.

It was observed that *Gigaspora* spore size was small, less than 100 µm, but it was larger than the other two species found with bulbous suspensors. The *Gigaspora* genera had the following characteristics: single spores in the soil, globose or subglobose; yellow, brownish yellow, to blackish; slimy white, golden yellow; the contents of the spore contain an oil-like fluid; lack of ornamentation; hyphae form bulbous suspensors or rounded hyphae holders; spores grow at the tip of the "bulbous suspensor". Another characteristic of *Gigaspora* spores was the presence of spiny appendages on the surface of the spores and thin walls. Usually, the *Gigaspora* spore was bigger than *Funneliformis* and *Acaulospora*.

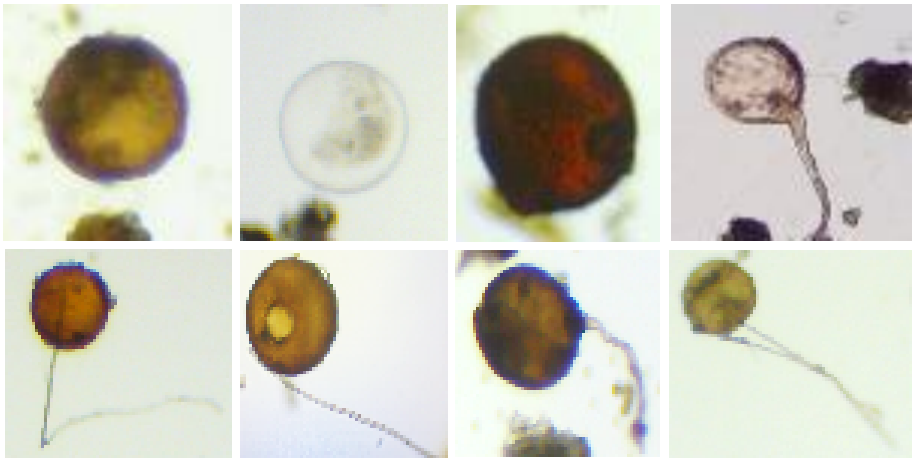

Figure 3. Spores of *Funneliformis* isolated from the rhizosphere

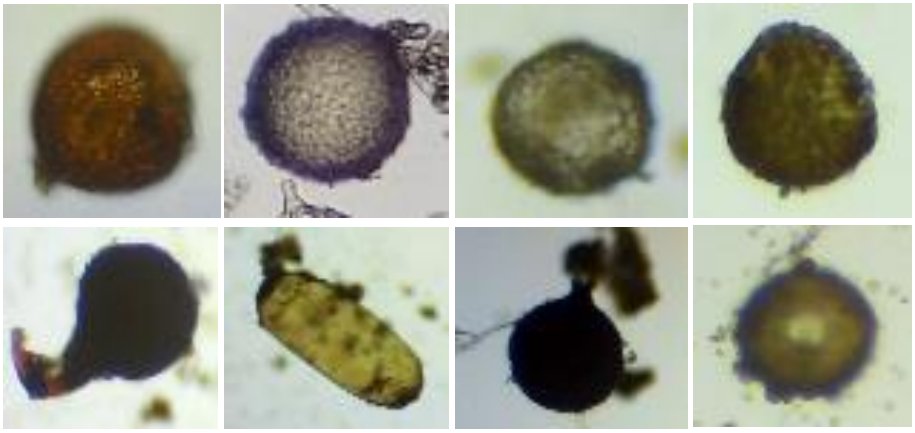

Figure 4. Spores of *Gigaspora* isolated from rhizosphere

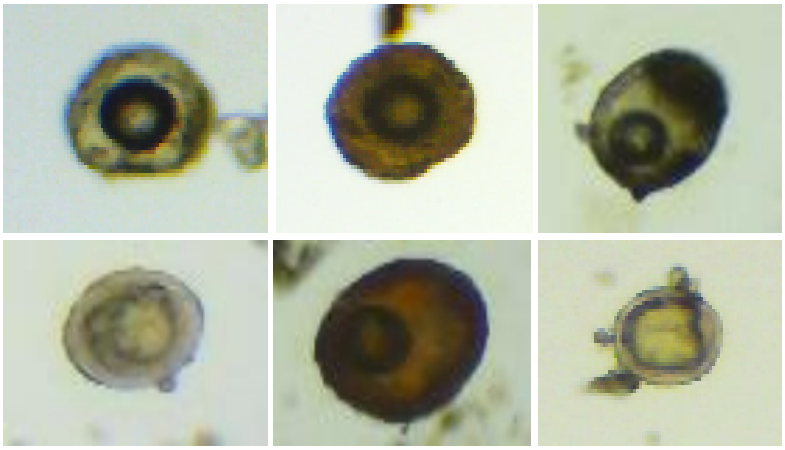

Figure 5. *Acaulospora* with several wall layers isolated from the rhizosphere soil of citrus seedlings

Acaulospora spore size was approximately 210 × 230 µm, and they were yellow with orange in the spore wall in the shape of a small hole spread uniformly (Munarti et al. 2019). Lobed vesicles were one of the characteristic of the *Acaulospora* species. Genus *Acaulospora* had several spore wall layers, giving the appearance of multiple layers of spore walls in a single spore. Next, identify AMF by molecular identification using PCR to ensure that AMF DNA was present in the roots.

PCR amplification with the universal primer-set of AML1/AML2 successfully amplified DNA fragments from AMF-colonized roots of four citrus species: 1. *C. limonia*, 2. *C. jambhiri*, 3. *F. japonica* cv. "Salam", 4. *C. reshni*. Further, the bright band was observed in the visualization on a UV transilluminator (Figure 6).

A PCR product of about 800 bp was produced by the AML1 and AML2 primers from each of the 23 distinct morphotypes of AMF spores and colonized roots. According to phylogenetic analysis, all of these products' sequences belonged to the phylum *Glomeromycota*. The small unit ribosomal gene of the AMF may be amplified effectively from field roots using the primers AML1 and AML2. The primers are substantially more specific to the *Glomeromycota* and offer higher coverage throughout the *Glomeromycota*, which has two significant advantages.

The percent identity and accession number of AMF species from the NCBI GenBank database are shown in Table 2. The data taken were the five species with the highest percent identity for each sample was sequenced. The sequence of the PCR products confirmed the highest similarity range from 83.7-99.0% to that of *Funneliformis*, followed by *Acaulospora* at a similarity range from 83.3-99.0% and found on all of the citrus species. The similarity sequence of the PCR products to that of *Gigaspora* range from 83.3-95.0% only found on *C. reshni* and *C. jambhiri*.

Of the 20 species in each sample, there were 11 species with the highest percentage identity. The 11 AMF species with the highest percent identity and 4 roots DNA samples were aligned into a phylogenetic tree to see the relationship between AMF species from four different citrus roots (Figure 7).

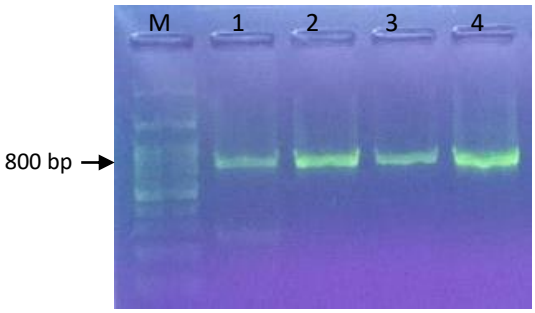

Figure 6. The amplification of DNA fragments by using AML1-AML2 primers from the total DNA of root samples. M: 100 bp DNA ladder Geneaid. Root sample of: 1. *C. limonia*, 2. *C.jambhiri*, 3. *F. japonica* cv. "Salam", 4. *C.reshni*

Table 2. Percent identity and accession number of AMF species from NCBI GenBank database

Citrus species	AMF species	Percent identity	GenBank accession number
F. japonica cv. "Salam"	*Funneliformis mosseae*	99.0%	NG_070284.1
	Acaulospora spinosa	99.0%	NG_062381.1
	Vinositunica ingens	99.0%	NG_070695.1
	Dentiscutata savannicola	99.0%	NG_070690.1
	Scutellospora calospora	99.0%	NG_070685.1
Citrus limonia	*Funneliformis mosseae*	95.2%	NG_070284.1
	Acaulospora spinosa	90.1%	NG_062381.1
	Scutellospora calospora	89.6%	NG_070685.1
	Acaulospora cavernata	89.6%	NG_062371.1
	Vinositunica radiata	89.4%	NG_070694.1
Citrus reshni	*Funneliformis mosseae*	83.7%	NG_070284.1
	Gigaspora candida	83.3%	NG_070692.1
	Racocetra castanea	83.3%	NG_070232.1
	Acaulospora cavernata	83.3%	NG_062371.1
	Diversispora celata	82.8%	NG_070908.1
Citrus jambhiri	*Funneliformis mosseae*	96.3%	NG_070284.1
	Spencermartinsiella europaea	96.2%	NG_061103.1
	Acaulospora spinosa	95.1%	NG_062381.1
	Gigaspora candida	95.1%	NG_070692.1
	Racocetra castanea	95.1%	NG_070232.1

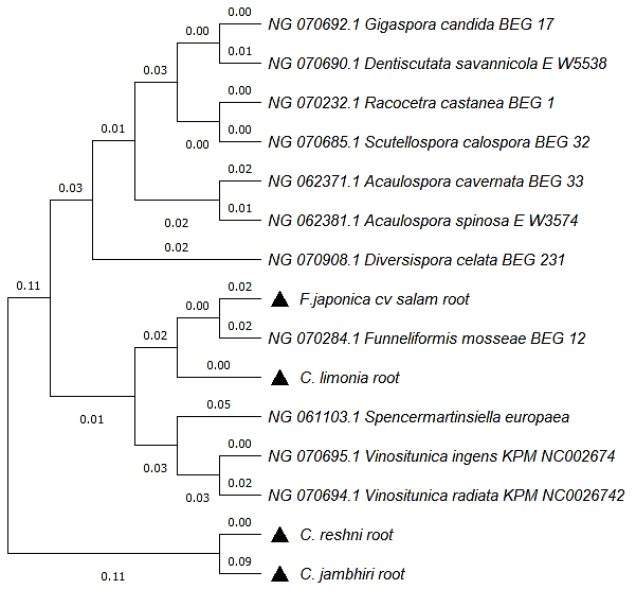

Figure 7. The phylogenetic tree of AMF colonized four citrus roots (▲) coverage by primers AML1/AML2

Table 3. Physio-chemical properties of soil

Citrus sp.	pH (H_2O)	C-organic (%)	P_2O_5 (ppm)
C. limonia	6.9	3.8	92
C. jambhiri	6.3	3.7	166
C. reshni	6.2	4.3	65
F. japonica cv. "Salam"	6.1	3.4	65

Based on the phylogenetic tree, the sample from *F. japonica cv salam* and *C. limonia* DNA roots was very close to the species *Funneliformis mossaeae* BEG 12 with accession number NG_070284. *Funneliformis mossaeae* BEG 12 was the basionym of *Endogone mosseae* and a homotypic synonym of *Glomus mosseae*. While *F. japonica* cv. "Salam" and *C. limonia* DNA roots were not closely related to the DNA samples of *C. reshni* and *C. jambhiri* roots.

Soil conditions and chemical composition were two factors that influence the proliferation and density of AMF spores. Table 3 shows the results of the rhizospheric soil chemistry compound.

The pH of water in the soil samples used in the study ranged from 6.1 to 6.9. The soil samples were classed as acidic to somewhat acidic based on the chemical composition of the soil. The activity of enzymes involved in plant germination, growth, and development can be influenced by the pH of the soil.

Discussion

The ability of citrus roots to produce AMF symbionts is widely documented (Song et al. 2015; Tian et al. 2021; Yang et al. 2021), with the higher number of *Glomeromycota* families, there are more diverse AMF species with higher spores. The *Glomeromycota* linked with the citrus in various species and environmental settings are represented in the present study in various ways. The AMF, which spontaneously colonized citrus seedlings in the nursery, was also included in this study.

In this study, the native AMF invaded all citrus root, forming vesicles and intracellular hyphae, which are characteristic AMF features. However, vesicles and intra-radical spores are rarely observed in most citrus roots (Beck et al. 2007). *C. limonia* had the highest root colonization, followed by *F. japonica* cv. "Salam", *C. reshni*, and *C. jambhiri*. Data on AMF colonization not significant statistically, while data on spore density did. Compared to other types, *C. jambhiri*, also known as Rough Lemon, has the highest spore density. AMF fungal spore density and root colonization were presumably selective adaptations toward the ecosystem. Additionally, it has been demonstrated that the community of AM fungi may influence the association and production of the host plant community (Powell and Bennett 2016; Wu et al. 2017; Sudová et al. 2020). On the other hand, it was shown by (Montoliu-Nerin et al. 2021) that each endophyte can multiply quite differently on various host plants and that there can be positive and negative feedback between a given host plant and its endophytes.

The spore morphological observation shows that spores of *Funneliformis* were light yellow, and hyphae were attached to the spore. This result relevant to recent study of AMF spores (Toh et al. 2018; Susila et al. 2022). In this study, glomus spores was small in size, less than 100 µm. *Funneliformis* spore forms were globose, ovoid, and ellipsoid, while the ornaments were smooth and verrucose. The distinctive characteristic of *Funneliformis* spore was that the spore wall visible, and hyphae tips were attached to the spore surface or subtending hyphae (Susila et al. 2022). Spores of this type of *Funneliformis* were most commonly found during observation under a microscope. This indicates that the citrus seedlings used as rootstocks have been colonized naturally by AMF dominated by the genus *Funneliformis* before being transplanted into polybags and grafted (Velázquez et al. 2020).

Spores of other genus, *Gigaspora* have the following characteristics: single spore in the soil, globose or subglobose; yellow, brownish yellow, to blackish; slimy white, golden yellow; the contents of the spore contain an oil-like fluid; lack of ornamentation; hyphae form bulbous suspensors or rounded hyphae holders; spores grow at the tip of the "bulbous suspensor" (Antoniolli et al. 2002; Beck et al. 2007; Munarti et al. 2019; Susila et al. 2022). Another characteristic of *Gigaspora* spore was the presence of spiny appendages on the surface of the spores and thin walls. Usually, the *Gigaspora* spore was bigger than *Funneliformis* and *Acaulospora*, and called the giant spore. In this study, we found a *Gigaspora* spore with a small size, less than 100 µm, but it was bigger than the other two species found, with a bulbous suspensor.

The last genus that found in the rhizosphere of citrus root was *Acaulospora*. *Acaulospora* spore size was approximately 210 × 230 µm, and they were yellow with orange in the spore wall in the shape of a small hole spread uniformly and relevan to recent study by Munarti et al.

(2019). Lobed vesicles were characteristic of the *Acaulospora* species (Morton and Msiska 2010; Redecker et al. 2013; Toh et al. 2018; Alimi et al. 2021). The hallmark of the genus *Acaulospora* was several spore wall layers so that one spore appears to have many layers of spore walls (Susila et al. 2022).

PCR amplification with the universal primer-set of AML1/AML2 successfully amplified DNA fragments from AMF-colonized roots of four citrus species. A PCR product of about 800 bp was produced by the AML1 and AML2 primers from each of the 23 distinct morphotypes of AMF spores and colonized roots. According to phylogenetic analysis, all of these products' sequences belonged to the phylum *Glomeromycota* (Lee et al. 2008). The small unit ribosomal gene of the AMF may be amplified effectively from field roots using the primers AML1 and AML2.

The result of molecular analysis revealed that the sequence of the PCR products confirmed the highest similarity range from 83.7-99.0% to that of *Funneliformis*, followed by *Acaulospora* at a similarity range from 83.3-99.0% and found on all of the citrus species. The similarity sequence of the PCR products to that of *Gigaspora* range from 83.3-95.0% only found on *C. reshni* and *C. jambhiri*. They present the first chance to examine the relative abundance of all recognized orders of *Glomeromycota* with a single instrument. The genus of *Funneliformis*, *Acaulospora*, and *Gigaspora* are included in the *Glomeromycota* phylum and class of *Glomeromycetes*. There are three classes of *Glomeromycota*: *Archaeosporomycetes*, *Glomeromycetes*, and *Paraglomeromycetes*, with five orders: *Archaeosporales*, *Diversisporales*, *Gigasporales*, *Glomerales*, and *Paraglomerales*, 14 families, 29 genera and approximately 230 species (Arofatullah et al. 2019).

Based on the BLAST analysis results, the genus *Funneliformis* and *Acaulospora* are highly similar, as indicated by their percent and found on all citrus species. Meanwhile, *Gigaspora* genus resembled *C. reshni* and *C. jambhiri* roots samples. According to the phylogenetic tree, DNA roots from *F. japonica* cv. "Salam" and *C. limonia* are highly similar to the *Funneliformis mossaeae* BEG 12 species, which had the accession number NG_070284 with taxonomy ID 27381. Species *Funneliformis mossaeae* BEG 12 was isolated from the Austrian Department of Botany collection. While *F. japonica* cv. "Salam" and *C. limonia* DNA roots were not closely connected to the DNA samples of *C. reshni* and *C. jambhiri* roots.

However, comprehensive studies utilizing morphological and molecular methods can aid in identifying the biodiversity of AMF, which naturally colonized the citrus seedlings in the nursery. Based on the results of this study, it may be necessary to increase AMF inoculation to improve AMF colonization of citrus plant roots by inoculation AMF biofertilizer. The activity of enzymes involved in plant germination, growth, and development can be influenced by the pH of the soil. The pH soil from the samples used in the soil study ranged from 6.1 to 6.9. The soil samples were classed as acidic to somewhat acidic based on the chemical composition of the soil. The AMF will be beneficial if applied on unfavorable soil (Susila et al. 2022). The fertility of the soil affects the availability of C-organic. Because one of the purposes of organic matter is to offer macro and micronutrients, in such a scenario, soil fertility is poor; a shortage of those nutrients defines it. While the symbiosis with AMF declines, the roots actively operate in fertile soil. This is connected to the mutualistic symbiosis principle, which states that plants won't seek assistance from other microbes, such as AMF, when soil fertility is high. The AMF symbiotic association majorly recognized to improve the growth of plant because AMF can change root system morphology, providing a better absorption area for water and nutrients in the rhizospheric soil (Nadeem et al. 2014; Goldschmidt 2014; Lü et al. 2018; Ishaq et al. 2021; Mitra et al. 2021, 2020; Yin et al. 2023). The colonization rate in the roots of citrus seedlings is classified as medium and low, so it is necessary to provide additional treatment or application of AMF biopesticides to increase the percentage of colonization (Verzeaux et al. 2017; Alimi et al. 2021). A recent study suggests that inoculation of AMF treatment in the early days of seedling growth can increase the percentage of colonization (Sulistiono et al. 2023), and the amount of AMF spore density in the plant rhizosphere may also grow as the proportion of colonization in the roots increases.

ACKNOWLEDGEMENTS

The author would like to thank the Educational Fund Management Institution (LPDP RI), Ministry of Finance, Republic of Indonesia, for scholarship funds during Doctoral education. The author also would like to thank The Australian Centre for International Agricultural Research (ACIAR), Project Number ACIAR HORT 2019/164, for including the author in the research project on Huanglongbing in Indonesia.

REFERENCES

Alimi AA, Adeleke R, Moteetee A. 2021. Soil environmental factors shape the rhizosphere arbuscular mycorrhizal fungal communities in South African indigenous legumes (*Fabaceae*). Biodiversitas 22: 2466-2476. DOI: 10.13057/biodiv/d220503.

Antoniolli Z, Facelli E, O'Connor P, Miller D, Ophel-Keller K, Smith S. 2002. Spore communities of arbuscular mycorrhizal fungi and mycorrhizal associations in different ecosystems, South Australia. Revista Brasileira de Ciência do Solo 26: 627-635. DOI: 10.1590/S0100-06832002000300007.

Arofatullah NA, Kabirun S, Fujiyama K, Widianto D. 2019. Molecular identification and in vitro propagation of arbuscular mycorrhiza from tea plant rhizosphere. Curr Res Environ Appl Mycol 9: 92-102. DOI: 10.5943/cream/9/1/10.

Beck A, Haug I, Oberwinkler F, Kottke I. 2007. Structural characterization and molecular identification of arbuscular mycorrhiza morphotypes of *Alzatea verticillata* (*Alzateaceae*), a prominent tree in the tropical mountain rain forest of South Ecuador. Mycorrhiza 17: 607-625. DOI: 10.1007/s00572-007-0139-0.

Cheng HQ, Giri B, Wu QS, Zou YN, Kuča K. 2022. Arbuscular mycorrhizal fungi mitigate drought stress in citrus by modulating root microenvironment. Arch Agron Soil Sci 68: 1217-1228. DOI: 10.1080/03650340.2021.1878497.

Gehlot P, Singh J. 2015. Arbuscular mycorrhizal fungi, *Glomus* spp. (*Glomeromycetes*), associated with drought tolerant plants of the Indian Thar desert. Austrian J Mycol 24: 15-23.

Goldschmidt EE. 2014. Plant grafting: New mechanisms, evolutionary implications. Front Plant Sci 5: 1-9. DOI: 10.3389/fpls.2014.00727.

Gusmiaty G, Larekeng S, Istigfaiyah L. 2019. Diversity and abundance of mycorrhizal fungi spores in *Gmelina arborea* Stand. ICOST 2019. Universitas Hasanudin, Makasar, 02-03 May 2019. DOI: 10.4108/eai.2-5-2019.2284693. [Indonesia]

Ishaq L, Adu Tae ASJ, Airthur MA, Bako PO. 2021. Effect of single and mixed inoculation of arbuscular mycorrhizal fungi and phosphorus fertilizer application on corn growth in calcareous soil. Biodiversitas 22: 1920-1926. DOI: 10.13057/biodiv/d220439.

Jacott CN, Murray JD, Ridout CJ. 2017. Trade-offs in arbuscular mycorrhizal symbiosis: Disease resistance, growth responses and perspectives for crop breeding. Agronomy 7: 1-18. DOI: 10.3390/agronomy7040075.

Lee J, Lee S, Young JPW. 2008. Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. FEMS Microbiol Ecol 65: 339-349. DOI: 10.1111/j.1574-6941.2008.00531.x.

Lü L-H, Zou Y-N, Wu Q-S. 2018. Relationship between arbuscular mycorrhizas and plant growth: improvement or depression? In: Giri B, Prasad R, Ajit V (eds.) Root Biology. Springer, New York. DOI: 10.1007/978-3-319-75910-4_18.

Mitra D, Djebaili R, Pellegrini M, Mahakur B, Sarker A, Chaudhary P, Khoshru B, Gallo MD, Kitouni M, Barik DP, Panneerselvam P. 2021. Arbuscular mycorrhizal symbiosis: Plant growth improvement and induction of resistance under stressful conditions. J Plant Nutr 44: 1993-2028. DOI: 10.1080/01904167.2021.1881552.

Mitra D, Khoshru B, Mohapatra PKD, Panneerselvam P. 2020. Beneficial interaction of arbuscular mycorrhizal fungi in plants to improve the uptake of phosphorus. Indian J Plant Soil 7: 10-12. DOI: 10.21088/ijps.2348.9677.7120.8.

Montoliu-Nerin M, Sánchez-García M, Bergin C, Kutschera VE, Johannesson H, Bever JD, Rosling A. 2021. In-depth phylogenomic analysis of arbuscular mycorrhizal fungi based on a comprehensive set of de novo genome assemblies. Front Fungal Biol 2: 1-13. DOI: 10.3389/ffunb.2021.716385.

Morgan K, Rouse R, Ebel R. 2016. Foliar applications of essential nutrients on growth and yield of "valencia" sweet orange infected with huanglongbing. Am Soc Hortic Sci 51: 1482-1493. DOI: 10.21273/HORTSCI11026-16.

Morton JB, Msiska Z. 2010. Phylogenies from genetic and morphological characters do not support a revision of *Gigasporaceae* (*Glomeromycota*) into four families and five genera. Mycorrhiza 20: 483-496. DOI: 10.1007/s00572-010-0303-9.

Munarti, Wulan A, Utami A. 2019. Exploration and identification of arbuscular mycorrhizal fungi from the rhizosphere of chili plants (*Capsicum annuum* L) in Bogor. J Sci Innovare 1: 50-53. DOI: 10.33751/jsi.v1i02.1001.

Nadeem SM, Ahmad M, Zahir ZA, Javaid A, Ashraf M. 2014. The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. Biotechnol Adv 32: 429-448. DOI: 10.1016/j.biotechadv.2013.12.005.

Paz C, Öpik M, Bulascoschi L, Bueno CG, Galetti M. 2021. Dispersal of arbuscular mycorrhizal fungi: Evidence and insights for ecological studies. Microb Ecol 81: 283-292. DOI: 10.1007/s00248-020-01582-x.

Powell JR, Bennett AE. 2016. Unpredictable assembly of arbuscular mycorrhizal fungal communities. Pedobiologia 59: 11-15. DOI: 10.1016/j.pedobi.2015.12.001.

Redecker D, Schüßler A, Stockinger H, Stürmer SL, Morton JB, Walker C. 2013. An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (*Glomeromycota*). Mycorrhiza 23: 515-531. DOI: 10.1007/s00572-013-0486-y.

Song F, Pan Z, Bai F, An J, Liu J, Guo W, Bisseling T, Deng X, Xiao S. 2015. The scion/rootstock genotypes and habitats affect arbuscular mycorrhizal fungal community in citrus. Front Microbiol 6: 1-11. DOI: 10.3389/fmicb.2015.01372.

Sudová R, Kohout P, Rydlová J, Čtvrtlíková M, Suda J, Voříšková J, Kolaříková Z. 2020. Diverse fungal communities associated with the roots of isoetid plants are structured by host plant identity. Fungal Ecol 45: 1-15. DOI: 10.1016/j.funeco.2020.100914.

Sulistiono W, Aji HB, Handoko S, Lase JA, Suryanti S, Apriyana Y, Molide R. 2023. Effect of arbuscular mycorrhizal fungi on early growth , root colonization , and chlorophyll content of North Maluku nutmeg cultivars. Open Agric 8: 1-12. DOI: 10.1515/opag-2022-0215.

Sun X, Feng J, Shi J. 2022. Stimulation of Hyphal ramification and sporulation in funneliformis mosseae by root extracts is host phosphorous status-dependent. J Fungi 8: 1-15. DOI: 10.3390/jof8020181.

Susila E, Rukmana S, Sagita O, Achmad BS, Maulina FP. 2022. Exploration and morphology identification of spores. J Appl Agric Sci Technol 6 (1): 20-30. DOI: 10.55043/jaast.v6i1.31.

Tian, L, Zou YN, Wu QS, Kuča K. 2021. Mycorrhiza-induced plant defence responses in trifoliate orange infected by *Phytophthora parasitica*. Acta Physiol Plant 43: 1-8. DOI: 10.1007/s11738-021-03216-2.

Toh SC, Lihan S, Chuan B, Yong W, Tiang BR, Abdullahi R, Edward R, Samarahan K, Ecology E. 2018. Selected plant roots and their rhizosphere soil environment. Malays J Microbiol 14: 335-343. DOI: 10.21161/mjm.144187.

Velázquez MS, Fabisik JC, Barrera M, Allegrucci N, Valdés FE, Abarca CL, Cabello M. 2020. Diversity and abundance of arbuscular mycorrhizal fungi (*Glomeromycota*) associated with *Ilex paraguarensis* in Northeastern Argentina. Rev Biol Trop 68: 1231-1240. DOI: 10.15517/rbt.v68i4.41543.

Verzeaux J, Nivelle E, Roger D, Hirel B, Dubois F, Tetu T. 2017. Spore density of arbuscular mycorrhizal fungi is fostered by six years of a no-till system and is correlated with environmental parameters in a silty loam soil. Agronomy 7: 1-9. DOI: 10.3390/agronomy7020038.

Willis A, Rodrigues BF, Harris PJC. 2013. The ecology of arbuscular mycorrhizal fungi. Crit Rev Plant Sci 32: 1-20. DOI: 10.1080/07352689.2012.683375.

Wu QS, Srivastava AK, Zou YN, Malhotra SK. 2017. Mycorrhizas in citrus: Beyond soil fertility and plant nutrition. Indian J Agric Sci 87: 427-443. DOI: 10.56093/ijas.v87i4.69308.

Yang L, Zou YN, Tian ZH, Wu QS, Kuča K. 2021. Effects of beneficial endophytic fungal inoculants on plant growth and nutrient absorption of trifoliate orange seedlings. Sci Hortic 277: 1-7. DOI: 10.1016/j.scienta.2020.109815.

Yin X, Zhang W, Feng Z, Feng G, Zhu H, Yao Q. 2023. Improved observation of colonized roots reveals the regulation of arbuscule development and senescence by drought stress in the arbuscular mycorrhizae of citrus. Hortic Plant J 6: 1-16. DOI: 10.1016/j.hpj.2023.04.006.