Colonization of arbuscular mycorrhizal fungi (AMF) in the rhizosphere of robusta coffee plant (*Coffea canephora*)

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Abstract. In this study arbuscular mycorrhizal fungi (AMF) colonization in the soil at the Bangelan Robusta coffee plantation, Malang, East Java was observed in two different locations with different plant ages, 49 and 10-year-old. The soil and root samples were collected from 0-50 cm and >100 cm from the plant base to observe the variation in the genus of AMF, the number of spores in the soil, and AMF infection in the roots of robusta coffee. A wet screening method was applied to observe the AMF spores in the soil. 250 µm, 125 µm, and 63 µm sieve meshes were used to collect the AMF spores from the 10 g soil sample. Root infection was observed using Philips and Hayman's staining roots method. The results show that AMF has a root infection in the coffee roots of both 49-year-old and 10-year-old coffee plants. A higher spore number trend in the soil was found in the coffee farm with 49-year-old coffee plants than in the soil with 10-year-old coffee plants. Higher root infection was found in the roots collected from 0-50 cm from the plant-based. Morphologically, the AMF genera found in the robusta coffee plantation were *Glomus* and *Acaulospora*, while the *Acaulospora* spore number was found to be higher than *Glomus*. The AMF infection rate in the robusta coffee roots was relatively low, ranging from 20% to 30%.

1 Introduction

Coffee is a perennial tropical crop widely cultivated in various countries, including Indonesia. Indonesia's most popular coffee plants have two types of coffee: Arabica and Robusta coffee. 71% of Indonesia's coffee production is dominated by robusta coffee (BPS, 2022). Farmers have adopted two coffee cultivation systems in Indonesia: organic and conventional. The cultivation systems affect the colonization of soil microorganisms, such as Arbuscular Mycorrhizal Fungi (AMF) (Azhar et al., 2023). AMF plays an important role in helping the plant roots absorb nutrients in the soil, especially phosphate (Doudi et al., 2018), increasing the availability of water for plants through hyphal extension, triggering root growth (Muhammad & Isnatin, 2019), increasing the reach or exploration of roots in the soil, increasing the stability of soil aggregates (de Novais et al., 2019), and increasing tolerance to drought stress, salinity stress (Khaliq et al., 2022).

AMF is naturally found in the roots of coffee plants (Muleta et al., 2007; Sugiarti, 2018). Previous studies found that in the rhizosphere layer of Arabica coffee, many AMF spores AMF of the species Acaulospora, Gigaspora, and Glomus were found (Dewi et al., 2016), while in an area cultivated with Robusta coffee, the rhizosphere layer obtained more AMF of the species Acaulospora, Gigaspora, and Glomus.

The information on AMF colonization in conventional coffee plantations still needs to be gathered. This study was conducted specifically to observed whether the distance of rhizosphere from the base of the stem is affected to the AMF colonization in the soil and coffee roots. The distance between the roots and the base of the coffee stem is associated with applying chemical fertilizer to the area close to the base of the plant stem. Therefore, this study can provide information on whether chemical fertilizer residues influence AMF colonization in the rhizosphere of coffee plants. In addition, the plants' different ages were also evaluated compared to the AMF infection. This study hypothesizes that AMF colonization and infection will be lower as the distance between the roots and the base of the coffee stem increases (≥100 cm away from the plant canopy).

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2 Materials and Methods

This study was conducted from August to November 2023 in Bangelan Robusta coffee plantation, Malang, East Java, Indonesia. The coffee clones cultivated are BGN371,300,372, BP308,254, and 358. The elevation of the Bangelan coffee plantation is 450-650 meters above seal levels (masl), covering an area of 883.20 ha with rainfall type C (Schmidt Ferguson).

2.1 Soil and Root Sampling from Bangelan Plantation

Two different sites were chosen, one area with 49-year-old coffee plants (the planting year 1974) and 10-year-old coffee plants (the planting year 2013). The soil and root samples were collected from two different distances: 0-50 cm and ≥ 100 cm from the base of the stem. The samples from each location with different plant ages and sampling distances from the plants were collected with five replications, respectively. Sampling illustration and vegetation of the sites can be seen in Figures 1 and 2.

Soil samples were taken in as much as 100 grams, and the soil samples were placed into ziplock plastic. A description of the sample number, plant age, and collection location was then given. Root samples were taken at the fibrous roots by cleaning the remains of wild plants around Robusta coffee plants; root samples were taken at a depth of 20 cm and placed into a centrifuge tube filled with alcohol 60% for preservation.

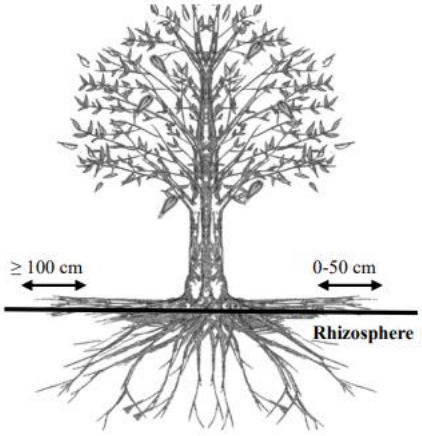

Figure 1. Illustration of soil and root sampling collected from the nearest shoot-based (0-50 cm) and away from shoot-based (≥ 100 cm)

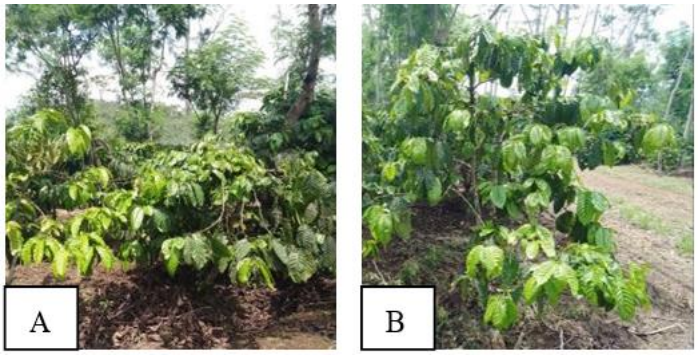

Figure 2. Sampling sites of soil and root sampling at Robusta Coffee (A.49 years) (B. 10 years).

2.2 Isolation and Identification of Mycorrhizal Spores in the soil

The soil samples were collected from two different plant ages, namely 49 years and 10 years of age with two different distances (0-50 cm) and (≥ 100 cm) from the base of the stem with a depth of ±20 cm. After the soil samples were completed, the isolation and identification stage continued. The technique used in isolating AMF spores is the method (Brundrett et al., 1996). The isolation process continued with several steps (1) mixing 10 grams of soil samples with 200-300 ml of water and stirring; (2) filtering in a set of sieves with sizes 250 µm, 125 µm, 63 µm sequentially from top to the bottom; (3) the material (supernatant) stored on the 63 µm sieve is transferred to a container in the form of a petri dish; (4) observed under a stereo microscope to count the spore population.

Identification based on shape and color. To observe spore identification by matching spore characteristics, look at the website: http: fungi.invam.wvu.edu/the-fungi/species-descriptions.html. Spore preparations were made using Melzer's dye to help accelerate the identification of spores to the genus level. However, no identification was carried out in this study using Melzer's coloring material. Spores were placed on a glass plate. Spore preparations were observed under a microscope.

2.3 Roots Staining for AMF Infection Identification in the Roots of Coffee

Staining coffee roots can be observed by using the method of Philips and Hayman (1970). (1) wash the roots thoroughly with running water; (2) soak the roots in 10% KOH solution for 12-24 hours or put in an oven at 90℃ for 15-30 minutes (depending on the type of root); (3) wash the roots with water 3-5 times using a tea strainer as a container; (4) if the roots are not clear, soak the roots in a commercial bleach solution that has been diluted 10 × dilution for 24 hours or put in an oven at 90℃ for 15-30 minutes (until the roots look clear) then wash with water 3-5 times washing; (5) soak roots in 2% HCl solution for 12 hours; (6) soak the roots in trypan blue solution for 12-24 hours or put them in a 90℃ temperature oven for 15-30 minutes and wash with water 2-5 times washing; (7) soak in destaining solution to remove excess trypan blue dye solution; (8) the roots are arranged on glass plate with as many as 10 pieces of 2 cm roots; (9) root pieces on the glass plate were observed under a microscope for each field of view; (10) fields of view that showed infected (external hyphae, internal hyphae, vesicles, and arbuscular) were marked (+) while those that were not infected were marked negative (-).

2.4 Calculating Mycorrhizal Infection Rate

Calculation of mycorrhizal infection rate using the following equation (Brundrett et al., 1996)

The infection root (%) = the number of infected roots/the number of observed roots × 100% The level of infection in the root was categorized into five classes according to (Rajapakse and Miller. 1992).

Table 1. AMF Infection Level

Percentage of infection	Category	Description
0-5	Class 1	Very low
6-25	Class 2	Lower
26-50	Class 3	Medium
51-75	Class 4	High
76-100	Class 5	Very high

3 Results and Discussion

3.1. Isolation and Identification of Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular mycorrhizal fungi (AMF) found in Robusta coffee plantation, Bangelan, Malang, East Java were identified based on spore characteristics such as shape and color. At the age of 49 years with both distances (0-50 cm) and (≥ 100 cm) from the base of the stem, the spores found were *Glomus* and *Acaulospora*.

Glomus spores were round and slightly round, brownish orange, and had hyphal mounts (Figure 3. A, B). According to INVAM (2023), *Glomus* spores are round, slightly round, and oval. Has several layers of spore walls. There is a straight hyphal holder (subtending hyphae) in a cylinder. Spore color varies from hyaline, pale white, brownish yellow, yellowish brown, light brown, brownish orange, to dark blackish brown.

The *Acaulospora* spores were round, slightly round, brownish yellow, and no hyphal mounts (Figure 3. C, D, E). According to INVAM (2023), *Acaulospora* spores are usually round slightly round, and oval. It does not have hyphal mounts (subtending hyphae). Spore colors range from hyaline, brownish yellow to dark brownish red.

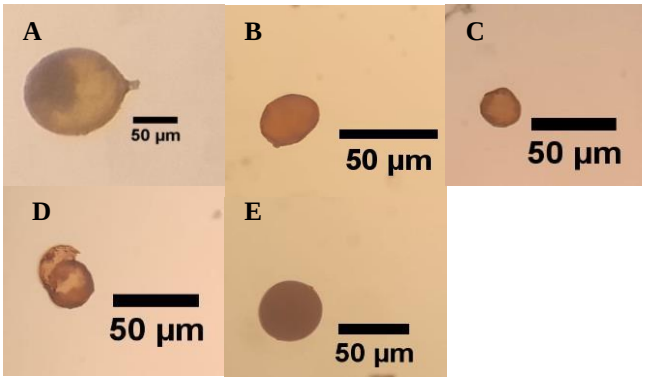

Figure 3. Arbuscular mycorrhizal spores obtained from soil samples at 49 years, bar = 50 µm. *Glomus sp.* (A, B), *Acaulospora sp.* (C, D, E).

The AMF colonization in the Robusta coffee plantation was identified based on spore morphological characteristics such as shape and color. Based on the analysis, *Acaulospora* and *Glomus* spores were found in a 10-year-old robusta coffee plant where the soil samples were collected at both distances, spores were *Acaulospora* and *Glomus*.

The *Acaulospora* spores were slightly round, yellow to dark brown, and no hyphal mounts were found (Figure 4. A, B, C). According to INVAM (2023), *Acaulospora* spores are usually round slightly round, and oval. It does not have hyphal mounts (subtending hyphae). Spore colors range from hyaline, brownish yellow to dark brownish red.

Glomus spores were round, brownish yellow, and had hyphal mounts (Figure 4. D). According to INVAM (2023), *Glomus* spores are round, slightly round, and oval. Has several layers of spore walls. There are straight hyphae stands (subtending hyphae) in a cylinder. Spore color varies from hyaline, pale white, brownish yellow, yellowish brown, light brown, brownish orange, to dark blackish brown.

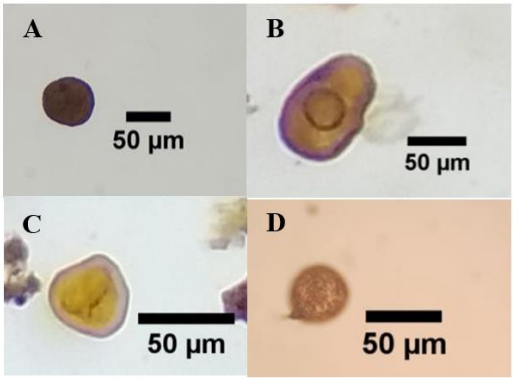

Figure 4. Arbuscular mycorrhizal spore obtained from soil samples at 10 years bar= 50 µm. *Acaulospora sp.* (A, B, C) and *Glomus sp.* (D).

Based on the observation, the *Glomus* and *Acaulospora* spores were found in 10 years old and 49 years old coffee populations and both (0-50 cm) and (≥ 100 cm) from the stem (Dewi et al., 2016) also reported that the AMF genus found most in the Robusta coffee rhizosphere is the *Acaulospora* genus. The same thing happened in the research results (Utami. 2021) regarding identifying AMF in Robusta coffee plants two genera were found *Acaulospora* and *Glomus*. Certain genus has a very wide distribution and some genera have a limited distribution (Miska et al., 2016). Inequality of location and age of plants causes differences in species diversity and AMF populations (Sundari et al., 2011; Ansiga et al., 2017).

3.2 Number of AMF Spores from the Rhizosphere of Robusta Coffee Plant

According to the Student t-test, in both locations there was not a significant difference (Figure 5).

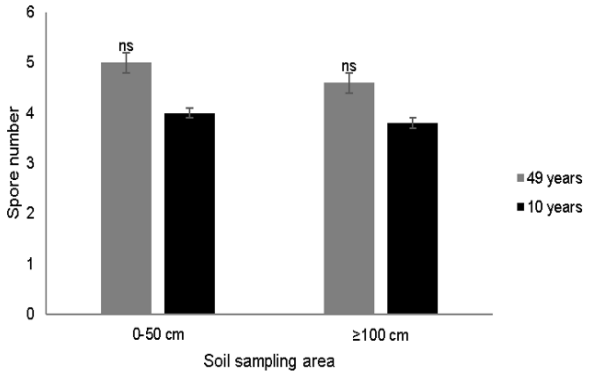

Figure 5. Spore counts in soil samples were taken in the rhizosphere of 49 years old (gray) and 10 years old (black). ns: not significant according to Student t-test (n=20)

This is consistent with the research (Syahputra et al., 2021) on Arabica coffee plants which explains that the number of spores has no significant effect on plant age. Differences in plant age affect AMF populations in the soil (Widiastuti 2006; Parlindungan 2017). The number of AMF spores between plant age and location had no significant effect. Spore number is also affected by soil pH, vegetation, and land use type (Miska et al., 2016). The highest number of spores was found in the soil sampling area with a distance of (0-50 cm) from the plant. This aligns with research (Rasyid et al., 2017) that the closer a plant is to the root zone, the more spores are found when compared to the number of spores far from the plant's root zone.

3.3 AMF Infection Rate

The results of the percentage level of AMF infection in the roots of Robusta coffee plants with different plant ages and areas of sampling location points are presented in (Figure 6).

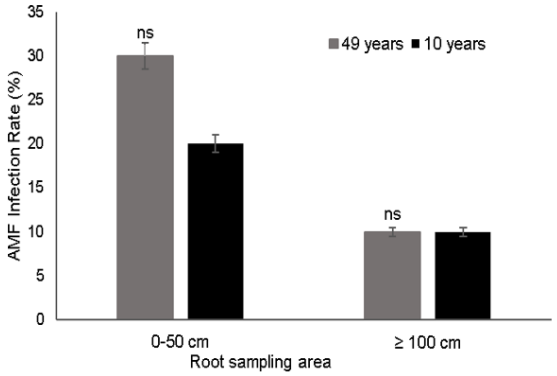

Figure 6. Percentage of AMF infection in roots of robusta coffee plants at 49 years and 10 years. ns: not significant according to Student t-test (*n*=20).

The percentage of AMF infection of Robusta coffee roots at 49 years and 10 years with the distance (0-50 cm) from the base of the stem has a percentage of 30% and 20% is included in the high category level if the percentage value of infection ≥ 50% (Rajapakse and Miller. 1992). This result found that the number of AMF infections between plant age and location has no significant effect. According to Research (Tuheteru 2003; Smith & Read 2008; Qomariah et al., 2017) stated that there was no relationship between the percentage of root infection and the number of spores produced, so a large number of spores did not necessarily have a high rate of root infection. According to (Sieverding 1986; Sari et al., 2017) different plant species will show different reactions to mycorrhizal infection and indirectly affect the development of AMF infection.

The high and low infection levels are influenced by several factors, such as the shape of plant roots in taproots or fibrous roots, root texture, root type, and environmental conditions where the plants grow (Qomariah et al., 2017).

3.4 Analysis of AMF Root Infection

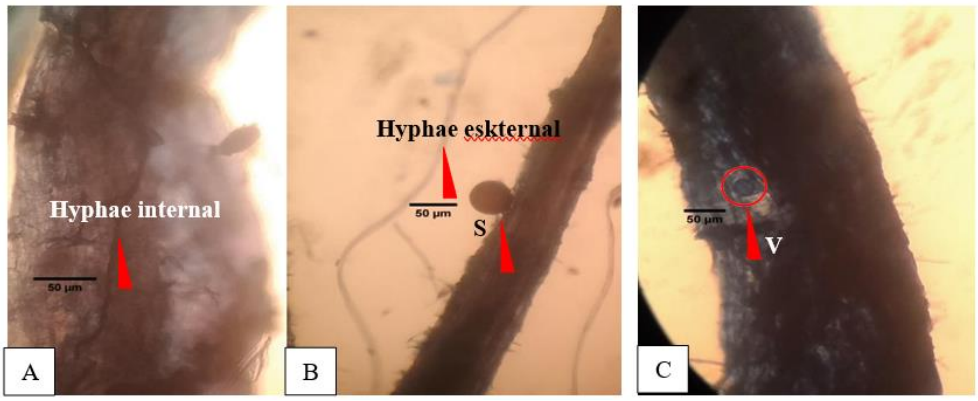

Figure 7. Photomicrograph of root samples obtained from Robusta coffee. AMF infection coffee roots (<1 mm). Vesicles (V), spores (S) with 20 time magnification

The results of staining on Robusta coffee roots by observation under a microscope with 20 times magnification taken at both plant ages and two different distances (0-50 cm) and (≥ 100 cm) from the base of the stem found the presence of infection in Robusta coffee roots characterized by the presence of AMF structures in the form of hyphae and vesicles (Figure 7. A, B). In addition, spores and external hyphae were found to infect Robusta coffee roots (Figure 7. C). According to (Allen 2001; Suharno et al., 2020) AMF symbiosis in the host can be seen by the presence of commonly found structures, namely external hyphae, internal hyphae, arbuscules, vesicles, and spores.

The vesicles and spores found in this study were ovoid. According to (Dewi et al., 2016; Suharno et al., 2020) vesicles and spores are ovoid structures. This research by (Dewi et al., 2016) on Robusta coffee plants found the structure of hyphae, vesicles, and inner spores with a total percentage of AMF infection of 30%. This is in line with the results of observations of AMF infection in the two different planting years, which found structures on infected roots in the form of hyphae, vesicles, and spores stated that the presence of these structures indicates infection or symbiosis of AMF with plant roots. According to (Sukmawati 2013; Syahputra et al., 2021) The number of spores had no significant effect on the infections in the host plant roots. The association between mycorrhiza and plant species can provide different

infections in the root system, and its impact on nutrient absorption is also different, not because of the number of spores contained in the rhizosphere but because plant genotypes and environmental factors influence this response (Lukitaningdyah 2013; Sari et al., 2017).

4 Conclusion

The AMF genera found in the Robusta coffee plantation were *Glomus* and *Acaulospora*. Morphology and visually *Acaulospora* spore was the dominant genus in the soil. The AMF infection rate in Robusta coffee roots was relatively low, range 20-30%.

Acknowledgments

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