



The arbuscular mycorrhizal fungi colonizing the roots of pre-nursery stage of oil palm seedlings: *Elaeis guineensis* in Malaysia

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ABSTRACT

Aims: Arbuscular mycorrhizal is an obligate mutualistic symbiosis fungus which survives by forming endomycorrhizal on plant roots. The arbuscular mycorrhizal fungi are not host-specific, allowing them to form a mutualistic symbiosis with a wide range of host plants including oil palm. In Malaysia, the arbuscular mycorrhizal fungi are used as a growth enhancer for the oil palm: *Elaeis guineensis*. The arbuscular mycorrhizal fungi are introduced only during transplantation to the field when the ages of the seedlings are approximately one year old. As such, this study is designed to investigate the ability of the arbuscular mycorrhizal fungi to form colonisation with pre-nursery oil palm seedlings.

Methodology and results: Here, the arbuscular mycorrhizal fungi were introduced at the pre-nursery stage oil palm seedlings. After inoculation, the seedlings were harvested on different days, i.e. on day-3, day-7, day-14, day-21, day-40 and day-60 to determine the colonisation of arbuscular mycorrhizal fungi. We found that the arbuscular mycorrhizal fungi are able to form a mycorrhizal association with the oil palm seedling at the pre-nursery stage after 40 days of inoculation, and the arbuscular mycorrhizal fungi that formed the association are *Glomus* sp. and *Scutellospora* sp.

Conclusion, significance and impact of study: This study suggested that the oil palm seedling can be made into a mycorrhizal plant as early as the nursery stage before transplanting them into the plantation.

Keywords: *Glomus* sp., *Scutellospora* sp., arbuscular mycorrhiza, fertilizers, oil palm

INTRODUCTION

Oil palm plantations cover a large area in Malaysia and contribute as a major economic income of Malaysia (Lindsay *et al.*, 2012). In particular, the development of oil palm plantations has helped alleviate poverty among landless farmers in Malaysia (Basiron, 2007). Palm oil is now a major source of sustainable and renewable raw material for the world's food, oleochemical and biofuel industries (Basiron, 2007). In addition, high expectation and demand for palm oil in biofuel research has strengthened the value of oil palm in the world's market (Koh, 2007). In order to focus on developing a sustainable country, the oil palm plantations in Malaysia should not only aim at improving the yield of the oil palm but also focus on the long-lived land usage for agriculture (Basiron, 2007). The arbuscular mycorrhizal (AM) fungi have played the vital role of creating an ideal ecological agriculture and also fulfilling the quantity and quality demands from the public (Smith and Read, 2008). The symbiosis of AM fungi and plant has existed for a long time and it is believed that the evolution and diversity of plant kingdom are highly related to the colonisation of AM fungi on plant root (Smith and Read, 2008). This is a

mutual symbiosis in which both the AM fungi and host plant benefit from each other (Peterson *et al.*, 2004; Smith *et al.*, 2011). This symbiosis confers benefits directly to the host plant's growth and development through the acquisition of phosphorus (P) and other mineral nutrients from the soil via the AM pathway (Harrier, 2001; Aggarwal *et al.*, 2011). In this symbiosis relationship, the AM fungi receive energy supplies in the form of carbon from its host plant (Ibjijsen *et al.*, 1996). The AM fungi can increase the tolerance ability of the host plant towards environmental stresses such as toxicity, drought condition and high salinity (Smith and Read, 2008).

The root structure of the oil palm shows a high association with AM fungi to obtain nutrients (Corley and Tinker, 2015). Most of the oil palm plantation owners apply an enormous dosage of fertiliser to increase the yield of palm oil (Smith and Read, 2008). In fact, without the aid of the AM fungi, the amount of nutrient absorption by oil palm is reduced (Corley and Tinker, 2015). Consequently, the excess fertiliser will be washed out and cause environmental problems such as eutrophication (Tilman *et al.*, 2002). In order to reduce the cost of a plantation to gain the highest profit, we should utilise natural beneficial fungi, i.e. the AM fungi to promote our

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agriculture industry (Smith and Read, 2008). One of the current practices conducted by some planters is the introduction of AM fungi to oil palms during the transplantation process of the palms to the plantation. As such, this study is designed to investigate the ability of pre-mature oil palm seedling to form a mycorrhizal association with AM fungi isolated from oil palm plantation, which will allow the planters to transplant 'ready-mycorrhizal' oil palm seedling to the plantation in the future. The stage of nursery seedling is more vulnerable but important to ensure good development and yield of the plant (Azcón-Aguilar and Barea, 1997).

MATERIALS AND METHODS

Soil sampling

The soil samples were collected from Sungai Kechil Ulu Bandar Baharu oil palm plantation, Perak, Malaysia (N05°06'33.9" E100°32'45.9") and the oil palms ages were three years old. This plantation is an open plantation area with good aeration.

Isolation of arbuscular mycorrhizal fungi spores

The wet-sieving technique was used to isolate the AM from soil samples as per Brundrett *et al.* (1996). The 100 g of soil was weighed and mixed thoroughly with water. The soil was then sieved through multiple sizes of sieves, starting with 1,000 µm, 500 µm, 250 µm, 75 µm and followed by 45 µm. The debris that had been sieved in 1,000 µm and 500 µm sieves were thrown away and the soil samples that were sieved using the 250 µm, 75 µm and 45 µm sieves were collected into 50 mL centrifugation tubes for centrifugation process. The samples were centrifuged for 3 min at 2,000 rotations per minute (rpm) to separate the spore from the soil samples. The floating debris with supernatant was discarded and the pellets were kept for the second centrifugation. The pellets were re-suspended in 50% sucrose and second centrifugation was conducted for 1 min at 2,000 rpm. A vacuum pump was used to filter the supernatant with a Whatman No. 42 filter paper. The spore observation was then conducted under the dissecting microscope. The spores were sorted out by their shapes, sizes and colour under the dissecting microscope. The different morphologies of the AM spores were selected and made into slides and the pictures were taken using camera Olympus BX41 attached to the compound microscope equipped with the software Cell A. The spores were identified based on their physical morphologies.

Arbuscular mycorrhiza propagation

Pot cultures with six pots of three months old oil palm nursery seedling were used to propagate the AM spores. The isolated AM spores were washed from the filter paper into the pot using distilled water. The three months old oil palm nursery seedlings were then left for two months to allow the AM spores to propagate.

Sowing the oil palm seedlings.

The oil palm seeds were sowed in heat-sterilised soil (150 °C for 24 h) with 4:1 of clay : sand ratio that were mixed together thoroughly. The mixed sterilised soil was filled into the seed tray tightly to prevent the soil leakage from seed tray. While filling in the soils, the soils were watered to increase the soil compaction. The soil was added into the seed tray up to 80% full. A hole of 2 cm depth was made on each container soil and the seeds were sowed into the hole. The radical of the seed was ensured to be directed down and the plumule of seed was directed upward and covered with the soil. The seeds were left for approximately two months to grow into pre-nursery seedlings. The pre-nursery seedlings are the seedlings that are less than 3 months old, while the main nursery seedlings are the seedlings that are between 3 to 4 months old (Yusoff *et al.*, 2013).

Preparation of the arbuscular mycorrhizal fungi inoculums

After three months of AM spores propagation in the pots of three months old oil palm seedlings (as section 2.4), the oil palm seedlings were harvested to be made into the AM inoculums. Enumeration of spores was conducted to ensure the success of propagation. The host plants were harvested and the number of spores was calculated. The soils from three pots of host plants were mixed together and the roots of the propagation AM spores host were chopped into small pieces and mixed into the entire propagated soil.

Transplantation and inoculation of arbuscular mycorrhizal fungi

Ninety pre-nursery seedlings were transferred into 15 cm × 23 cm (6 inch × 9 inch) polybags after two months of germination in a mixture oven-sterilised soil of clay, sand and organic soil in the ratio of 4 clay : 1 sand : 1 organic soil. During the transplantation, 100 g of inoculum soil had been weighed and added into 45 pots of oil palm seedlings. Another 45 oil palm seedlings were potted as controlled pots with no inoculums soils added. The seedlings were harvested on different days, i.e. on day-3, day-7, day-14, day-21 and day-40 after the introduction of AM inoculums.

Roots staining

The harvested roots were cut and immersed in 10% KOH (w/v) at 90 °C for 2-3 h in a water bath using a modified method from Phillips and Hayman (1970). KOH in the staining functions as the bleaching agent to remove dark pigments on the root surface in order to ease the staining process (Phillips and Hayman, 1970). The roots were then acidified in 5% HCl prior to the staining as the HCL will neutralise the alkaline of the bleached roots (Galindo-Castañeda and Romero, 2013). The roots were stained in 0.05% w/v trypan blue in lactoglycerol in a 90 °C water

bath for 12 to 60 min (Bevege, 1968; Phillips and Hayman, 1970; Brundrett *et al.*, 1996) for root colouration. The stained roots were stored in 50% glycerol before observation, as recommended by Brundrett *et al.* (1996).

RESULTS

Identification of arbuscular mycorrhizal fungi spores

Figure 1 shows the fresh *Glomus* sp. spores with aggregated spore arrangement under a dissecting microscope. The *Glomus* sp. spores have attached to the subtending hyphae and showed no cell wall (Figure 1c-1f). Under the compound microscope, the structure of the subtending hyphae is simpler (Figure 1e) in comparison to the swollen subtending as *Scutellospora* sp. and *Gigaspora* sp. The *Glomus* sp. spores showed the colour of 60:50:80 CYM (Cyan:Yellow:Magenta) value as suggested by International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) colour chart designed by Morton (1992).

Figure 2 shows the isolated fresh *Scutellospora* sp. spore and the squashed *Scutellospora* sp. spore under a dissecting microscope and a compound microscope, respectively. The fresh *Scutellospora* sp. spore was attached to a swollen subtending hypha. The

Scutellospora sp. spore showed singly spore aggregation. The squashed *Scutellospora* sp. spore showed two layers of cell walls, i.e. the inner wall and outer wall. The germination shield was seen in the squashed *Scutellospora* sp. spore. The *Glomus* sp. spores showed a colour of 20:70:80 CYM (Cyan:Yellow:Magenta) value according to the INVAM colour chart designed by Morton (1992).

Colonisation of arbuscular mycorrhizal fungi in pre-nursery oil palm roots and development of arbuscular mycorrhizas on oil palm roots

The AM-treated oil palm seedlings were harvested on various days to observe the development of AM colonisation on oil palm. Figure 3a-x show the development of AM morphologically. After 3 days of AM introduction, there was no sign or symptom of colonisation by AM fungi on the seedling roots showed in Figure 3a and b. The stained secondary and tertiary roots did not show any sign of colonisation and the plant cells had no hyphal infection.

For oil palm seedlings harvested after 7 days, there were signs of colonisation of AM fungi as shown in Figure 3c and d.

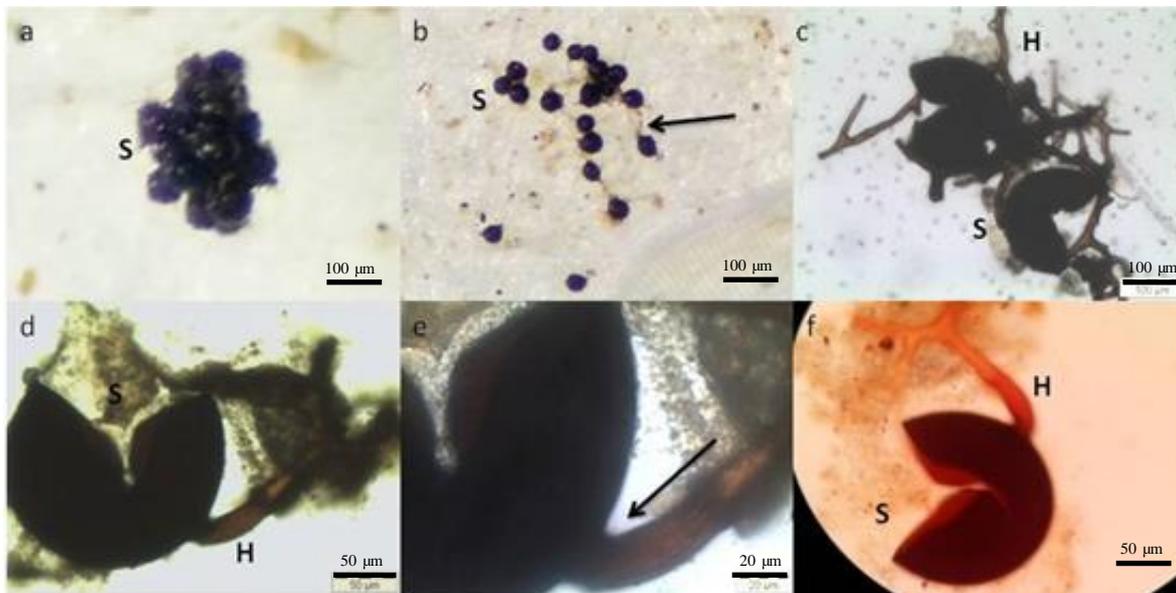


Figure 1: The *Glomus* sp. spores isolated from the sampled soil. (a and b) The fresh and unsquash *Glomus* sp. spores observed under dissecting microscope. (c, d and e) The squashed *Glomus* sp. spores observed under compound microscope. (a) The fresh *Glomus* sp. spores with aggregated spore arrangement. (b) Each of the separated fresh spores are attached to the subtending hyphae (arrow). (c) The squashed spores attached to each other and formed aggregation arrangement. (d) The squashed *Glomus* sp. spore attached subtending hypae. (e) Simple subtending hyphae attached with *Glomus* sp. spore. (f) The *Glomus* sp. spores showed the colour of 60:50:80 CYM value. S, spore; H, hyphae.



Figure 2: The *Scutellospora* sp. spore. (a) The fresh *Scutellospora* spore attached to a swollen subtending hypha. (b) The squashed spore showing its inner wall (red arrow), outer wall (black arrow) and germination shield. S, spore; H, hyphae; G, germination shield. The *Scutellospora* sp. spores showed a colour of 20:70:80 CYM value.

The hyphae development was depicted by the blue stain in Figure 3c. The hyphae can be differentiated from the secondary and tertiary roots cell given that trypan blue will only stain the fungi structures (Phillips and Hayman, 1970). Figure 3d shows a seedling with an extraradical hyphal network. The development of AM fungi in 14 days is as shown in Figure 3e and f. The intraradical hyphal network developed from an entry point in the exodermis cell is shown in Figure 3e. Figure 3f shows the appressorium as the initial fungal penetration on a host plant.

Figure 3g shows the stable colonisation of AM given the various types of the hyphal network formed in the plant cells after 21 days of AM introduction into oil palm seedlings. Figure 3h-k show the infection started by the appressorium of AM fungus and the formation of entry points. The entry points grew and branched into intraradical hyphae in the exodermis cells and cortex cells. Figure 1.3l shows the extraradical and intraradical hyphal network which grew densely and compactly by surrounding the tertiary roots for further infection.

Figure 3m-o show the various sizes and huge numbers of vesicles developed in plant cells from the oil palm seedlings harvested after 40 days of AM introduction. The vesicles had been stained and attached with hyphae. Figure 3n shows that the vesicles were connected to each other through the hyphal network. Figure 3o shows that the vesicles grew extracellularly between the plant cells. The thickened wall of vesicles is a typical structure of *Glomus* sp.

Figure 3p-x show the formation of arbuscules observed in the oil palm seedlings harvested after 60 days. There were symptoms of two species of AM fungi, i.e. the *Scutellospora* sp. and *Glomus* sp. The hyphae of *Scutellospora* sp. is thick and is stained dark in Figure 3r. The hyphae grew intercellularly, entered the cell and formed intracellular looping hyphae. The arbuscules

appeared wispy due to the curving hyphae as shown in Figure 3s. Based on these characteristics, the AM fungal species is suspected to be *Scutellospora* sp. based on Figure 3r and s. The arbuscules formation was highly branched and compact in the plant cells as shown in Figure 3t-x. The arbuscules was branched from the intercellular hyphal network.

The mature arbuscules showed trunk which developed from intercellular hyphae as shown in Figure 3x. Also, the hyphae grew by densely surrounding the inside and outside of the plant cells as shown in Figure 3t-w. The parallel spread of intercellular hyphae resulted in a linear appearance of the AM hyphae. The linear (*Arum*) series of AM showed hyphae proliferation by growing longitudinally between the intercellular airspace of a host plant cells as shown in Figure 3u. Based on these characteristics, the AM fungal species is suspected to be *Glomus* sp.

DISCUSSION

There are two genera of AM fungi collected and isolated from the field soils, i.e. the *Glomus* and the *Scutellospora*. The genus *Glomus* is the dominant AM fungi found in most of the host plants. A study showed that the *Glomus* sp. was the most frequently occurring AM fungi species found in the rhizosphere of the potential commercial tree species, *Octomeles sumatrana* and *Anthocephalus chinensis* in Niah, Sarawak (Chubo *et al.*, 2009). Spore identification was done in reference to Brundrett *et al.* (1996). There are many morphological aspects that can be used to identify the AM spores. In this project, the sporulant arrangement, subtending hyphae, the wall layer and the colour of the spores were considered during the spore identification. The spore colour value had been determined in reference to the modified version of the INVAM colour chart by Morton (1992).

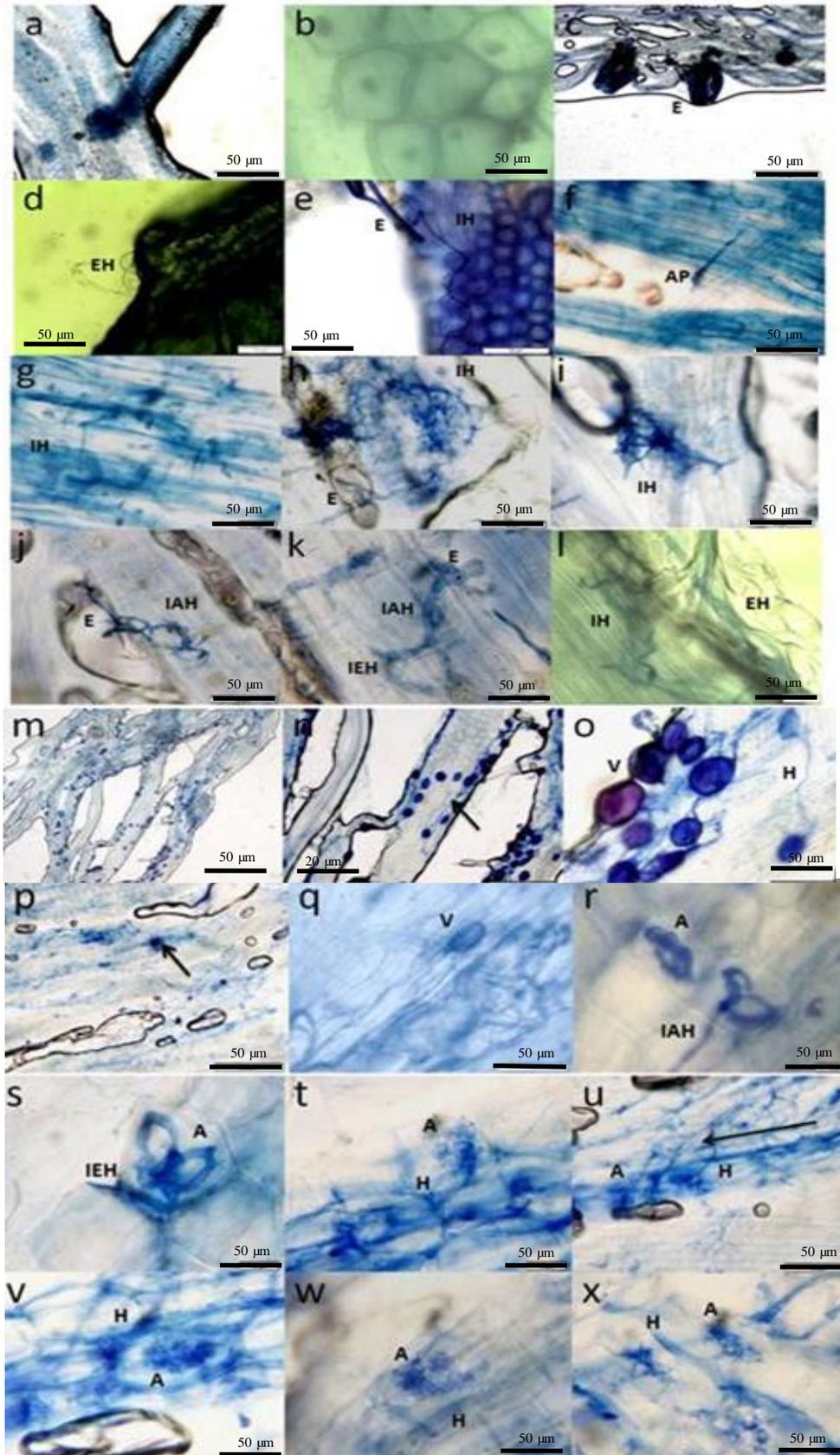


Figure 3: Colonisation of arbuscular mycorrhizal fungi in pre-nursery oil palm roots. (a and b) After 3 days of AM introduction into oil palm seedlings. (c and d) After 7 days of AM introduction into oil palm seedlings. (e and f) After 14 days of AM introduction into oil palm seedlings. (g-l) After 21 days of AM introduction into oil palm seedlings. (m-o) After 40 days of AM introduction into oil palm seedlings. (p-x) After 60 days of AM introduction into oil palm seedlings. (a) Secondary and tertiary roots without colonisation point. (b) Clear plant cell without staining effect. (c) Secondary and tertiary roots with a staining effect. (d) Seedling with an extraradical hyphal network. (e) Intraradical hyphal network development from an entry point in exodermis cell. (f) Appressorium as the initial fungal penetration on a host plant. (g) Various types of hyphal network formed in plant cells. (h) Dense intraradical hyphal network formed via the entry point in cortex cell. (i) Intraradical network developed from the main hyphae. (j) Intracellular hyphae developed from the entry point. (k) Hyphae developed from the entry point to form intracellular hyphae and extracellular hyphae. (l) Strong and dense extraradical and intraradical hyphal network formed in tertiary roots. (m-o) Various sizes and huge numbers of vesicles shown under different magnifications of a compound microscope. Each vesicle attached with hyphae and connected each other with a hyphal network (arrow). (o) Vesicles grew extracellularly and the vesicles had thickened wall (arrow). (p) Formation of arbuscules (arrow). (q) A few vesicles. (r-s) *Scutellospora* sp. arbuscules. (r) Thick looping hyphae grew intracellularly and wispy arbuscules formed in the cells. (s) Extracellular hyphae grew in the plant cell and formed looped and wispy arbuscules. (t-x) *Glomus* sp. Arbuscules. (t-w) Dense hyphal surrounded in and out of the plant cells and compact fine branch hyphae formed arbuscules in the plant cells. (u) A parallel spread of intercellular hyphae resulting in a linear (*Arum*) appearance of the AM hyphae (arrow). (x) Mature arbuscules showing trunk which developed from intercellular hyphae. AP, appressorium; E, entry point; H, hyphae; EH, extraradical hyphae; IH, intraradical hyphae; IAH, intracellular hyphae; IEH, intercellular hyphae; T, trunk; V, vesicle; A, arbuscules.

Figure 1a and c show the fresh spores and the squashed spores forming sporulate aggregation. The sporulate aggregation is one of the characteristics of *Glomus* sp. spores and the spore arrangement is normally found in the field soil. This is because the *Glomus* sp. spores need to accumulate sufficient energy to form the aggregation (Brundrett *et al.*, 1996). Besides, the identification was done based on the hyphae attachment of spores. The subtending hypha of *Glomus* sp. spores was attached in a simple way, in contrast to the bulbous subtending hyphae in *Gigaspora* spore and *Scutellospora* spore.

In Figure 2, the characteristics of *Scutellospora* spores were observed. The spore arrangement is totally different from the *Glomus* spore in which the *Scutellospora* spore appear singly and has no sporulant aggregation. The subtending hyphae of *Scutellospora* spore are swollen or bulbous, similar to *Gigaspora*. However, the germination shield showed in Figure 2b show the difference between *Scutellospora* and *Gigaspora*, i.e. the germination shield is not present in the *Gigaspora* sp. spore. The layered walls shown in Figure 2b is also one of the characteristics of the *Scutellospora* spores.

Besides that, the morphology of arbuscules can be used for AM fungi identification. The main arbuscular characteristics of *Glomus* sp. and *Scutellospora* sp. are shown in Figure 3, which is in reference to Brundrett *et al.* (1996). However, the AM fungi are identified based on the morphology of the spores and arbuscules. To strengthen the evidence, a molecular test on AM spores should be done in further studies.

Host plant physical response towards AM fungi colonisation

In Figure 3a and b, there was no colonisation sign of infection on the oil palm roots, indicating that the AM spore inoculums need more than three days to begin colonising the oil palm roots. The AM colonisation on oil palm roots initiated with the signs of a hyphal network from day-7 to day-60. The extraradical hyphal network developed first in AM colonisation as shown in Figure 3d, which is on day-7. A study conducted by Galindo-Castañeda and Romero (2013) showed that the AM colonisation initiated with the evidence of the formation of an intraradical hyphal network take longer, approximately one month after planting the pre-nursery oil palm seedlings.

Although there were various types with an abundant of hyphal network colonised on the oil palm roots from day-7 until day-21 (Figure 3c-l), the signs of AM colonisation was not complete. Figure 3l and m show that the thick and high root mass are the obvious morphologies of a mycorrhizal root. These characteristics relate to the coevolution of the plant and the arbuscular mycorrhizae given that the thick and slow-growing dimorphic roots will maximise the colonisation of AM fungi on root cortex area (Brundrett, 2002).

Interaction of AM fungi among the species

In Figure 3m-o, the vesicles were abundant in the oil palm roots on day-40. In other words, the population of *Glomus* sp. was high. The sign of infection of *Scutellospora* sp. was not observed in the plant root on day-40 but the arbuscules were present in the day-60 oil palm seedling roots (Figure 3r-s). Figure 3q shows the *Glomus* vesicles were weaker on day-60 relative to the

vesicles on day-40. There was competition between *Glomus* sp. and *Scutellospora* sp. under a stress competition. Previously, *Glomus* sp. was the main AM fungi in day-40 oil palm roots, but the succession had been disturbed by the *Scutellospora* sp. The competition brings positive effects on AM fungi diversity in oil palm seedling roots. A study by Bever *et al.* (1996) showed that the different sporulation rate between the different AM fungi creates a diverse AM fungi community within a host plant. A high diversity of AM fungi community constructs a diverse plant community structure (Vandenkoomhuysen *et al.*, 2003).

CONCLUSION

This study showed that the arbuscular mycorrhizae was able to form colonisation on the pre-nursery oil palm seedling roots. The colonisation needs at least three days after inoculation to be initiated with an extraradical hyphal network formation. To achieve a stable colonisation, it needed less than 40 days of inoculation to develop the mycorrhizal association. *Glomus* sp. and *Scutellospora* sp. had colonised the oil palm roots successfully. Various signs were formed on the oil palm roots as an indication of colonisation, i.e. the hyphae, vesicles and arbuscules. This study suggested that the oil palm seedling can be made into a mycorrhizal plant as early as the nursery stage before transplanting them into a plantation. As such, to enhance the plant growth and to strengthen the plant, it is suggested the arbuscular mycorrhizae should be applied at younger oil palm before transplanting the seedling into the field given the benefits of arbuscular mycorrhizae fungi to the plant.

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