ROLE OF MYCORRHIZA HELPER BACTERIA ON MYCORRHIZAL COLONIZATION AND NEMATODE *Pratylenchus coffeae* INFECTION

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ABSTRACT

The coffee nursery is susceptible to endoparasitic *Pratylenchus coffeae*. Application of biological method in the nursery is suggested to control the nematode population and maintain the seedling health. The objectives of this study were to observe the ability of Arbuscular Mycorrhiza Fungi (AMF) *Glomus* spp. and liquid inoculant of Mycorrhiza Helper Bacteria (MHB) consortium *Pseudomonas diminuta* and *Bacillus subtilis* for increasing AMF colonization and reducing the infection *P. coffeae* in Arabica coffee seedling and their growth. A pot experiment was conducted using a Completely Randomized Block Design with four treatments and five replications. The treatments were *Glomus* spp. spore inoculation without and with two concentrations of MHB. The control treatment did not receive *Glomus* spp. and MHB. The seedlings were growing in the greenhouse for three months. The results indicated that *Glomus* spp. and MHB consortium significantly reduced the nematode total number in soil and roots by approximately 30%; and infection degree of *P. coffeae* by 50%. The application of *Glomus* spp. significantly increased root colonization by mycorrhizal fungi, but MHB inoculation did not affect the mycorrhizal colonization. Seedlings treated with MHB had higher shoot length compared to the plant without MHB and control; but the leaves number and shoot dry weight of seedlings were not affected by all treatments. Even though the root fresh weight was reduced after MHB treatment, the lateral roots growth of MHB-treated seedling visually was improved. The experiment demonstrated that MHB was efficient to reduce *P. coffeae* infection of Arabica seedling.

Keywords: *Glomus* spp., infection degree, nematode population, root colonization, seedling growth

INTRODUCTION

Arabica coffee is the valuable agriculture commodity in global market. Brazil is still the first coffee producer worldwide while Indonesia is the 4th largest producer and exporters of coffee beans. In 2020, the coffee beans production in Indonesia was 12.0 million of 60-kg bags. In growing coffee many challenges occur and have to be dealt with endoparasitic *Pratylenchus coffeae* which well adapted to warm tropical and subtropical agroecosystem (Thiep *et al.* 2018; Tarno *et al.* 2021). *Pratylenchus* are root lesion nematode; mainly infecting the cortical parenchyma which inhibit the water and nutrients absorption and thereby, cause serious root damage and limit plant growth (Yu *et al.* 2012). In general, this problem was resolved by applying chemical and soil disinfectant as well as shading. Nowadays, the use of Arbuscular Mycorrhizal Fungi (AMF) is suggested in order to reduce the harmful effect of chemicals on the agricultural environment.

The AMF is well known as biofertilizer, which inoculation become a prominent way to save phosphorous fertilizer and increase coffee tree growth in tropical agroecosystem (Sewnet & Tuju 2013; Hernández-Acosta *et al.* 2018). To date, researcher have reported that other advantage of mycorrhizal formation in roots is to reduce nematodes in coffee tree seedlings as

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well as replanted coffee fields (Asyiah *et al.* 2015; Pham *et al.* 2020). Moreover, biocontrol strategy using AMF may protect the plant form nematode pathogen and induce plant resistance against this pathogen (Veresoglou & Rillig 2012; Poveda *et al.* 2020).

Among the AMF species, the natural occurrence of *Glomus* spp. in soil and rhizosphere of coffee tree are reported (Prates Júnior *et al.* 2019). The effect of AMF on coffee growth were recorded. Introducing AMF on coffee nursery resulted in increment of height, foliar area and root volume Arabica coffee seedling (Hernandez-Acosta *et al.* 2018). Coffee tree inoculated by AMF spores combined with chemical fertilizer showed better mycorrhizal colonization and tree growth (Daras *et al.* 2015).

Certain soil bacteria have been reported to develop positive interaction with AMF, which bacteria are known as Mycorrhiza Helper Bacteria (Frey-Klett *et al.* 2007). The bacteria increase fungal spore germination and mycelium growth in soil and root-mycelium recognition (Deveau & Labbé 2016). The ability of Gram-negative Proteobacteria *Pseudomonas* and a Gram-positive *Bacillus* to help mycorrhizal establishment has been reviewed (Frey-Klett *et al.* 2007; Labbé *et al.* 2014). Both bacteria are well known Plant Growth Promoting Rhizobacteria (PGPR) which induce plant growth by the mechanisms of phytohormones production, biocontrol of soilborne diseases, arbuscular mycorrhiza stimulation and acquired systemic resistance induction (Jankiewicz & Kołtonowicz 2012; Patel & Saraf 2017; Hashem *et al.* 2019).

We have developed Mycorrhiza Helper Bacteria (MHB) liquid inoculant of *Bacillus* as well as *Pseudomonas*. In previous research, single application of *B. subtilis* and *P. diminuta* suppressed the population of nematode *P. coffeae* in coffee seedlings by 71.3% and 64.2%, respectively compared to the untreated seedlings (Asyiah *et al.* 2015). Nonetheless, the ability of mixed cultures of both bacteria to induce mycorrhizal formation and repress *P. coffeae* infection have not been studied. Controlling coffee pest in the nursery is an important step to enhance tree growth and coffee production in the field. Improving the quality of coffee seedlings is essential to support the sustainability of coffee plantation. The objectives of this study were to determine the ability of mycorrhiza *Glomus* spp. and the liquid formula of MHB consortium to enhance AMF colonization and reduce *P. coffeae* infection, as well as to observe the effect of *Glomus* spp and liquid formula of MHB consortium on the growth of coffee seedlings.

MATERIALS AND METHODS

The experiment was carried out in the greenhouse of Coffee and Cocoa Research Institute (CCRI) at Jember, East Java Province, Indonesia which is located in the tropical area at 60 m asl. The range of annual temperature and humidity in year 2019 were 23.0 - 29.9 °C and 67 - 86%, respectively. Preparation of MHB and *P. coffeae* inoculants was conducted at the Microbiology Laboratory of Universitas Jember. The spores of *Glomus* spp. (Zygomycetous fungi) were provided by Laboratory of Mycology at the Faculty of Agriculture, Universitas Gadjah Mada, while the Arabica coffee seedlings were obtained from CCRI.

Table 1 Major essential nutrient composition of soil before experiment

Parameters	Methods[a]	Value
pH_{H2O}	Potentiometry	5.6
Organic C	Walkey and Black	2.39%
Total N	Kjeldahl	0.24%
C to N Ratio	Calculation	9.95
Available P	Spectrophotometry	14.65 mg/kg
Available K	Spectrophotometry	79.82 mg/kg

Note: [a] = Based on AOAC proximate analysis method (2012).

The top soil of Inceptisols with loam texture (Prastowo *et al.* 2013) was collected from the agricultural field at CCRI. The soil properties showed that the soil was average in organic carbon (C) and total nitrogen (N), low in available phosphor (P) and high in potassium (K) (Table 1). The C to N ratio of soil was low indicating the nitrogen was available for plants.

Preparation of *Glomus* spp. and MHB Inoculants

Arbuscular mycorrhizal fungi propagation was carried out in zeolite-based media with corn as host plant. A 150-g sterilized zeolite were poured into 250-mL transparent cup and

saturated with 0.5% NaCl solution. Then 100 *Glomus* spp. spores were spread evenly on the surface of zeolite prior to sow two corn seeds and covered with 50 g of zeolite. Substrate humidity was maintained by adding 30 mL water per day. The first fertilization was carried out a week after sowing by applying 30 mL of liquid mixed fertilizer at a concentration of 1 g/L; similar fertilization was done twice a week. Two months later watering was reduced gradually in order to induce spore formation. The corn plants were eliminated and the growth substrates containing *Glomus* spp. spores were used as AMF inoculant.

The production of bacterial liquid inoculant was carried out using molasses-based broth. Molasses containing 19.58 C/N ratio, 0.18% P_2O_5, and 0.39% K_2O was obtained from Probolinggo Sugar Factory. Each MHB species was grown separately in 2% molasses-based broth for 2 days at a temperature of 30 °C, then two volumes of *B. subtilis* were mixed with three volumes of *P. diminuta*. The liquid bacterial consortium was then put on a shaker at room temperature for three days. The final concentration of mixed MHB liquid inoculant was 10^9 colony forming unit (cfu)/mL.

Experimental Design and Set Up

The pot experiment was conducted in completely randomized block design composed of four treatments and six replications. The Arabica coffee seedlings were treated with *Glomus* spp. inoculant without MHB consortium (MHB-0) and with 10^8 cfu/mL MHB inoculant (MHB-1) and 10^9 cfu/mL MHB inoculant (MHB-2). Control plants received neither *Glomus* spp. nor MHB inoculant. The mature *P. coffeae* was applied to all treatments.

Plant substrate consisting of air-dried soil, sand and commercial compost in balanced composition was sterilized at 115 °C for 30 min and placed overnight at room temperature. As much as 5 kg plant substrate was put into opaque polyethylene pot with 5 drainage holes on its bottom and stored at the greenhouse. An 8-cm depth planting hole with 5 cm diameter was made in each pot prior to growing 3-month old coffee seedlings. The average shoot height and leaves number of seedlings at planting time were 11.2±0.08 cm and 4.2±0.05, respectively. Soon after transplanting, 1 g of Urea fertilizer was applied into 2-cm depth hole with a distance of 5 cm away from the stem seedlings. Then, the holes were covered with the growth media.

A week after transplanting, 50 mature *P. coffeae* was spread on 4-cm depth circular band with a distance of 5 cm from plant stem and then covered with thin layer of substrate. Subsequently, 9.1 g *Glomus* spp. inoculant containing 100 spores were spread over similar circular band and covered with substrates. Inoculation of mixed MHB were carried out by pouring the liquid inoculant evenly around the seedling stem. The MHB-treated plants were inoculated with 1 mL of bacterial inoculant which was diluted in 99 mL distilled water, while control plants were watered with 100 mL distilled water. All seedlings on potted soil were grown in the greenhouse for 3 months.

Parameters Measurement and Statistical Analysis

Plant height and leaves number were measured once a month until 3 months after treatment. Fresh weight of shoot and root as well as shoot dry weight were measured at the end of the experiment. The root dry weight was not analyzed since the roots were used for nematode enumeration. The shoot dry weight was determined after being oven-dried at 70 °C for two days. After 3 months of treatment, the number of nematodes in root and soil were counted by extracting the nematodes from 10 g of soil sample and 100 mL of root extract following the method of Baermann funnel (van Bezooijen 2006). The soil and roots extracts samples were passed through a different set of filters with an opening size of 40 mesh and followed by the 325 mesh before counting the nematodes under a light microscope at 100x magnification.

Determination of Root Colonization degree (RC) by AMF was carried out following the method of Kormanik dan Mc Graw (1982). The roots were cut into 2-cm length root segments and subsequently soaked in 2% KOH at 80 °C, followed by staining the roots with acid fuchsin. A total of 10 stained root segments were placed in object glass and covered with cover glass. The cover glass then pressed manually for flatting the roots and removing excess liquid. The number of infected roots was observed using a light microscope at 400 x magnification.

The infected roots were determined by the hypha, vesicle and arbuscule occurrence in root segments. The RC (%) was calculated by dividing the number of infected root segments by number of observed root segments.

At the end of experiment, the Infection Degree (ID) for estimating disease severity due to *P. coffeae* was calculated using a scale of five classes (Table 2) by using the Townsend-Heuberger formula described by Scalzo *et al.* (2012):

$$ID\ (\%) = \frac{\Sigma_{1-i}\ (n_i.v_i)}{(N.V)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

where:
v_i = infection class;
n_i = number of seedlings in one class;
N = total seedling number;
V = the highest class;
i = the number of classes.

Table 2 The classes for scaling *P. coffeae* infection based on leaves and roots conditions

Class	Description
0	Healthy seedling, the roots are normal without color alteration; leaves are green without yellow spots.
1	Less than half leaves had a yellow spot but the leaves number was similar to healthy seedling; roots had altered their color to yellow and brown.
2	At least half of leaves number showed yellowing symptom and seedlings had some fallen leaves; roots are not healthy with rotten-lateral roots.
3	The seedling is dominated by yellow leaves and the number of intact leaves were less than half of healthy seedling; their root system was not fully developed and rotted.
4	Seedling had 1-2 yellow leaves and their roots were badly damaged indicated by brown in color and rotten-lateral roots.

All data were subjected to analysis of variance ($P \leq 0.05$). If the effect of treatment on the parameter was significant then the Least Significant Difference (LSD) test was carried out at $P \leq 0.05$. Statistical analysis was performed using SPSS 16 statistical program.

RESULTS AND DISCUSSION

Root Colonization and Infection Degree

Without *Glomus* spp. inoculation, the roots did not show any root colonization (RC) by AMF (Fig. 1a), while inoculation of *Glomus* spp. resulted in RC higher than 80%. The result verified that seedlings without MHB showed similar RC compared to seedlings treated with lower concentration of MHB inoculant (MHB-1). Furthermore, seedlings inoculated with higher concentration of MHB (MHB-2) caused significant RC reduction up to 12.4%.

Low nematode Infection Degree (ID) was demonstrated by seedlings grown with the application of *Glomus* spp. inoculation compared to control (Fig. 1b). The application of MHB combined with *Glomus* spp. inoculation enabled to decrease ID up to 50% compared to the control. Seedlings inoculated with MHB containing 10^8 cfu/mL (MHB-1) demonstrated lower ID even though it was not significantly different from the results showed by the inoculation of MHB-2 (10^9 cfu/mL) based on LSD test.

Microscopic observation revealed that nematode *P. coffeae* infected roots of Arabica coffee at 3 months after treatment (Fig. 2). Moreover, mycorrhizal colonization in the root cell was clearly showed by arbuscular and vesicular formations.

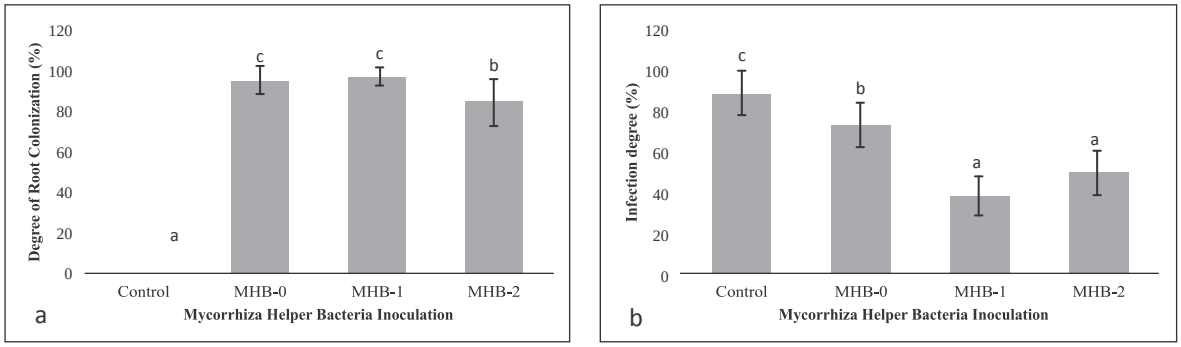

Figure 1 Effect of Mycorrhiza Helper Bacteria consortium on (a) root colonization by AMF and (b) infection degree caused by *P. coffeae* on coffee seedlings treated with *Glomus* spp. at 3 months after treatment
Note: Control = without *Glomus* spp. and MHB inoculations.

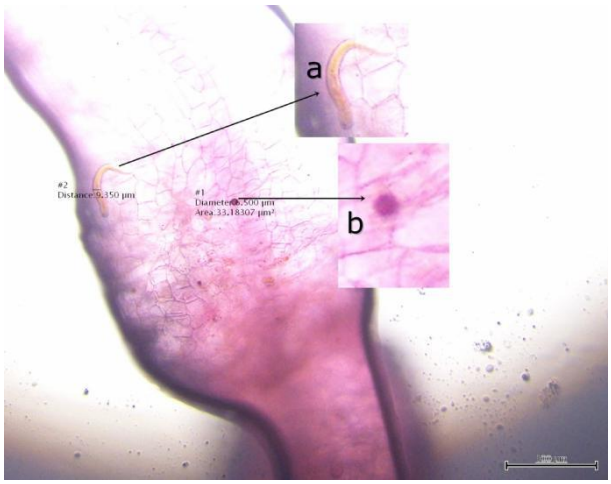

Figure 2 Mature nematode in coffee root cell of coffee seedling infected by (a) *P. coffeae* and (b) AMF colonization resulted in vesicular formation in the root cells
Note: Photo source: Elena F.L.Lilipaly, using Olympus digital camera.

The *Glomus* spp. inoculation with and without MHB lowered the nematode population compared to the control even though the differences were not statistically significant (Table 3). Regardless of statistical analysis, lower nematode population in roots was shown by seedlings with *Glomus* spp. and MHB. The lowest nematode population in soil was shown by seedlings treated with low concentration of MHB inoculant even though this decline was not statistically different based on LSD test. In general, the significant reduction of total nematode count was performed by seedlings treated with 10^8 cfu/mL and 10^9 cfu/mL of MHB liquid inoculants.

Results of this experiment found that *Glomus* spp. inoculation with or without MHB increased root colonization by AMF. The soil was sterilized before experiment and considered free from indigenous AMF. Therefore, only exogenous *Glomus* spp. colonized the roots. This result verified that MHB did not induce mycorrhizal formation in roots and MHB slightly reduced root infection. Microscopic observation showed that the presence of external hyphae, arbuscular and vesicular were only detected in *Glomus*-treated roots. The lower RC in MHB-treated seedling shown in this experiment disagrees with the induction of AMF spore germination, mycelium growth, and root-mycelium recognition proposed by Deveau & Labbé (2016). The failure of MBH to increase RC might be related to the inability of MHB to perform those roles. The Arabica coffee seedlings were responsive to *Glomus* spp. inoculation which relates to soil acidity and available P in soil. Before experiment the soil acidity was 5.6. In general, soil fungi showed optimal mycelial growth at pH of 4.5 to 5.5. Certain *Glomus* spp. species produce the highest number of spores in soil pH of 6.5 (Costa *et al.* 2013). The acidic and slightly acidic soil condition was suitable for the growth of *Glomus* spp. and hence, their colonization in roots (Rohyadi *et al.* 2004).

Table 3 Effect of mycorrhiza *Glomus* spp. and Mycorrhiza Helper Bacteria consortium on *P. coffeae* population in roots and soils at 3 months after treatment

Treatments	Nematode Number			Nematode Total Reduction (%)[a]
	Root	Soil	Total	
Control	221±29.6[a]	214±54.1[a]	435±72.0[a]	-
Glomus spp. without MHB	281±60.8[a]	104.4±4.4[a]	385.4±66.0[a]	11.4
Glomus spp. + MHB-1	200.6±77.5[a]	74.4±12.5[a]	275±77.6[a]	36.7
Glomus spp. + MHB-2	181.8±27.9[a]	112.2±18.1[a]	294.2±37.6[a]	32.4

Note: Values followed by the same letter in the same column are not significantly different according to LSD test at $P \leq 0.05$.

AMF colonization in roots treated with *Glomus* spp. is promoted by low available P content in soil. Before experiment, the soil contained as low as 14.65 mg/kg of available P. The growth media composed of soil-sand-compost without inorganic P fertilizer. High P in soil and high rate of P fertilizer can inhibit AM colonization (Linderman & Davis 2004). In contrast, adding only moderate inorganic P fertilizer enhanced the root colonization and diversity of AMF communities (Higo *et al.* 2020).

The experiment found that inoculation of *Glomus* spp. combined with MHB resulted in the significant reduction of nematode population up to 32 - 36% and ID by 43 - 56%. Both MHB produce phytohormone that play an essential role to activate the mechanisms of plant defence response against unfavourable condition (Egamberdieva *et al.*, 2014). Chitinase is probably produced by MHB *Pseudomonas* and *Bacillus* as described by some researchers (Zhong *et al.* 2015; Amar *et al.* 2016). This chemical substance might limit nematode pathogen development. In the root knot of nematode, chitinase produced by *Paenibacillus illinoisensis* was capable to lysis nematode eggshell and inhibit egg hatching (Jung *et al.* 2002). However, the mechanism of chitinase to repress *P. coffeae* infection is remained unclear.

Increased root colonization by AMF in *Glomus*-treated seedlings might be related to low ID of *P. coffeae*. Mycorrhiza can suppress the population of *P. coffeae* through symbiont competition on space, carbon and nitrogen (Bell *et al.* 2021) and induce the lignification of root endodermic cells that possibly decreased nematode pathogen infection (Linderman 1994; Elsen *et al.* 2001). The decrease of nematode population in our experiment agrees with the 68% of Pratylenchus population reduction in soil of apple seedling after *Glomus intraradices* inoculation (Ceustermans *et al.* 2018).

Plant Growth

Glomus spp. inoculation with or without any concentration of MHB liquid inoculant did not affect plant height and number of leaves at one and two weeks based on LSD test (Fig. 3a & 3b). The application of *Glomus* spp. and MHB enhanced plant height at 3 months after treatment, with decreasing number of leaves compared to control plants. This experiment showed that mixed inoculation of *Glomus* spp. and MHB significantly increased plant height up to 22% compared to seedlings inoculated with *Glomus* spp. without MHB (Fig. 3a). The highest number of leaves was demonstrated by seedlings inoculated by Glomus spp. without MHB (Fig. 3b).

The fresh and dry weight of shoots had not altered due to MHB inoculation (Table 4). In contrast, growing coffee seedlings with *Glomus* spp. but without MHB inoculation resulted in higher root weight over MHB-treated seedlings. Inoculation of MHB clearly increased plant height at 3 months after treatment (Fig. 3) but their dry weight was not different.

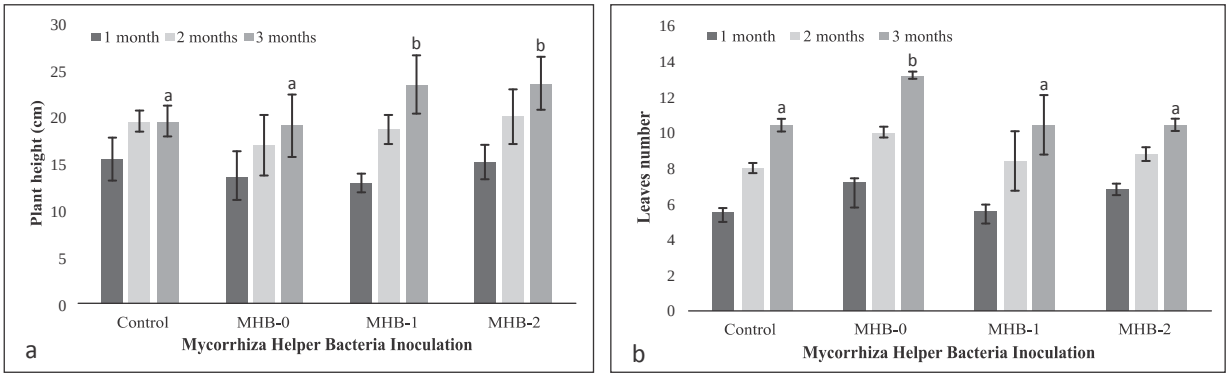

Figure 3 Effect of Mycorrhiza Helper Bacteria inoculation on plant height and number of leaves of coffee seedlings treated with *Glomus* spp. at 1, 2 and 3 months after treatment
Note: Control = without *Glomus* spp. and MHB inoculations.

Table 4 Effect of mycorrhiza *Glomus* spp. and Mycorrhiza Helper Bacteria inoculation on weight of shoot and root of coffee seedlings at 3 months after treatment

Treatments	Shoot weight (g)		Root fresh weight[a]
	Dry Weight	Fresh weight	
Control	0.44 ± 0.25[a]	1.60 ± 1.09[a]	0.43 ± 0.28[b]
Glomus + without MHB	0.49 ± 0.26[a]	1.93 ± 1.11[a]	0.36 ± 0.26[b]
Glomus + MHB-1	0.44 ± 0.05[a]	1.64 ± 0.60[a]	0.11 ± 0.04[a]
Glomus + MHB-2	0.42 ± 0.08[a]	1.49 ± 0.36[a]	0.14 ± 0.08[a]

Note: Values followed by the same letter in the same column are not significantly different according to LSD test at $P \leq 0.05$.

Based on visual observation, the performance of seedlings treated with *Glomus* spp. was better than that of the control plant (Fig. 4). Despite lower root fresh weight, seedlings inoculated with *Glomus* spp. combined with MHB liquid in general had more rigorous lateral roots than plants without being inoculated with *Glomus* spp. and MHB (Fig. 5). Moreover, the tap root of control seedlings and seedlings without MHB are likely thicker than roots of seedlings inoculated with MBH.

Significant increase in shoot height was observed in the seedlings inoculated with *Glomus* spp. combined with MHB (*B. subtilis* and *P. diminuta*) at 3 months after treatment. Shoot elongation is determined by several growth factors including the regulation of cell division and enlargement that affected by sufficient nutrient and plant growth substances. In the photosynthate partitioning, the stems are the sink that demands the energy (sugar) to elongate, while leaves are the source that produces the starch through photosynthesis. The sugar allocation from the leaves and essential ion taken by the roots were not suffice for shoot development. A plant growth hormone called auxin influences and integrates in plant growth (Sachs 2005).

Figure 4 Shoot performance of Arabica coffee after being infected by *P. coffeae* and inoculation by *Glomus* spp. and MHB
Note: Control = without *Glomus* spp. and MHB inoculations.

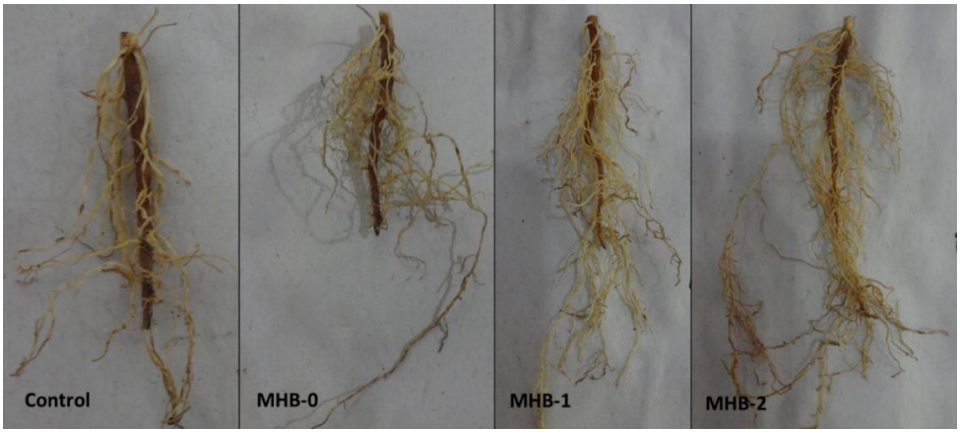

Figure 5 Root performance of Arabica coffee after being infected by *P. coffeae* and inoculated with *Glomus* spp. and MHB
Note: Control = without *Glomus* spp. and MHB inoculations.

Plants produce auxin in the shoot tissues and has an essential role in initiating root growth and inhibiting primary root development (Sachs 2005; Alarcón *et al.* 2019). Visual observation showed better growth of the seedlings' lateral roots growth inoculated with *Glomus* spp. combined with MHB compared to roots without *Glomus* spp. and MHB inoculations. (Fig. 3). MHB liquid inoculant of *P. diminuta* and *B. subtilis* consortium contained 4.01 mg/L of IAA, a group of auxin hormone that is capable of improving lateral root growth and subsequently facilitating better nutrient uptake and shoot growth. The results of this experiment agree with a study showing an increase in cucumber rooting after the application of low concentrations of exogenous auxin (Balliu & Sallaku 2017). The intensive growth of lateral roots in MHB-treated seedlings might cause low fresh weight of root because the primary roots become thin.

In relation to root colonization by *Glomus* spp., better root growth provides more physical contact establishments between fungi and roots. The mycorrhizal fungi are obligate symbiont characterized by forming arbuscular and/or vesicular in root cells. Both particular and functional structure are found in *Glomus*-treated coffee seedlings. This experiment found that *Glomus* spp. are compatible with coffee seedlings as host plant and possibly build a positive interaction with the community of indigenous AMF. Both *Pseudomonas* and *Bacillus* in this experiment are not endophytic bacteria, so they do not compete with AMF for infection sites. The precise mechanisms by which *P. diminuta* and *B. subtilis* decrease the infection degree of *P. coffeae* and repress nematode population in the presence of AMF have to be studied.

CONCLUSION

Mixed liquid formula of Mycorrhiza Helper Bacteria (MHB) consisted of *P. diminuta* and *B. subtilis* combined with spore of arbuscular mycorrhiza fungi *Glomus* spp. significantly reduced the total number of *P. coffeae* in roots and soil by 30%. Both microbial inoculations decrease the infection degree (ID) of *P. coffeae*. Introducing 10^8 cfu/mL and 10^9 cfu/mL MHB cells to the Arabica coffee seedlings increased the ID up to 50%. MHB has no significant role on the enhancement of root colonization (RC) by *Glomus* spores in root seedlings inoculated with *Glomus* spp. Slightly but significant reduction of RC was shown in roots treated with MHB. A combination of MHB and *Glomus* spp. increased shoot height at 3 months after treatments without affecting its dry and fresh weight. The decrease of root weight was observed in seedlings inoculated with MHB and *Glomus* spp., with thinner primary roots and more intensive lateral roots. We suggest to introduce the inoculation of *Glomus* spp. spores and MHB at the coffee nursery in order to minimize *P. coffeae* infection.

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