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Mycorrhizal fungi *Glomus* spp. propagation in zeolite enriched with mycorrhiza helper bacteria for controlling nematode in coffee

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In-situ kuskus conservation strategy in Wakasihu Village, West Leihitu District, Central Maluku District

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Mycorrhizal fungi *Glomus* spp. propagation in zeolite enriched with mycorrhiza helper bacteria for controlling nematode in coffee

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Abstract. Arbuscular mycorrhizal fungi (AMF) play a role in suppressing the nematode *Pratylenchus coffeae*. Mycorrhizal helper bacteria (MHB) can increase the effectiveness of AMF to control the diseases. The experimental purpose was to increase the spore population of AMF *Glomus* spp. in zeolite-based formulation inoculated with liquid consortia of *Pseudomonas diminuta* and *Bacillus subtilis* as MHB. The experimental design was a completely random design with six treatments consisted of 10^6, 10^7, $10^{8,}$ and 10^9 CFU/mL MHB liquid inoculants. The control treatments were water and 2% molasses. All treatments were replicated four times. A total of 300 mL/pot Liquid inoculant of MHB have been inoculated a three day before transplanting the maize seedling to the Zeolite inoculated with *Glomus* spp. in the pot. One month after MHB inoculation, *Glomus* formulation in Zeolite with different levels of MHB increased the degree of infection. Three months after MHB inoculation, spore content in Zeolite increased. The density of *P. diminuta* and *B. subtilis* in zeolite-based mycorrhizal inoculant increased at the end of the experiment. Liquid inoculant MHB contained 10^8 CFU/mL enhanced spora number fourth times compared to the control. This experiment suggests that *P. diminuta* and *B. subtilis* were effective to increase the spore density of AMF inoculant.

1. Introduction

Many publications describe mycorrhizae's role in inhibiting parasitic nematodes' penetration and development [1, 2, 3, 4, 5]. Mycorrhizal symbiosis is considered an interaction between plant roots and fungi and must include supporting other organisms. Mycorrhizosphere is a mutual influence that produces what is known as the "mycorrhizosphere" [6, 7, 8]. The mycorrhizosphere is composed of mycorrhizae, external mycelium, and supporting organisms [9]. This mycorrhizosphere effect can lead to increased nutrition, growth, and plant disease resistance [10, 8].

Usually, the AMF and its supporting organisms (bacteria) apply as biofertilizers. Bacteria that can increase mycorrhizal development are defined as Mycorrhizal Helper Bacteria, MHB [11]. Bacteria isolated from mycorrhizal fungi can stimulate mycorrhizal infection, spore production, and plant pathogens' resistance [12, 13].

The MHB is a term used for endophytic bacteria that can help mycorrhizae carry out their role. The bacteria must be in one part of the mycorrhizal body and play a role in developing mycorrhizae. Mycorrhizal symbiosis is not only a relationship between the mycorrhizal-forming fungi and the host plant but involves other supporting organisms such as bacteria.

The bacteria excretes beneficial organic substance that often stimulates the germination of fungal spores. Most bacteria from the AMF spore cell walls were able to increase *Glomus clarum* spores' germination when there was direct contact between spores and bacteria, while some bacterial isolates inhibited spore germination by producing volatile antagonists [14].

Moreover, MHB affects the concentration of antagonistic compounds produced by mycorrhizal fungi [15]. They found that the bacteria were able to detoxify the liquid media from inhibiting fungal metabolites. MHB bacteria may also be able to suppress the production of toxic compounds by soil microbes. Vivas [16] reported that MHB bacteria has positive impact on the spore germination and growth of presymbiotic fungi in a broth contaminated with heavy metals. Bacterial inoculation not only decreased the destruction of *G. mosseae* hyphae but even resulted in increment of root growth by 95% (without Cd) to 254% (with Cd). This effect was as strong as in the Zn treatment, where mycelium growth ranged from 125% (without Zn) to 232% (with Zn solution).

Nunang [17] showed that there were 12 bacteria isolated from AMF spores, seven bacteria from *Gigaspora* sp. and five bacteria from *Glomus* sp. Eight types of bacteria, namely: *P. diminuta, B. licheniformis, B. laterosporus, E. hormaechei, B. brevis, B. subtilis, B. cereus,* and *B. firmus,* can stimulate the development of mycorrhizal hyphae. Seven bacteria have the potential for cellulase and protease activity, namely: B. *subtilis, B. cereus, B. laterosporus, B. pasteurii, P. penneri, B. firmus,* and *B. cereus*. There are four bacteria (*B. subtilis, P. diminuta, P. penneri*, and *E. hormaechei*) which can inhibit the growth of pathogens *Rhizoctonia* sp., *Sclerotium* sp., and *Ganoderma* sp.

The *B. subtilis* and *P. diminuta*) has been reported to increase the degree of mycorrhizal infection in the roots of coffee up to 98.4% [5]. Furthermore, *Pseudomonas diminuta* 10^8 increased the degree of infection by 93.6%; even the density of *P. diminuta* $2x10^8$ increased mycorrhizal infections by up to 98.4 %. Based on the results of this study, we can conclude that mycorrhizal propagation will be more effective with the help of MHB. To develop a more effective mycorrhiza inoculant, the formulation of the inoculant integrated with MHB inoculation is needed. In general, arbuscular mycorrhiza (AM) inoculants are developped by using corn as the fungal host since the mycorrhizal fungal is a host-depend microbes. Our previous experiment demonstrated that molasses-based liquid inoculant of MBH *B. subtilis* and *P. diminuta* supported their cell count up to 10^9 CFU/mL. This liquid inoculant will be utilized to improve the quality of AM inoculant. The objective of this greenhouse experiments was to evaluate the effect of liquid inoculant consortia of *P. diminuta* and *B. subtilis* as MHB on the spores density of AMF *Glomus* spp. in zeolite-based AM inoculant as well as the infection of AMF on the roots of host plants, corn.

2. Methodology

2.1 Materials and Methods

This study used spores of *Glomus* spp; a collection of the Faculty of Agriculture Universitas Gajah Mada. The MHB consortium liquid formula composed of *B. subtilis* and *P. diminuta* with a ratio of 2:3 with the cell count of 10^9 respectively at day three was prepared by Biology education University of Jember Laboratory. The arbuscular mycorrhiza inoculant developed using corn grown in Zeolite with size 1-2 mm. A selective medium of either *B. subtilis* or *P. diminuta* has been used to count bacterial cells in the zeolite-based AMF inoculant.

2.2. Experimental setup

The study was conducted in a greenhouse in a completely randomized design consisting of 6 treatments and four replications. The treatment included an MHB consortium solution with cell densities of 10^6, 10^7, $10^{8,}$ and 10^9 CFU/mL. The control treatment was water and 2% molasses. Each solution is given three days before planting corn seeds as much as 30 ml per pot. The 7-day old corn seeds are planted in

polyethyelene pots containing 200 g Zeolite media; each pot consists of two corn seeds. A total of 100 mycorrhizal spores inoculated on maize seedlings after seven days of transplanting.

The corns were watering every day as much as 30 ml/pot. Fertilization of plants using Hyponex (25-5-20) with a 1g/L concentration at a dose of 30 ml/pot a week after planting. Furthermore, fertilization is repeated twice a week with the same amount until the plants are two months old. In the third month, watering is gradually reduced for the stressing process to stimulate spore formation. In the first week, water every other day with a dose of 30 ml of water; on the second week of watering every other day with a dose of 20 ml of water; in the 3rd week of watering every other day with a dose of 10 ml of water, and in the last week of watering is not done watering at all [18].

2.3. Observed Parameters

Observations consisted of 1) the degree of mycorrhizal infection in maize roots, calculated after planting for one month, refers to the Kormanik and Mc Graws method [19]; 2) MHB cell viability by counting the number of bacterial colonies in a selective medium *B. subtilis* and *P. diminuta*, respectively, which observed one month and three months after application refers to Schinner methode [20]; and 3) the number of spores, calculated after planting for three months (harvest) using the extraction method per 10 grams.

2.4. Statistical analysis

All data were subjected to analysis of variance (5% F test) and Duncan Multiple Range Test of 5% by using SPSS program.

3. Results

The addition of the MHB consortium increased the average degree of mycorrhizal infection and bacteria density at one month after treatment, as can be seen in Table 1. The effect of the acquisition of MHB on the propagation medium mycorrhiza on the number of spores and bacterial density (CFU) at harvest time is depitected in Table 2.

Table 1. The percentage of mycorrhizal infection degree and bacterial density in AMF inoculant at one month after treatment

Treatments	Degree of mycorrhizal infection (% ± SD) *	Bacterial density (CFU/g) x10^7	
		P. diminuta	*B. subtilis*
Water	78.00 ± 11.1355 a	-	-
Molase 2%	81.33 ± 11.3725 a	-	-
MHB 10^6	90.67 ± 4.1633 b	58.67	12.33
MHB 10^7	92.67 ± 4.1633 b	46.75	65.17
MHB 10^8	93.33 ± 3.0551 b	15.25	74.67
MHB 10^9	94.67 ± 5.0332 b	26.42	102.33

Note: * the mean number followed by the same letter is not significantly different based on the Duncan test at the 95% confidence level.

Table 2. The number of spores of *Glomus* spp. and bacterial density in AMF inoculant at harvest time

Treatments	Spore number per g inoculant *	Bacterial density (CFU/g) x10^7	
		P. diminuta	*B. subtilis*
Water	33,05 a	-	-
Molase 2%	28,28 a	-	-
MHB 10^6	90,81 b	122,17	257,00
MHB 10^7	115,67 bc	198,75	123,25
MHB 10^8	160,58 c	234,00	107,00

MHB 10^9	144,43 c	276,50	113,33

Note: * the mean number followed by the same letter is not significantly different based on the Duncan test at the 95% confidence level.

4. Discussions

This experiment showed that *B. subtilis* and *P. diminuta* are symbiotic bactera with fungi for carrying out their roles to increse mycorhizal infenction of host plant. The results of this research supported by Nunang [17], which states that several types of MHB were found, including *P. diminuta,* can help develop mycorrhizal hyphae and inhibits several types of pathogens. The *B. subtilis,* assist the development of fungal hyphae and has the potential for cellulase and protease enzymatic activity as well as inhibit the growth of certain pathogens. Garbaye [11] also stated that some bacteria could colonize roots well, for example, various kinds of *Pseudomonas* spp. able to live around the surface of the fungal hyphae. These bacteria live in the mycorrhizal bodies and also around the roots of host plants (rhizosphere).

Bacteria also has a benefit from their symbiosis with mycorrhizal fungi. Linderman [21] state that there is a synergy between bacteria, fungi, and plants. Bacteria get a source of nutrition from root exudates because of their chemical composition due to mycorrhizae's role, so that root exudates contain bacteria suitable. Root exudates are a source of nutrients that are essential for the survival of bacteria. Most of the bacteria in symbiosis with fungi will complete their fungi' life cycle [22]. Bianciotto [23] showed that active bacterial division occurs in the mycelium.

The mechanism of MHB in helping mycorrhizae to infect roots is as follows: 1) increase the rate of roots to mycorrhizal formation, bacteria initiate the formation of IAA hormones to induce short and roots, then bacteria produce enzymes to catalyze the softening of root cell walls prior to AMF-root interaction; 2) Furthermore, MHB plays a role in mediating root biomolecules and also fungi. Roots and fungi can interact based on enzymes or chemical substances produced by fungi or roots, so the part of MHB is to facilitate the introduction of enzymes or chemicals between them by creating certain compounds such as auxins and other enzymes. Mycorrhizal and root interactions can occur because of the presence of *myc* factors released by fungi to be recognized by plants and plants to acknowledge the potential of mycorrhizal symbiosis with strigolactones released by roots along with other root exudates such as sugars, fats, acids amino acids, fatty acids, and hormones growth.

The research showed that mycorrhizal infections in the maize roots were more than 70%. According to [24], the minimum mycorrhizal infections in roots that can increase plant growth and development is 70%. If the degree of infection was less than 70%, then the infection is not optimal. Meanwhile, according to [25], the roots of many host plants with a high degree of root infection by AMF indicate an excellent AMF inoculum source. The exudate produced by MHB will stimulate the germination of fungal spores. Besides, bacteria in symbiosis with fungi can influence plant physiology by increasing the permeability of root cells [10, 11].

P. diminuta and *B. subtilis* have several functions, namely: 1) increasing the effectiveness of mycorrhizal infections against the roots of host plants, 2) as biological control agents, and 3) being able to increase plant growth [5]. These prominent functions are because of the role of two bacteria as Plant Growth Promoting Rhizobacteria (PGPR) as well as a solubilizing phosphate.

5. Conclusions

The conclusions of this study were: 1 Inoculating liquid culture of MHB consortium consisting of *P. diminuta* and *B. subtilis* with a ratio of 2: 3 on mycorrhizal propagation media increased the degree of infection and the number of mycorrhizal spores *Glomus* spp., 2) The MHB consortium liquid culture with a cell density of 10^8 CFU/mL increase the number of spores by 162.067 per gram of Zeolite, 3) The MHB cell viability can survive in Zeolite media for up to / more than three months.

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