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Enhanced mycorrhiza helper bacterial inoculant for improving the health of Arabica coffee seedlings grown in nematode-infected soil

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Abstract. *Hindersah R, Asyiah IN, Amaria W, Fitriatin BN, Mudakir I, Banerjee S. 2025. Enhanced mycorrhiza helper bacterial inoculant for improving the health of Arabica coffee seedlings grown in nematode-infected soil. Biodiversitas 26: 127-124.* The Arbuscular Mycorrhizal Fungi (AMF) and Mycorrhiza Helper Bacteria (MHB) combine to combat the *Pratylenchus coffeae* nematode infection on coffee plantations sustainably and synergistically. Additionally, AMF facilitates the availability of phosphorus in plants. The objectives of present study are to formulate an enhanced MHB liquid inoculant containing *Bacillus subtilis* and *Pseudomonas diminuta*, and to test its efficacy in controlling *P. coffeae* in roots, improving P status in soil and plants, and promoting the growth of Arabica coffee seedlings infested with the nematodes. MHB liquid inoculant was enhanced by optimizing molasses, nitrogen, phosphorus, and MHB concentrations. The five treatments were used, and five replications were in a randomized block-design greenhouse experiment to investigate the AMF *Glomus agregatum* and MHB inoculant. The improved substrate for MHB liquid inoculant comprised 2% molasses, 0.05% NH_4Cl, and 0.1% KH_2PO_4, with a 2:3 initial volume ratio of *B. subtilis* and *P. diminuta*. Scaling up the MHB inoculant in the 2 L reactor boosted the bacterial population to 10^{10} CFU/mL and the P content to 100 mg/kg. Applying 200 AMF spores and 10^9 CFU/mL MHB increased leaf number, plant P uptake, and soil P while decreasing root damage and nematode population in soil and roots. Combined AMF and MHB reduced *P. coffeae* infestation in roots by 70.79% and increased P content in soil and plants by 57.2% and 61.9%, respectively.

Keywords: *Bacillus*, liquid culture, plant growth, *Pratylenchus coffeae*, *Pseudomonas*

Abbreviations: AMF: Arbuscular Mycorrhizal Fungi; MHB: Mycorrhiza Helper Bacteria; N: Nitrogen; P: Phosphorus

INTRODUCTION

According to the International Coffee Organization (2022), global coffee consumption increased by 3.3% in 2021-2022, reaching 175.6 million bags. This growth follows last year's more modest 0.6% increase. Arabica coffee accounted for 56.21% of global coffee consumption in 2021-2022. Arabica thrives in tropical climates at altitudes ranging from 800 to 1700 meters above sea level, with the highest quality beans typically harvested at 1300 meters. Coffee is cultivated on 89,219 ha of plantations in East Java, Indonesia, producing 45,913 tons of coffee (BPS 2023). However, global warming and agricultural and industrial activities have exacerbated the vulnerability of high-altitude tropical regions. Temperature and rainfall changes affect crop water availability and groundwater recharge in hilly areas (Banerjee et al. 2016). These changes and human activity increase the vulnerability of agricultural and food systems (Beroya-Eitner 2016; Dias et al. 2024). Climate change is expected to impact Arabica coffee through altered rainfall patterns, higher temperatures, and more frequent droughts (Bunn et al. 2015). Climate change also threatens coffee production in Africa, where the coffee berry disease caused by *Colletotrichum kahawae* has become widespread (Ayalew et al. 2024).

The nematode *Pratylenchus coffeae* is a significant limiting factor in Arabica coffee production in tropical climates. This endo-parasitic nematode inhabits the soil and infects coffee roots at all developmental stages, from juvenile to mature (Asyiah et al. 2015; Jackson-Ziems 2016). *Pratylenchus coffeae* reduces NO_3^- and NH_4^+ absorption by 56% and 24%, respectively, causing visible nitrogen deficiency symptoms in coffee plants (Vaast et al. 1998). Nematicides, the traditional method of controlling *P. coffeae*, suppress root population density for up to two years following application (Barbosa et al. 2024). However, recent studies indicate that applying nematicides at a rate of 3 L/ha can suppress *P. coffeae* population by 94.3% (Cepeda-Siller et al. 2018). Biological methods, such as inoculation with Arbuscular Mycorrhizal Fungi (AMF), are emerging as an alternative to chemical control. These fungi establish symbiotic relationships with over 70% of plant species, including coffee (Brundrett 2009; de Beenhouwer et al. 2015). They are essential in low-input agricultural

systems requiring minimal chemical fertilizers and pesticides (Verbruggen et al. 2010).

AMF protects plants from nematode attacks by competing for space and resources, enhancing plant tolerance, triggering systemic resistance, and modifying microbial composition in the rhizosphere (Schouteden et al. 2015). However, the effects of various AMF species on *Pratylenchus* populations range from unfavorable to neutral to beneficial (Gough et al. 2020). Mycorrhizal Helper Bacteria (MHB), primarily *Bacillus* and *Pseudomonas*, promote AMF establishment by boosting spore germination and mycelial growth (Asyiah et al. 2021). These MHB also enhance plant growth, nutrient uptake, and soil conductivity while protecting against pathogens and improving plant defense mechanisms. *Bacillus* and *Pseudomonas* are well-known Plant Growth Promoting Rhizobacteria (PGPR) that provide Nitrogen (N), Phosphorus (P), and phytohormones to plants (Li et al. 2017; Kalayu 2019; de Andrade et al. 2023; Fitriatin et al. 2023).

MHB liquid inoculants require a cost-effective carrier providing adequate nutrients for bacterial growth. Molasses, a byproduct of sugarcane production, is readily available and has been utilized as a substrate for MHB cultivation. Previously, MHB were cultured in 2% molasses-based broth for two days, yielding a final cell concentration of 10^9 colony-forming units (CFU)/mL (Hindersah et al. 2022a). While seedlings treated with MHB had longer shoots, there were no significant effects on leaf number, shoot dry weight, or mycorrhizal colonization. Despite its value, molasses contains minimal nitrogen and phosphorus levels, essential for bacterial growth. Thus, to optimize MHB growth, additional nitrogen and phosphorus sources need to be added to the molasses-based formulation. Although studies on the N and P content of MHB inoculants are limited, this reformulation aims to improve the proliferation of MHB and assess their effectiveness in promoting mycorrhizal formation, regulating *P. coffeae*, and enhancing the growth of Arabica coffee seedlings.

MATERIALS AND METHODS

Biological materials

The Laboratory of Microbiology at Faculty of Teaching Training and Education-Universitas Jember (FTTE-UNEJ) supplied the pure culture of *P. diminuta*, the AMF *Glomus agregatum*, and the endo-nematode *P. coffeae*, and the Laboratory of Soil Biology at Faculty of Agriculture Universitas Padjadjaran provided *B. subtilis* (Figure 1). The pure cultures of both bacteria were maintained at 4°C in nutrient agar plates. The inoculant for host-dependent AMF was developed using maize grown on a zeolite-based substrate. The spore count in the AMF inoculant was 50 spores/g. The *P. coffeae* nematodes were extracted from the roots of nematode-infected coffee plants using a modified Baermann method (Hindersah et al. 2022a). They were reared in a greenhouse using Arabica coffee seeds planted in field soil to obtain a nematode capable of infecting coffee seedlings.

Determination of media composition

Molasses, NH_4Cl, and KH_2PO_4 were added at varying amounts to create the eight liquid culture media compositions. Molasses concentrations ranged from 1% to 2%, whereas NH_4Cl and KH_2PO_4 concentrations were 0.1% and 0.05%, respectively. Molasses at 1% and 2% concentrations were used as controls. Approximately 100 mL of broth from each formula was transferred into 250 mL Erlenmeyer flasks and sterilized in an autoclave at 121°C for 20 min before being stored overnight at room temperature.

The pure culture of *P. diminuta* and *B. subtilis* were prepared by spreading one loop of the colony on a nutrient agar slant and storing it at 30°C for 72 h. The liquid inoculant for each species was generated by pouring 10 mL of sterilized 0.85% NaCl into a slant of each bacterial pure culture and vortexing it for 30 min. Approximately 0.1% of each bacterium's liquid culture was added to 100 mL of each media in a 250-mL Erlenmeyer flask and incubated at room temperature for 72 h. The assay was carried out in duplicate. The bacterial population was assessed using the serial dilution plate method, which utilized *Pseudomonas* agar base (Sigma-Aldrich) and *Bacillus* agar (Sigma-Aldrich). The Kjeldahl method measured total N, and available P was determined using spectrophotometry following concentrated HCl (25%) extraction. The liquid media for *P. diminuta* and *B. subtilis* were selected based on the bacterial population and the culture's N and P content.

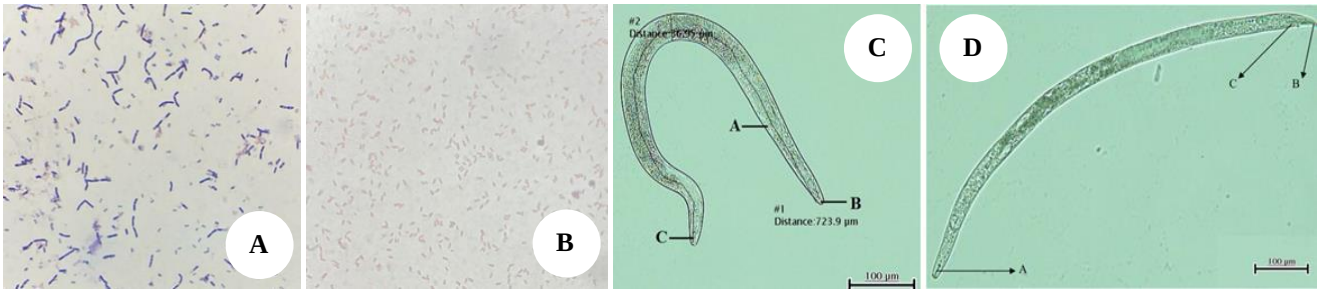

Figure 1. Cell morphology of: A. *B. subtilis*; B. *P. diminuta*. *P. coffeae* isolated from: C. Arabica coffee roots female: A. Vulva, B. Stylet, C. Tail; D. Male: A. Stylet, B. Tail, C. Spicules

Table 1. Combination treatments of endoparasitic nematode, AMF, and MHB in the pot experiment

Code	Treatments
C (Control)	Without *P. coffeae*
PC	*P. coffeae*
PC+AMF	*P. coffeae* and AMF *G. agregatum*
PC+AMF+MHB$_1$	*P. coffeae*, AMF *G. Agregatum*, and MHB 10^8 CFU/mL
PC+AMF+MHB$_2$	*P. coffeae*, AMF *G. Agregatum*, and MHB 10^9 CFU/mL

Determination of bacterial composition

The experiment used a Completely Randomized Design with five liquid inoculant ratios of *P. diminuta* and *B. subtilis*, including 1:1, 1:2, 2:1, 2:3, and 3:2 (v/v). The *Pseudomonas* and *Bacillus* liquid inoculants were prepared based on the media composition data. Both cultures were combined in a 500 mL Erlenmeyer flask to make a final volume of 300 mL, which was then shaken at 115 rpm for 48 h at room temperature. Each treatment was replicated four times. The bacterial density and pH were determined at the end of the experiment. All data underwent analysis of variance (F-test; $p<0.05$) and Duncan Multiple Range Test (DMRT) at $p<0.05$. The optimal bacterial composition was used for liquid inoculant production in a laboratory-scale reactor.

Liquid inoculant production

Using the optimal recipe for *P. diminuta* and *B. subtilis* based on the initial formulation step, the liquid inoculants of both bacteria were scaled up. Approximately 0.1% of the liquid pure culture of bacteria was cultured in 1000 mL of Nutrient Broth (NB) in a 2 L bioreactor for 72 h at room temperature with 115 rpm agitation. The *P. diminuta* and *B. subtilis* inoculants were combined based on the results of the second formulation step and stored for 7 h. The bacterial density, acidity (pH), available N and P, and *E. coli* and *Salmonella* sp. presence in the mixed inoculant were investigated. The liquid inoculant of *P. diminuta* and *B. subtilis* was mixed in the volume composition based on the formulation results and stored in a sterile Erlenmeyer flask with a screw cap at room temperature. The pH, total N, available P, and *E. coli* and *Salmonella* sp. contaminants from single and mixed inoculants were analyzed.

Greenhouse experiment

The experiment was conducted in the Biology Education Study Program greenhouse at FTTE-UNEJ, located 60 m above sea level in the tropical region. The average annual temperature and humidity during the experiment were 23.0-29.9°C and 67-86%, respectively. The sterilized-potted soil we used was Inceptisols (pH 5.6, organic C 2.39%, total N 0.24%, C to N ratio 9.95, available P 14.65 mg/kg, and available K 79.82 mg/kg) collected from the field soil at Indonesian Coffee and Cocoa Research Institute (ICCRI) in Jember, East Java. The ICCRI supplied the two-month-old Arabica coffee cv. Kartika seedlings. The four bioagent treatments and the control were arranged in a randomized block design with six replications, each with five potted plants (Table 1).

AMF had a spore density of 20 spores/g, while *P. diminuta* and *B. subtilis* in mixed MHB had cell counts of 1.1×10^{10} and 8.7×10^9 CFU/mL, respectively. The seedlings were transplanted onto a five-kg substrate mix of soil, sand, and manure (1:1:1).

One week after transplantation, 50 mature *P. coffeae* were placed 5 cm from the plant stem in a circular hole. Coffee seedlings were infected by all nematode life stages (juveniles, male and female adults) since all stages of this nematode can infect and damage coffee roots. The pot with AMF treatments received 10 g of AMF inoculant containing 200 spores, which was applied in a similar circular band, and the hole was promptly covered with the substrate. Liquid MHB inoculation was applied to the seedling stem. MHB-treated plants received 1 mL of inoculant mixed with 99 mL of distilled water, whereas untreated plants received 100 mL of distilled water. Each plant received 5 g of NPK compound fertilizer, which was deposited in a two-cm-deep hole 5 cm away from the seedling stems. All seedlings were maintained in the greenhouse for nine weeks and watered regularly. The weeds were removed from the pots and avoided using pesticides on the plants.

Stem thickness, plant height, and leaf count were measured every two weeks. Disease symptoms were also observed during the experiment. At the end of the experiment, the shoot and root dry weight, Root Colonization (RC) by mycorrhizae, Infection Degree (ID) of roots caused by *P. coffeae*, nematode population in the soil and roots, and P concentration in plant shoot and soil were analyzed.

Mycorrhizal RC was determined after soaking the roots in 2% KOH at 80°C and staining with acid Fuchsin (Kormanik and McGraw 1982). The Baermann funnel (van Bezooijen 2006) was used to count nematodes in 10 g of soil and 100 mL of root extract. The soil and root extract samples at the funnel's tip were passed successively through 40 mesh and 325 mesh filters to collect the nematodes, which were then counted under a light microscope with 100 times magnification. Root ID was determined using the Townsend-Heuberger method (Scalzo et al. 2012), while the classes for scaling *P. coffeae* infection were based on root performance to determine the ID (Hindersah et al. 2022a).

Statistical analysis

All data sets were subjected to a one-way Analysis of Variance (ANOVA) to examine the significant effects of the treatment on parameters. The treatment means were compared using the Duncan Multiple Range Test (DMRT), with $p<0.05$. Statistical analyses were performed using Minitab, version 5.02.

RESULTS AND DISCUSSION

Broth composition

The broth compositions affected *Pseudomonas* and *Bacillus* counts, albeit the population change was non-significant (Table 2 and Table 3). The vegetative cell population in *Pseudomonas* and *Bacillus* liquid cultures was approximately log 8 and 7, respectively. The broths

with D composition (1% molasses, 0.05% NH_4Cl, 0.05% KH_2PO_4) and F composition (2% molasses, 0.1% NH_4Cl, 0.05% KH_2PO_4) had the highest *Pseudomonas* count (Table 2).

The total N and available P levels vary depending on the broth composition (Table 2). The A and F compositions in *Pseudomonas* liquid culture contained higher total N, while the A and C compositions had higher available P. The D and F compositions facilitated improved bacterial growth, as evidenced by a higher bacterial count. However, the F composition had more total N and available P than the D composition. It had the highest total N of all the broth compositions but had less available P than the A and B compositions.

The A media composition promotes *Bacillus* proliferation. This culture possessed a higher total N than other compositions, even though its available P was not as high (Table 3). The total N and available P concentrations were proportional to the concentration of NH_4Cl and H_2PO_4 added to the growth media. The G formula, which included 2% molasses, 0.05% NH_4Cl, and 0.1% KH_2PO_4, had the highest available P but the smallest *Bacillus* population. We also observed that the acidity of *Pseudomonas* and *Bacillus* cultures reduced from neutral to very acidic (pH 3-4).

Initial ratio of *P. diminuta* and *B. subtilis* inoculant

After 72 h of incubation at room temperature, all MHB cultures were allowed to rest for an hour (Figure 2) before cell counting. Table 4 shows that the liquid inoculant containing a 2:3 PD to BS volume ratio exhibited higher *Pseudomonas* and *Bacillus* counts after 72 h. Each bacterial population grew compared to the single culture, as depicted in Tables 2 and 3. Regardless of the PD-to-BS ratio, the mixed culture had a pH of less than 4.

Improved quality of MHB liquid inoculant

The quality of the liquid inoculants for *P. diminuta* and *B. subtilis* produced separately in a 2 L reactor at room temperature with 115-rpm agitation (Table 5). A single culture of *P. diminuta* and *B. subtilis* contained approximately 10^9 and 10^{10} CFU/mL viable cells.

Response of coffee seedlings to AMF and MHB inoculation

Inoculation of AMF with or without MHB altered the plant height of coffee seedlings for up to 7 weeks but did not affect that parameter after 9 weeks (Table 6). The AMF without MHB most significantly increased plant height 1-5 weeks after inoculation compared to other treatments. AMF treatment also increased plant height at 7-9 weeks, but the values were not statistically significant. Plants infected with *P. coffeae* showed a reduction in height one week after planting. By the 9th week, *P. coffeae* -infected plants treated with and without bioagents had similar heights as plants without *P. coffeae*. Surprisingly, the *P. coffeae*-infected plant had a higher leaf count than the control and other treatments after 3 and 5 weeks (Table 7). Meanwhile, infected plants treated with AMF and a lower concentration of MHB demonstrated the lowest leaf number at 9 weeks.

Table 2. Effect of NH_4Cl and KH_2PO_4 on *Pseudomonas* population, total N, and available P in molasses-based media after 72 h of incubation

Media composition Molasses+NH_4Cl+KH_2PO_4 (%)	*Pseudomonas* count (Log CFU/mL)	Total N (%)	Available P (mg/kg)
A (1+0.1+0.1)	8.14^{ab}	0.05^{d}	92.80^{f}
B (1+0.1+0.05)	8.29^{ab}	0.06^{e}	62.84^{d}
C (1+0.05+0.1)	8.18^{ab}	0.04^{c}	89.80^{f}
D (1+0.05+0.05)	8.39^{b}	0.03^{bc}	53.40^{c}
E (2+0.1+0.1)	8.22^{ab}	0.03^{bc}	72.82^{e}
F (2+0.1+0.05)	8.35^{b}	0.05^{d}	51.71^{c}
G (2+0.05+0.1)	8.25^{ab}	0.04^{c}	75.19^{e}
H (2+0.05+0.05)	8.15^{ab}	0.03^{b}	46.88^{b}
I (control: molasses 1%)	8.08^{a}	0.02^{a}	4.10^{a}
J (control: molasses 2%)	8.24^{ab}	0.03^{b}	3.60^{a}

Note: Means in each column followed by the same letters are not significantly different based on DMRT at $p<0.05$

Table 3. Effect of NH_4Cl and KH_2PO_4 on *Bacillus* population, total N, and available P in molasses-based media after 72 h of incubation

Media composition (%) Molasses–NH_4Cl–H_2PO_4	*Bacillus* count (Log CFU/mL)	Total N (%)	Available P (mg/kg)
A (1+0.1+0.1)	8.14^{b}	0.55^{f}	78.71^{bcd}
B (1+0.1+0.05)	7.85^{ab}	0.09^{b}	82.79^{cd}
C (1+0.05+0.1)	7.78^{ab}	0.08^{b}	93.31^{d}
D (1+0.05+0.05)	7.96^{ab}	0.02^{a}	63.39^{b}
E (2+0.1+0.1)	7.89^{ab}	0.15^{d}	131.57^{e}
F (2+0.1+0.05)	7.98^{ab}	0.09^{b}	81.77^{cd}
G (2+0.05+0.1)	7.59^{a}	0.11^{c}	157.20^{f}
H (2+0.05+0.05)	7.62^{ab}	0.12^{c}	76.72^{bc}
I (control: molasses 1%)	8.11^{ab}	0.03^{a}	0.73^{a}
J (control: molasses 2%)	7.88^{ab}	0.21^{e}	0.12^{a}

Note: Means in each column followed by the same letters are not significantly different based on DMRT at $p<0.05$

Figure 2. Liquid inoculant MHB with varied initial ratio of *P. diminuta* and *B. subtilis*

The treatments did not significantly affect root and shoot biomass (Table 8). Nematode-infected plants treated with AMF and MHB had significantly increased root colonization by AMF and a lower degree of nematode infection of roots. Plants treated with AMF *Glomus aggregatum* exhibited a lower nematode ID in their roots. Surprisingly, the shoot dry weight did not decrease after nematode inoculation. Moreover, AMF and MHB applications did not change the root and shoot dry weights. Although bioagent treatments did not affect plant height, single AMF inoculation and combined AMF and MHB treatment significantly reduced nematode count in root and soil (Table 9). The AMF application reduced nematode populations in root and soil by 3.2% and 50.2%, respectively, compared to the control. The combined inoculation of AMF and MHB decreased the nematode count in root and soil by 67.6% and 75.2%, respectively.

The combined inoculation of AMF and MHB raised P levels in soil and shoot (Table 10). The AMF, along with a higher concentration of MHB (10^9 CFU/mL), modestly increased the available P in the soil compared to the *P. coffeae* -infected soil without biological agents. Both MBH concentrations (10^8 and 10^9 CFU/mL) combined with AMF enhanced total P_2O_5 in the soil compared to other treatments. Despite a minor increase in available P in soil, the total P_2O_5 in soil treated with MHB increased by 88.9% and 99.8%, respectively, compared to the *P. coffeae*-infected soil without biological agents. The AMF inoculation into *P. coffeae*-infected soil had little effect on shoot P uptake, while *P. coffeae*-infected plants that received AMF combined with high concentrations of MHB showed increased P uptake.

Table 4. Effect of *P. diminuta* and *B. subtilis* composition on bacterial population and acidity after 72h incubation

P. diminuta [1] **to** ***B. subtilis*** [2] **volume ratio**	**Bacterial count (log CFU/mL)**		**pH**
	Pseudomonas	***Bacillus***	
1:1	10.27^{ab}	9.92^{ab}	3.46^{c}
1:2	10.12^{a}	9.65^{a}	3.71^{d}
2:1	10.31^{ab}	10.04^{ab}	3.24^{a}
2:3	10.45^{b}	10.25^{b}	3.67^{d}
3:2	10.16^{a}	10.05^{ab}	3.38^{b}

Note: Means in each column followed by the same letters are not significantly different based on DMRT at p<0.05. [1]: *P. diminuta*; [2]: *B. subtilis*

Table 5. Quality of MHB liquid inoculants after 72 h in a laboratory scale reactor at room temperature

Culture	**Bacterial count (CFU/mL)**	**pH**	**Total N (%)**	**Available P (mg/kg)**	**Bacterial contaminants (MPN/mL)[1]**	
					E. coli	***Salmonella* spp.**
P. diminuta	4.5×10^9	3.6	0.03	136.54	0	$<10^3$
B. subtilis	1.77×10^{10}	3.3	0.03	109.17	0	$<10^3$
Mixed culture (*P. diminuta*:	PD: 1.1×10^{10}	3.6	0.03	117.32	0	$<10^3$
B. subtilis of 2:3)	BS: 8.7×10^9					
Minimum threshold[2]	10^7	3.8	-	-	$<10^3$	$<10^3$

Note: [1]Most Probable Number; [2]Indonesian Ministry of Agriculture Decree No. 261 year 2019

Table 6. Effect of AMF *G. agregatum* and MHB on plant height of Arabica coffee seedlings

Treatments	**Plant height (cm) at week**				
	1	**3**	**5**	**7**	**9**
Without *P. coffeae* (Control)	12.62^{b}	13.62^{a}	15.95^{bc}	19.25^{b}	22.23^{a}
P. coffeae (PC)	11.24^{a}	13.03^{a}	15.99^{bc}	19.09^{b}	21.91^{a}
PC and AMF	13.36^{b}	15.67^{b}	17.08^{c}	20.05^{b}	23.13^{a}
PC, AMF[1], and MHB[2] 10^8 CFU/mL	12.07^{ab}	13.53^{a}	15.46^{ab}	17.55^{a}	20.84^{a}
PC, AMF, and MHB 10^9 CFU/mL	12.05^{ab}	13.93^{a}	14.58^{a}	17.27^{a}	21.27^{a}

Note: Means in each column followed by the same letters are not significantly different based on DMRT at p<0.05. [1]Arbuscular mycorrhizal fungi; [2]Mycorrhizal helper bacteria

Table 7. Effect of AMF *G. agregatum* and MHB on the leaf number of Arabica coffee seedlings

Treatments	**Leaf number at the week of**				
	1	**3**	**5**	**7**	**9**
Without *P. coffeae* (Control)	4.00^{a}	6.00^{a}	8.00^{a}	8.00^{a}	10.00^{c}
P. coffeae (PC)	4.00^{a}	7.00^{b}	9.00^{b}	9.00^{b}	9.00^{b}
PC and AMF	4.00^{a}	5.67^{a}	7.33^{a}	9.17^{b}	10.00^{c}
PC, AMF, and MHB 10^8 CFU/mL	3.50^{a}	5.50^{a}	7.33^{a}	7.50^{a}	7.50^{a}
PC, AMF, and MHB 10^9 CFU/mL	3.50^{a}	5.50^{a}	7.50^{a}	7.50^{a}	9.67^{c}

Note: Means in each column followed by the same letters are not significantly different based on DMRT at p<0.05

Table 8. Effect of AMF *G. aggregatum* and MHB *P. diminuta* and *B. subtilis* on plant biomass, root colonization, and infection degree of nematode in nine-week-old coffee seedlings

Treatments	Root dry weight (g)	Shoot dry weight (g)	RC[1] (%)	ID[2] (%)
Without P. *coffeae*	0.30[a]	0.57[a]	-	-
P. coffeae (PC)	0.25[a]	0.51[a]	-	45
PC and AMF	0.20[a]	0.48[a]	96	28
PC, AMF, and MHB 10^8 CFU/mL	0.24[a]	0.51[a]	96	30
PC, AMF, and MHB 10^9 CFU/mL	0.26[a]	0.49[a]	99	13

Note: Means in each column followed by the same letters are not significantly different based on DMRT at p<0.05. [1]RC: Root Colonization by AMF; [2]ID: Infection Degree of roots by nematode

Table 9. Effect of AMF *G. agregatum* and MHB *P. diminuta* and *B. subtilis* on nematode count in the root and soil of coffee seedlings nine weeks after treatment

Treatments	Nematode count (individual/g)		Decrease in nematode count (%)	
	Root	Soil	Root	Soil
Without *P. coffeae*	0	0	-	-
P. coffeae (PC)	154.67[b]	143.33[b]	-	-
PC and AMF	149.67[b]	65.67[ab]	3.2	50.2
PC, AMF, and MHB 10^8 CFU/mL	65.67[a]	37.57[a]	57.5	73.8
PC, AMF, and MHB 10^9 CFU/mL	50.00[a]	35.57[a]	67.6	75.2

Note: Means in each column followed by the same letters are not significantly different based on DMRT at p<0.05

Table 10. Effect of AMF *G. aggregatum* and MHB *P. diminuta* and *B. subtilis* on soil P and P uptake in seedling shoots nine weeks after treatment

Treatments	Soil P		P uptake
	Available P (mg/kg)	Total P_2O_5 (me/100 g)	
Without *P. coffeae* (Control)	9.89[a]	16.34[a]	9.30[a]
P. coffeae (PC)	11.45[b]	19.14[b]	10.97[b]
PC and AMF	11.12[b]	29.71[c]	10.81[b]
PC, AMF, and MHB 10^8 CFU/mL	10.50[b]	36.17[d]	11.23[bc]
PC, AMF, and MHB 10^9 CFU/mL	12.14[bc]	38.25[d]	11.61[c]

Note: Means in each column followed by the same letters are not significantly different based on DMRT at p<0.05

Discussion

Pseudomonas and *Bacillus* thrive in molasses-based media since they are heterotrophs. They use various organic carbon sources to generate energy by metabolizing complex carbohydrates and sugars through the glycolytic pathway. Molasses, which contains sucrose (38%), fructose (8.4%), glucose (7.8%), minerals, and amino acids (0.1-0.87%), including tyrosine, glycine, proline, glutamic acid, and valine (Khairul et al. 2022). The amino acids most likely act as microbes' N source. However, the C/N of molasses in this study was high (108), indicating a lack of available N for microbial growth.

Specific NH_4Cl and KH_2PO_4 concentrations were applied to molasses-based bacterial monocultures, resulting in a moderate increase in bacterial populations, total nitrogen content, and available phosphorus levels compared to the control. However, each bacterium had a cell density of approximately 10^8 CFU/mL. The rise in N and P also aids bacterial proliferation after 72 h, maintaining cell viability. Enriching molasses with NH_4Cl and KH_2PO_4 can reduce media C/N and C/P ratios, increasing available N and P for bacterial metabolism. Nitrogen (N) is critical in cell proliferation and amino acid synthesis. It promotes bacterial cell elongation and is an essential nutrient for several biomolecules, including amino acids that comprise proteins and enzymes. Phosphor is critical for energy transfer across all metabolic pathways. Carbon metabolism pathways involve phosphorus (P), which interferes with N in the storage and transfer of energy in cell metabolisms (Sui et al. 2024).

The pH decreases to 3.3-3.6 in the *P. diminuta* and *B. subtilis* liquid culture could be attributed to the bacteria's organic matter degradation pathway. During fermentation, the breakdown of organic matter through the glycolytic pathway produces organic acid and lower pH (Yan et al. 1996; Jojima and Inui 2015). *Pseudomonas* and spore-forming *Bacillus* grow at different pH ranges. *Pseudomonas* growing at pH 5 had an 11% longer doubling time than bacteria growing in neutral media (Mozaheb et al. 2023). *Bacillus subtilis* and *B. pumilus* populations reached 10^5 and 10^8 CFU/mL at pH 4 and 6, respectively (Andriani et al. 2017). Lower pH (2-3) in *Bacillus* cultures induces spore formation (Xu et al. 2021). Even though this study did not consider the spore count, the spore formation in MHB *Bacillus* could increase cell viability (spore germination) when applied to the soil. In the previous experiment, without N and P enrichment, the pH was slightly reduced to 6.1-6.4 (Hindersah et al. 2022b).

A 2:3 volume ratio of *P. diminuta* to *B. subtilis* in mixed cultures achieved a 10^{10} CFU/mL bacterial population density. The larger Erlenmeyer flask enhanced oxygen availability, which aided bacterial proliferation because both bacteria are aerobic. However, *B. subtilis* populations decreased compared to *Pseudomonas*, consistent with *Pseudomonas* sp. dominance in growth under various glyphosate concentrations (Enemuor 2014). Competition for nutrients and oxygen likely contributed to this outcome. Research suggests that *Bacillus* and *Pseudomonas* co-cultures frequently exhibit competitive or amensal interactions induced by bioactive natural products (Lyng and Kovács 2023).

Organic acid generation, indicated by a pH decline in liquid cultures, is beneficial for agricultural applications because it solubilizes phosphorus in soil, increasing its bioavailability to plants. This mechanism is particularly critical for tropical soils, which are typically phosphorus-deficient. Indonesian guidelines require liquid biofertilizers to maintain a bacterial population of 10^7 CFU/mL. The *P. diminuta* and *B. subtilis* mixed culture surpassed the

benchmark, with bacterial counts corresponding with reported biofertilizer levels of 10^6-10^9 CFU/mL (Bhavya et al. 2017; Raimi et al. 2020). Enriching molasses with NH_4Cl and H_2PO_4 effectively enhanced the bacterial inoculant's total nitrogen and phosphorus levels, highlighting their role in supporting bacterial metabolism and plant health (Suryanti and Santiasa 2020).

The most effective bioassay treatment was a combination of AMF *G. aggregatum* and MHB at 10^9 CFU/mL. The treatment increased leaf count, total and available phosphorus in the soil, and phosphorus uptake by shoots while reducing root infections and *P. coffeae* soil populations. MHB treatment decreased AMF root colonization and nematode infection rates. However, plant height and shoot biomass (fresh and dry weight) remained unaffected, indicating that autotrophic metabolism shifted resources toward maintaining plant health and minimizing pathogen effects. MHB and AMF synergistically provided nitrogen and phosphorus, reducing nematode-induced stress and enhancing plant disease resistance (Tripathi et al. 2022).

AMF and MHB inoculation boosted phosphorus availability, which the plants absorbed directly. Treatments combining AMF with high MHB concentrations (10^9 CFU/mL) showed significant improvements in available phosphorus levels. This is consistent with the findings of Guarnizo et al. (2023), who reported that combining MHB with mycorrhizae enhances macronutrient availability and promotes plant growth. Furthermore, increased root volume caused by vigorous growth likely contributed to enhanced secretion of root exudates such as organic acids or phosphorus-solubilizing compounds, which improved phosphorus status (Yan et al. 2024). Nasslahsen et al. (2022) reported that MHB supplementation improved mycorrhizal systems' soil quality and plant fitness.

The optimized MHB formulation boosted bacterial cell densities and AMF colonization, reducing nematode infection rates in roots and soil. Roots sustained AMF proliferation, improving plant defense by competing for and nutrients, even while *Pratylenchus* nematodes had minimal influence on root architecture (Vaast et al. 1998; Schouteden et al. 2015). MHB promotes AMF symbiosis through improved fungal compatibility, spore survival, and mycelial growth (Turrini et al. 2018; Sangwan et al. 2023). Notably, this study demonstrated a 13% reduction in nematode infection degree and a 67.6% and 75% drop in nematode populations in roots and soil, respectively, surpassing previous formulations (Hindersah et al. 2022a). *Pseudomonas* and *Bacillus* serve a natural role in repressing biotic stress in coffee seedlings. An enhanced formulation of MHB with *P. diminuta* and *B. subtilis* could be a promising biological agent for controlling nematode *P. coffeae* infection in coffee roots.

The combination of MHB and AMF provides a reliable strategy for disease management in coffee plantations, mitigating growth retardation and yield losses while reducing reliance on chemical pesticides. This approach is particularly relevant given the imminent threat of climate change, which is expected to increase nematode generations in *Coffea arabica* plantations between 2050 and 2080 (Ghini et al. 2008). Future research should focus on scaling up bacterial inoculants in industrial-scale bioreactors while optimizing variables like temperature, agitation, and oxygen supply for mass production.

In conclusion, molasses enrichment with NH_4Cl and KH_2PO_4 improved the *Pseudomonas* and *Bacillus* count, N, and P content of the MHB liquid inoculant. The optimal ratio for both bacterial species to increase the MHB liquid inoculant quality was 2:3. The MBH inoculant produced in a 2 L reactor contained bacterial cells with a pH that met Indonesian biofertilizer standards. Seedlings treated with 200 spores of AMF and 10^9 CFU/mL MHB showed increased leaf number, plant P absorption, and soil P levels. This treatment effectively decreased root damage and nematode population in soil and roots. The results verified the significance of N and P in the MHB formulation and the potential of employing MHB and AMF to lower the endoparasitic nematode *P. coffeae* in the soil and roots of Arabica coffee seedlings. Both biological agents can be applied in the eco-friendly Arabica coffee nursery to reduce the use of pesticides.

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