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Abundance of Beneficial Soil Microbes in the Rhizosphere of Coffee Plants Infected by *Pratylenchus coffeae*

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Abstract. Enzymatic transformation in the Nitrogen (N) and phosphorus (P) cycles in soil can only be carried out by microbes. The latest approach in coffee cultivation is to utilize soil microbes to enhance plant growth, also to reduce the attack of the nematode *Pratylenchus coffeae* on coffee roots. This exploratory study aimed to observe the attack of *P. coffeae* on coffee tree, and the presence of N-fixing bacteria (NFB) and P- solubilizing microbes (PSM) in the coffee rhizosphere which have the potency to be used as biofertilizer and bioprotectants in coffee plantation. The study was conducted in Arabica and Robusta coffee plantation of PT Perkebunan Kalibendo, East Java; on immature plants (IP) and mature plants (MP). This exploration explained that the IP and MP of arabica and robusta coffee plantations were attacked by *P. coffeae*. The NFB and PSM were successfully isolated from the rhizosphere of both coffee plantations types. The population of NFB Azotobacter in IP was lower than in MP, but the PSM population in IP rhizosphere was not different from that in MP. Based on morphological and biochemical characterization, three isolates of *Azotobacter* bacteria, six species of P- solubilizing bacteria and six species of phosphate-solubilizing fungi were obtained. This exploration confirmed that the microbes involved in the N and P cycle colonized the rhizosphere of coffee which was attacked by *P. coffeae*. Further research is recommended to observe the effectiveness of microbes as biological fertilizers and bioprotectants for coffee plants.

Keywords: *Azotobacter*; biofertilizer; bioprotectant; nitrogen fixing bacteria; phosphate solubilizing microbes

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INTRODUCTION

Indonesia is the largest coffee-producing country in Southeast Asia and the third largest in the world after Brazil and Vietnam. Indonesia's coffee exports in 2021 reached 384,510 tons and worths US$ 849,370,000. In spite of being the largest coffee plantation area in the world with 1.3 million hectares, Indonesia could only produce 0.5 - 0.77 tons per ha. This productivity is a lot lower than that of Vietnam which produce up to 2.8 t/ha with the total coffee plantation area of 705,000 ha. The challenges of intensifying coffee plantations in Indonesia to achieve an equal figure with other producers include fertilization management related to soil fertility and disease control (Tran et al., 2021; Sunanto et al., 2019).

A common problem of plant nutrition in the tropics is low level of C, N and P in the soil; this obstacle is so far overcome by chemical fertilization which has several drawbacks. The limited raw materials for making chemical fertilizers due to the Russian-Ukrainian war markedly increased the price of chemical fertilizers between 50% -238%. The efficiency of using N and P fertilizers is also low due to successive leaching and evaporation, as well as adsorption of P by soil minerals (Jadon et al., 2018; Hanyabui et al., 2020).

The nematode *Pratylenchus coffeae* is an important disease of robusta and arabica coffee that attacks most coffee plantations in Indonesia (Wiryadiputra and Tran, 2008). Robusta coffee plantation yield losses due to *P. coffeae* attack can reach 78%, with an average of around 57%. The decline of Arabica coffee production in the Kintamani District, Bali due to *P. coffeae* reached 32.5%, although only 8.27% are in light attacks (Arsadja et al., 1996). The *Pratylenchus coffeae* attack destroyed 95% of Arabica coffee in Java (Sulistyowati et al., 2012). For six years (1981-1986) the nematode attack of *P. coffeae* caused an average yield loss of 56.84%, or about 150 tons of coffee per year. In addition to reducing the quantity, nematode attacks can also reduce the quality of coffee (Mustika, 2005).

In line with the concept of sustainable agriculture and green economy, nutrition management and plant disease control are starting to shift to the use of non-pathogenic microbes.

Biofertilizers start to be recommended to replace some chemical fertilizers. A number of microbes, especially non-pathogenic soil bacteria and fungi have the potential to be developed as disease control agents in plant roots. Two groups of beneficial microbes that affect the availability of plant nutrients are the non-symbiotic nitrogen (N_2)-fixing bacteria of *Azotobacter* and phosphate solubilizing microbes (PSM). In coffee cultivation, the Arabica coffee seedlings showed better morphological and biochemical characters after inoculated with *Azospirillum* singly or in multiples by coinoculation with the arbuscular mycorrhizal fungi *Glomus* and *Azotobacter* N fixer (Adriano-Anaya et al., 2011). *Bacillus* is one of the important genuses of the phosphate-solubilizing group of bacteria (Lindang et al., 2021). Microbial application of *Bacillus* sp. on IP robusta coffee can increase leaf chlorophyll content and coffee plant height (Subakti et al., 2021).

The reduction of diseases caused by the nematode *P. coffea* has the potential to be carried out by biological agents that act as bioprotectants. The root colonization by arbuscular mycorrhizal fungi (AMF) is important for coffee health; and AMF infection can be enhanced by Mycorhiza Helper Bacteria (MHB) (Asyiah, et al., 2015a). The role of PSM to enhance P uptake and growth and even yield of plants has been well documented. Several genera of P solubilizing bacteria such as *Bacillus* and *Pseudomonas* are also known to act as MHB (Asyiah et al., 2021, Hindersah et al., 2022) and are able to control *P. coffeae* (Asyiah et al., 2015a, b).

Both *Azotobacter* and PSM can be isolated by specific media, namely N-free Ashby and Pikovskaya respectively. The two are aerobic, heterotrophic and mesophyll microbes that live in the rhizosphere of various agricultural crops. *Azotobacter* is a nonsymbiotic N_2-fixing bacteria. These rhizobacteria are Gram negative that form capsules but do not form endospores which can provide N of 15 - 60 kg N/ha/year (Bhattacharyya and Jha, 2012; Saha et al., 2017). The P-solubilizing bacteria (PSB) that are commonly found is including *Acinetobacter, Pseudomonas, Massilia, Bacillus, Arthrobacter, Stenotrophomonas, Ochrobactrum,* and *Cupriavidus* (Wan et al., 2020), while some important P-solubilizing fungi (PSF) are belong to the genera of *Aspergillus, Penicillium, Pseudeurotium* and *Trichoderma* (Kalayu, 2019).

Plant health is related to the adequacy and balance of nutrients in the soil to be absorbed by roots and then used for growth. ~~As~~ It has been explained that one of the causes of nematode infestation is nutritional deficiency. In nutrient deficiency (abiotic stress), the presence of nematodes in roots will increase stress (Misram et al., 2020) because nematodes take nutrients from plant tissue with stylets and dead plants (Bahadur, 2021)

Exploration of *Azotobacter* and PSM is important to obtain bioresources which can be formulated as biological agents with dual roles as biological fertilizers and *P. coffeae* control agents. Therefore, the aim of this study was to observe the attack of *P. coffeae* and the presence of N-fixing bacteria (NFB) and P- solubilizing microbes (PSM) in the coffee rhizosphere which in turn have the potential to be used as biological fertilizers and bioprotectants of coffee plants. This exploration also determined mycorrhizal colonization and the incidence of nematode infection in coffee roots.

METHODS

Microbial exploration was carried out in the immature plants (IP) and mature plants (MP) areas of Arabica coffee and IP areas of Robusta. The research location is PT Perkebunan Kalibendo Banyuwangi at an altitude of 600 and 825 MASL. The location was determined purposively with the inclusion factors 1) visual symptoms of parasitic nematode attack were found, especially in the canopy and 2) plant growth uniformity. Furthermore, one stretch (limited area?) was established in the IP area and MP area with four alley plants consisting of 30 plants (Figure 1).

Soil and root samples were taken compositely; determination of 5 plant samples was carried out by randomized method. Prior to exploration, soils at depths of 30 and 60 cm were analyzed to determine the pH, C, N and available P; and soil texture (Table 1). All analytical methods were carried out based on the *Association of Official Analytical Chemists* (AOAC, 2012). The soil pH was measured by pH meter. The C and N analysis method were Walkley and Black, and Kjeldahl respectively. The available P analysis was performed with Bray II method. The exchangeable cation determines by Percolation method. Soil texture was determined by Hydrometer.

Figure 1. a. Immature Plant (IP) Arabica coffee (var. Kartika); b. IP Robusta Coffee

Table 1. Chemical and physical characteristics of the soil before the study

No	Samples	pH H_2O	pH KCl	Soil Texture fraction (%)			Organic Carbon (%)	Total Nitrogen (%)	Available Phosphor (mg/kg)	Exchangeable Cations (cmol/kg)			
				Silt	Clay	Sand				K-ex	Ca-ex	Na-ex	Mg-ex
1	Soil-Arabica IP 30-cm deep	4.97	4.70	45	7	48	5.96	0.58	98.22	0.83	9.29	0.38	0.89
2	Soil-Arabica IP 60-cm deep	4.49	4.53	33	5	63	4.88	0.48	49.71	0.74	5.96	0.35	0.52
3	Soil-Arabica MP 30 cm	5.16	4.47	27	9	64	2.57	0.24	33.78	1.12	4.33	0.43	0.4
4	Soil-Arabica MP 60 cm deep	4.93	4.47	34	7	59	2.71	0.18	101.32	0.93	4.25	0.35	0.3
5	Soil-Robusta 30-cm deep	4.42	4.56	45	11	44	5.61	0.40	62.91	0.79	5.92	0.67	0.51
6	Soil-Robusta 30 cm deep	4.51	4.12	41	13	47	5.02	0.48	61.33	0.60	5.23	0.36	0.36

From each plant sample, 200 g of soil around the roots were taken from 4 subsample points. The soil from the 5 samples was mixed and stirred evenly; and 200 g of soil was taken for the isolation of *Azotobacter* bacteria, phosphate solubilizing bacteria and fungi, and nematode populations. A total of 200 g of lateral root samples were taken randomly from 5 sample plants and mixed evenly and 200 g were collected for analysis of the degree of mycorrhizal infection and the incidence of *P. coffeae* disease.

The isolation of *Azotobacter* bacteria was carried out using N-free Ashby Manitol media through two stages, namely enrichment to form biofilms in Ashby broth and isolation using the streak method on agar plates with the same media (Jiménez et al., 2011). The *Azotobacter* colonies are characterized by round, convex, transparent and slimy shapes. PSB and PSF were isolated by serial dilution plate method on Pikovskaya media (Susilowati and Syekhfan, 2014). PSM colonies are shown by the formation of halozone around the colonies. Bacterial colonies characterizing *Azotobacter* and PSM were purified. Determination of the genus of the *Azotobacter* and PSB groups was carried out based on biochemical characteristics, while the PSF group was based on the morphology of the generative structure.

The number of nematodes in roots and soil was calculated by extracting method from 10 g of soil samples and 10 ml of root extract using a Baermann funnel (van Bezooijen, 2006) modified by Asyiah et al. (2015a). Determination of the degree of root colonization by AMF followed the method of Brundett et al. (1994). Roots were cut to 2 cm long; after soaking the roots in 2% KOH at 80 0C the roots were stained with acid fuchsin.

RESULT AND DISCUSSION

Symptoms of nematode attack are leaves yellowing but they are not accompanied by wilting. In contrast to chlorosis due to N deficiency, yellowing does not only occur on young leaves but spreads throughout the leaves (Figure 2). In accordance with the symptoms of nematode attack plant, especially young leaves, but nematodes infection were detected in roots since the juvenile and adult nematodes were successfully isolated from the roots.

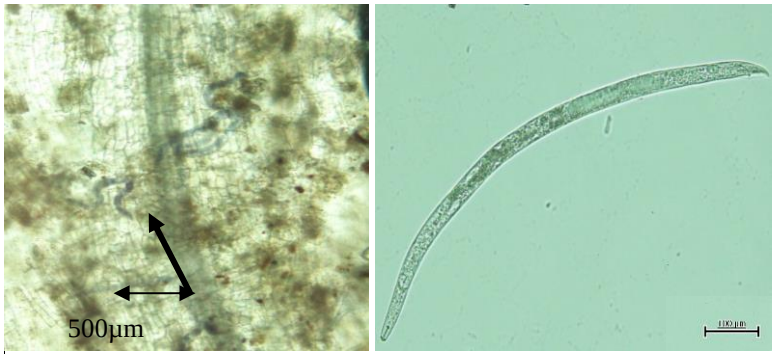
Figure 2. Symptoms of nematode attack marked by yellowing of leaves in mature plant (MP) of arabica coffee (a) and immature plant (IP) of robusta (b)

Nematode infection was demonstrated by the presence of juvenile and adult *P. coffeae* nematodes both in soil and roots (Figure 3). In general, the populations of *P. coffeae* in soil and roots were 148 and 224.6 individual, respectively (Table 2). However, coffee plants have also been infected with indigenous AMF with an average infection rate of 42.6%; this is small but quite significant by considering that coffee plantations have never been inoculated with AMF either in the nursery or in the field.

Table 2. Population of *P. coffeae* in soil and roots; and the degree of AM infection in the roots of immature Arabica coffee plants and mature plants

Sample Origin[1]	Population of *P. coffeae*		Degree of infection AM (%)
	Soil (individu/100 g)	Roots (individu/ g)	
IP Arabica	160	216	46
MP Arabica	142	223	32
IP Robusta	144	235	50

[1]IP: Immature Plants; MP: Mature Plants



a b

Figure 3. a. *P. coffeae* in coffe root (magnification 500 µm); b. *P. coffeae* from soil

Isolation on agar plates resulted in a number of separate colonies of MPF with the surrounding halozone, as well as non-symbiotic nitrogen fixing bacteria (NFB) (Figure 4). The microbes were successfully isolated from each sample; A total of 14 NFB isolates, 15 PSB isolates and 17 PSF isolates have been purified (Table 3).

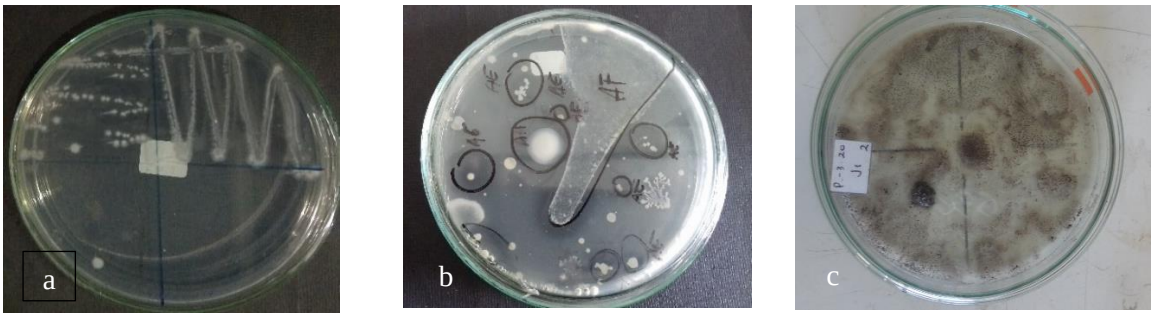


Figure 4. a.Non-symbiotic nitrogen fixing bacteria (NFB) colonies on N-free media; b. the halozone formed around the P-solubilizing bacteria (PSB); and c. the P-solubilizing fungi (PSF) colonies on Pikovskaya media.

Table 3. Number of different bacterial colonies on agar plates for non-symbiotic nitrogen fixing bacteria (NFB) and phosphate solubilizing microbes (PSM)

Sample Origin[1]	Biofertilizer Group		
	NFB	PSB	PSF
IP Arabica	3	5	5
MP Arabica	6	3	7
IP Robusta	5	7	5

[1]IP: Immature Plants; MP: Mature Plants

Based on the morphological and biochemical identification, of the 14 NFB isolates, only two bacteria were identified as *Azotobacter*, namely RI and F3 isolates (Table 4). Both isolates have the morphological and biochemical properties of *Azotobacter*; the cell wall is Gram negative, cocci or rod in cell shape, secretes the enzymes catalase and oxidase, uses mannitol sugar as a carbon source in aerobic metabolism, and uses glucose to produce acids that change the color methyl red (MR) from yellow to red as described by Holt et al (1994). In addition to fix the N_2 and produce phytohormones to stimulate plant growth (Hindersah et al., 2020), *Azotobacter* also induces plant resistance to Fusarium wilt disease in bananas (Proboningrum et al., 2019). However, research on Azotobacter as bioagents to control nematodes is still limited.

Table 4. Characteristics of non-symbiotic nitrogen fixing bacteria (NFB) isolates isolated from coffee stands based on morphological and biochemical tests

No	Isolate	Gram	Cell shape	Capsule	Catalase	Oxidase	Mannitol	Indole/VP1	MR2	t 50 ^{0}C
1	RI	-	Coccus	+	+	+	+	+	+	-
2	20A	-	Coccus	-	+	-	-	-	-	-
3	RH	+	Coccus	+	-	+	+	-	+	-
4	RK	+	Coccus	+	+	-	+	-	+	-
5	RA	+	Oval	-	+	-	-	-	+	-
6	RJ	+	Coccus	-	-	+	+	-	-	-
7	13K	-	Coccus	-	+	+	-	-	-	-
8	20F	-	Coccus	+	-	+	+	-+	+	-
9	13M	+	Coccus	+	-	-	+	-	+	-
10	20J	-	Coccus	-	-	+	+	-	+	-
11	13D	-	Oval	-	-	+	+	+	+	-
12	H2	-	Coccus	-	+	-	-	-	-	-
13	C3	+	Coccus	-	-	-	+	-	-	-
14	F3	-	Oval	+	+	+	+	+	+	-

[1]VP: Voges Proskauer; [2]Methyl red

During the purification process, five PSB isolates could not be further cultured on Pikovskaya media and were declared as "unculturable bacteria". The characterization of the 10 PSB isolates had various of biochemical characteristics (Table 5). Based on 13 types of biochemical tests, 8 isolates were suggested the PSB and then their species have been characterized (Table 6).

Table 5. Biochemical characterization of P-solubilizing bacteria isolated from soil under Arabica and Robusta coffee trees

Character	Isolates									
	C1	I1	520	C2	H1	A1	C1	M1	B2	D1
Gram	-	+	-	-	+	+	-	+	+	+
Cell Shape[1]		B	C	C	C	B	C	B	B	C
Motility	-	-	+	-	-	-	-	-	-	+
Catalase	+	+	-	+	+	+	+	+	+	+
Oxidase	-	+	-	+	-	+	+	+	-	+
O/F[2]	F	F	F	F	F	F	F	F	O	F
Citric	-	-	+	-	-	-	-	+	+	-
Gelatin	-	-	-	-	-	-	-	-	-	-
Urea	-	+	-	-	-	-	-	-	-	-
Indole/VP[3]	-	-	-	-	-	-	-	-	-	-
Starch	+	-	+	+	+	+	+	+	+	+
Protease	+	+	-	+	+	+	+	-	-	-

[1]B: Bacil, C: coccus; [2]O/F: Oxidation/Fermentation; [4]VP: Voges Proskauer;

Table 6. Bacterial genus various BPF isolates from coffee stand soil

No	Isolate	Bacterial genus
1	I1	*Staphylococcus*
2	520	*Acinetobacter*
3	C2	*Flavobacaterium*
4	H1	*Aerococcus* (pathogen)
5	A1	*Bacillus*
6	M1	*Bacillus*
7	B2	*Micrococcus*
8	D1	*Staphylococcus*

Bacillus and *Staphylococcus* have been reported to form halozone in media with insoluble P (Lindang et al., 2021) while *Acinetobacter* produces phosphatase and phytase which are important for degrading soil organic P (He and Wan, 2021). The genus *Micrococcus* has not been used as a P-solvent biofertilizer, but this bacterium interacts with cowpea (*Vigna unguiculata*) to dissolve P (Dastager et al., 2010). The capacity of *Flavobacterium* as PSB isolated from legume rhizosphere has also been described by Purwaningsih et al. (2018). However, the pathogenic *Aerococcus* has never been reported as a PSB. Among these bacterial genera, *Bacillus* has been reported to play a role in reducing disease incidence caused by the nematode *P. coffeae* (Asyiah et al., 2017; Fitriatin et al., 2022) and other plant-parasitic nematode *Radopholus duriophilus* (Duong et al., 2022).

The classification of fungi was carried out based on the morphology of the generative body, sexual reproduction, especially the generative hyphae, as well as spores and sporangium. The generative hyphae structure of the six isolates is in accordance with the classification of fungi according to Alexopoulos et al. (1996). Among the 16 isolates, only isolates 13 III AL -4 1 and R J F -4 1 were not identified at the genus level (Table 7). This research has confirmed the presence of PSF *Fusarium*, *Penicillium*, *Aspergillus*, *Paecilomyces* and *Trichoderma*; and the species *Acremonium charticola* and *Aspergillus niger* (Table 7 and Figure 5).

Table 7. Characterization of phosphate solubilizing fungi based on colony color and generative body morphology.

No	Isolate	Colony color	Genus/Species
1	P -4 E R 1	White	*Fusarium*
2	13 III A0 -4 1	Crystal white	*Penicillium*
3	P -5 A 20 1	Black	*Aspergillus*
4	P -4 F 20 2	Black	*Aspergillus*
5	P -5 20 B 1	Black	*Aspergillus*
6	P -5 A R 1	Black	*Aspergillus*
7	13 III AL -4 1	Light brown	-
8	P -4 R C 1	Black	*Aspergillus*
9	R J F -4 1	White	-
10	R J B -5 2	Green	*Paecilomyces* sp.
11	P -3 J 1 20 2	Black	*Aspergillus*
12	R J 6 -4 1	Green	*Trichoderma*
13	P -3 H 20 1	White	*Acremonium charticola*
14	P -4 E 20 2	Black	*Aspergillus*
15	P -4 G 20 2	Black	*Aspergillus niger*
16	P -3 B 20 1	Black	*Aspergillus niger*

Figure 5. shows the sexual structure of the six species based on macro- and microconidia. *Aspergillus* is known as a pathogen but non-pathogenic *Aspergillus* has been reported to be able to dissolve P and control diseases as a nematophagus (Jain et al., 2012; Akhtar et al., 2015). *Paecilomyces* is a cosmopolitan fungus that is primarily known for its nematophagous capacity, but is also reported to be parasitic in insects and controls several types of pathogenic fungi and bacteria (Moreno-Gavíra et al., 2020). *Trichoderma* sp. is a species of soil fungus known as a decomposer and biological controller of plant diseases. In vitro, *T. harzianum* inhibited the growth of soil-borne pathogens *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (Kalay et al., 2018). In this study, the P-solubilizing *Fusarium* fungus which is well known as a plant pathogen was obtained. However, the presence of P-solubilizing *Fusarium* in the rhizosphere of agricultural plants was lower than that of *Aspergillus* spp. and *Penicillium* spp. (Elias et al., 2016). The *Acremonium charticola* is found in soil but it is also found in fermented cassava; reported to produce antibacterial and antioxidant for poultry feed. However, the potential of *A. charticola* in dissolving P and disease control agents has not been reported.

In current study soil contained high organic matter, total-N, and available P_2O_5; which is prominent soil properties for fungal and bacterial growth as well as nematode proliferation. High organic carbon reflected the organic matter abundance in soil. The microbes isolated from the study area were aerobic and heterotrophic. Therefore, organic matters are prominent as the only carbon and energy source for P-solubilizing microbes and N_2-fixing bacteria (Rathore, 2014; Blesh, 2019). Moreover, the soil organic matter enhances soil porosity (Surya et al. 2017) that reflect the oxygen (O_2) availability for aerobic organisms determined in current study. Dependence of *Pratylenchus* in organic-matter-rich soil have been reported (Chałańska et al. 2016**).** The growth of soil organism included nematode and microbes depend on N, P, and K as their major nutrient as well as the presence of exchangeable cation (K^+, Ca^+, Na^+, Mg^{+2}). In this study, the existence of soil bacteria and fungi were supported by the sufficient nutrients depicted in Table 1.

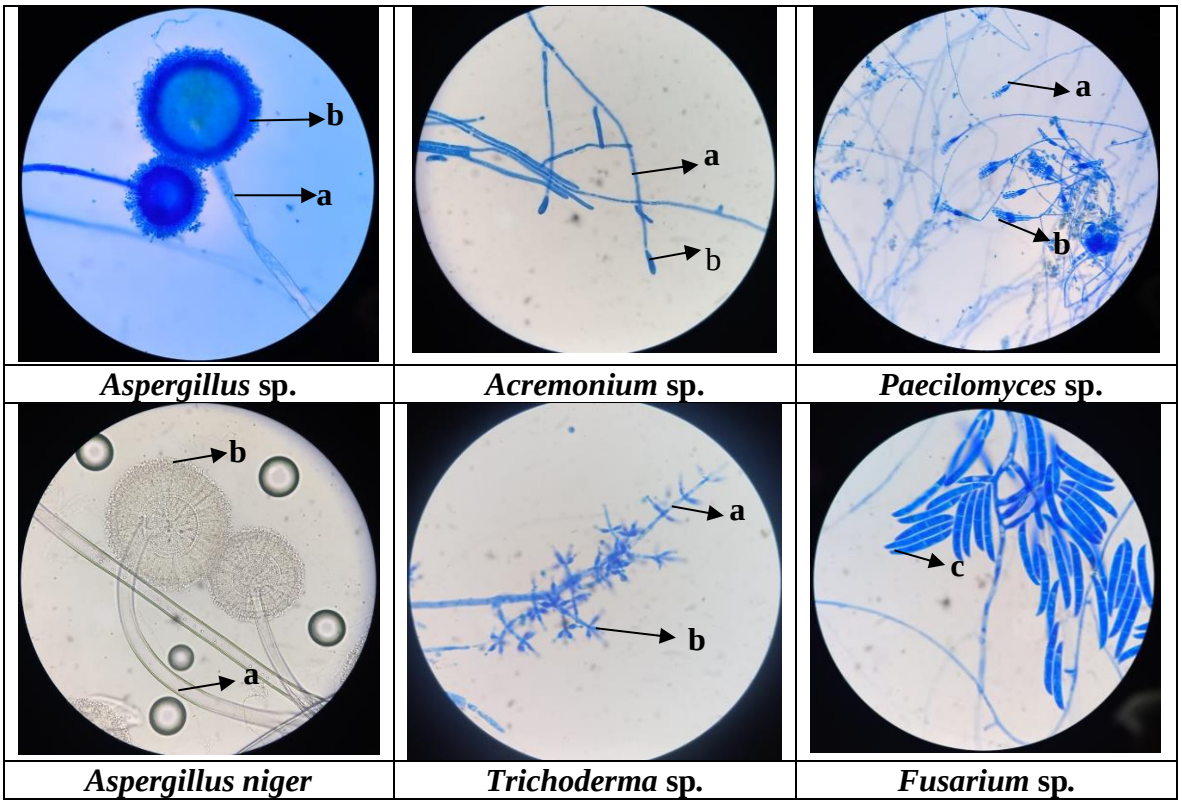


Figure 5. Generative body morphology of various species of phosphate solubilizing fungi a: Conidiophores, b: conidia; c: macroconidia

Despite the ability of soil bacteria to help arbuscular mycorrhizal formation in the roots that in turn reduce the parasitic nematode growth, the simultaneous identification of N-fixing bacteria and P-solubilizing bacteria in soil infested by *P. coffeae* is limited. In general, researchers report the existence of parasitic nematode without counting on these important beneficial soil bacteria and fungi. The current research verified that those bacteria enable to grow side-by-side with parasitic nematode. This founding is necessary to provide various isolates of N-fixing bacteria and P-solubilizing microbes that enable to induce AM formation in the soil infested by parasitic nematode. Nevertheless, enzymatic N_2 fixation and P solubilization maintain the biologically available N and P in the soil for coffee growth. Further, the mycorrhiza helper bacteria inoculant will have double role: as bioprotectant and biofertilizer.

CONCLUSION

The descriptive research under Arabica and Robusta coffee stands revelaed that Arabica Coffee Plants both that have and have not produced beans and the Robusta Coffee that have not produced beans were attacked by *P. coffeae*. The number of nematodes in 100 g of soil and root reached 148.6 and 224.6 respectively. Microbial isolation from the IP and MP Arabica and Robusta coffee provided 14 NFB isolates, 15 PSB isolates and 16 PSF isolates. Based on morphological and biochemical characterization, two isolates of *Azotobacter*, six species of PSB and six species of PSF were obtained. The presence of arbuscular mycorrhizae in coffee roots with an infection degree between 32-50% proved that indigenous mycorrhizal fungi infect coffee roots. This exploration confirmed that the microbes involved in the N and P cycle colonized the coffee rhizosphere which was attacked by *P. coffeae*. Further research is recommended to identify other isolates and observe the effectiveness of microbes as biological fertilizers and bioprotectants for coffee plants.

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