

Effectiveness of Endophytic Bacterial Consortium of Coffee Plant on Mortality of *Pratylenchus Coffeae* in Vitro

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Abstract

Bacteria live in wild in form of a consortium. Use of microbial consortium tends to give better results than single isolate, because the action of enzyme of each type of microbe can complement each other in order to survive. This study aimed to study the effectiveness of bacterial endophytic consortium from coffee plant on plant growth and mortality of parasitic nematodes in coffee. Isolation of bacteria is conducted by growing the crushed roots, stems and leaves of coffee on 20% TSA media, then testing their hemolysis and hypersensitivity reaction. Selected isolates were tested on their effect on the growth of seedling and *Pratylenchus coffeae* mortality, as well as their chitinolytic, proteolytic, lipolytic, HCN production, dissolution of phosphate (P) and fixation of nitrogen (N₂) abilities. The results showed that from 27 isolates of the consortium, 23 isolates showed negative reaction to hypersensitive test and 9 isolates to hemolysis test. The highest mortality rate was shown by K6 isolate (65.8%). The highest growth was shown by K15 and K 21 isolates while the highest root length by K21 isolate. Further analysis showed that 100% of the isolates could hydrolyze proteases, lipid, and produce HCN, while chitinolytic activity was shown by 78% isolates which could fix N₂ and 11% of isolates could dissolve phosphate.

Keywords: endophytic bacteria consortium, *Pratylenchus coffeae*, hydrolysis enzyme, growth promoters

INTRODUCTION

Pratylenchus coffeae nematodes is the most harmful nematodes on coffee plantations, both on Arabica coffee and Robusta coffee. *P. coffeae* is classified as endoparasitic sedentary or sedentary endoparasites (migratory). Female nematodes lay eggs in the root cortex tissue, especially young root tissue and eventually damage the roots. Root damage will be more widespread with the growing population of nematodes, and finally the nematodes will move out of the roots and look for other healthy roots (CABI, 2015).

P. coffeae attack on Robusta coffee and may cause a decrease in production up to 57%.

R. similis attack together with *P. coffeae* on Arabica coffee and can result in more severe damage reached to 80% and the plant will die at the age of less than three years (Wiyadiputra, 1998). This is due to that *P. coffeae* lives in the area of soil depth of 30 cm while *R. similis* at a depth of 50 cm (Hulupi & Mulyadi, 2007). According to Wiryadiputra & Atmawinata (1998), the decline in production by *P. coffeae* on Robusta coffee ranged from 28.7 to 78.4%. Until the age of five years, the plant mortality rate can reach 30% of the population of coffee plantations in certain farms (Nursol *et al.*, 2006). *P. coffeae* distribution in Indonesia is quite extensive, including all main coffee producing areas (Wiryadiputra & Atmawinata, 1998).

Nematode control has been carried out with technical culture (use of nematode free planting materials), nematicides, resistant varieties (CABI, 2015), coffee replanting after 5 years period of fallow (Trinh, 2011) and biological agent using endophytic bacteria (Vega *et al.*, 2005; Harni & Khaerati, 2013).

Endophyte is a micro organism that live inside plant tissues which provide a neutral, beneficial or detrimental effects (Sikora *et al.*, 2007). Use of endophytic bacteria as biological control has been studied in many plants, including coffee plants. Harni & Khaerati (2013) found that from 442 isolates, 12.3% were antagonistic and 14.2% can stimulate plant growth. In addition, 3 obtained isolates were potentially suppress population of *P. coffeae* with mortality about 94.8%. Vega *et al.* (2005) have isolated *Bacillus*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Escherichia*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas* from fruits, leaves and stems of coffee.

The previous research on the ability of endophytic bacteria as biocontrol agents as described above, was the result of exploration in form of single isolate. According to Baker & Cook (1974), microbes in nature, live in a niche or the same niche, as a group of endophytic bacteria in form of a consortium, to create biological buffer area which supports biological balance, resulting in stable relationship and mutual benefit and ecosystem balance. Munif *et al.* (2015) had isolated and tested the potential of consortium of endophytic bacteria, from a forest, plant as biocontrol agents and plant growth promoters of tomatoes. The exploration of endophytic bacterial consortium from coffee plant and their potential as growth promoton and biological agents has not been carried out. Therefore, the aim of this research was to explore and use of endophytic bacterial consortium to suppress nematode on coffee.

MATERIALS AND METHODS

Samples of plant roots infected by *P. coffeae* parasitic nematode, were obtained from a multiplication carried out in Indonesian Coffee and Cocoa Research Institute, Jember. Two months old coffee seedlings were inoculated with *P. coffeae* as many as 50 nematodes/plant which was later used as source of nematodes for inoculation.

P. coffeae nematodes were extracted from nematode infected roots with mist chamber method. Infected roots were cut about 1-2 cm and placed in a funnel covered with a filter cloth. Under the funnel a nematode reservoir container was placed connected with sprayer. The funnel filled with roots was put in a mist chamber equipped with a nozzle which produced water mist continuously. Harvesting performance of nematodes took 2-3 days after nebulization in a mist chamber. Harvesting was done by taking the solution in the container and filtered with a 500 mesh sieve. The nematodes were then counted as required.

Isolation of Endophytic Bacterial

Isolation of a consortium endophytic bacteria was conducted following the modified method of Hallmann *et al.* (1997). Samples of the coffee plants in form of roots, stems and leaves were washed with water and weighed as mach as one g fresh. Sterilization of tissue surface by soaking in 70% alcohol for 2 minutes, followed by immersion in NaOCl 3% + 3 drops of Tween 20 for 4 minutes, and rinsed with sterile distilled water three times. The samples were then crushed using a sterile mortar until fine size and diluted up to 10^{-3} . Suspension of 10^{-2} dilution was grown on TSA 20% and incubated for 24 hours at room temperature. As a comparison, or control, is conducted an etching at the roots, stems and leaves were

that has been surface sterilized (before the ground) on the TSA media 20%. If in the control there was no growth of bacteria then the bacterial colony was assumed as a result of endophytic isolation.

Harvesting bacterial endophytic consortium from each source (roots, stems and leaves) was done by pouring 2 mL of TSB medium 100% + glycerol 20% on a petri dish. The colonies were isolated from solid medium using a sterile ose needle. The suspension of consortium endophytic bacteria was then stored in 1.5 mL Eppendorf tube at 4°C.

Hypersensitivity and Hemolysis Tests

Hypersensitivity test of the endophytic bacterial consortium was conducted to determine its pathogenicity using a healthy tobacco plant. The endophytic bacterial consortium was propagated in TSB medium 100%, incubated in a shaker with the speed of 100 rpm for 24 hours. Suspension of bacterial endophytic consortium was injected using a syringe on tobacco leaves each 2 mL with three replicates for each endophytic bacteria, then incubated for 24 hours to 48 hours and observed the occurrence of chlorosis/necrosis on the leaves. The bacterial consortium which showed a negative reaction (no necrosis) was used for further testing.

Consortium of endophytic bacteria that showed a negative reaction to the hypersensitive, was tested for its ability to hydrolyze red blood cell. The 24 hours old culture of consortium bacteria was grown at Blood Agar media, then incubated for 24 hours and observed for its clear zone formation.

Plant Growth Promoting Test

Selection of endophytic bacterial consortium as a plant growth promotor was carried

out by modified method of Harni & Khaerati (2013). Hot water was applied on tomato seeds (as control). Tomato seeds were soaked in sterile distilled water at a temperature of about 55°C for 20 minutes, followed by immersion in a suspension of endophytic bacteria consortium for approximately one hour, then drained on sterile tissue paper. Further, the seeds were planted on moistened sterile filter paper and stored in a closed sterile tray. As a control, the tomato seeds were soaked with sterile water and planted on the same medium. Observations carried out 4 days after planting.

Nematodes Mortality Test

The ability of endophytic bacterial consortium in inhibiting *P. coffeae* was carried out by following the modified method of Harni & Khaerati (2013). Nematode test inoculum was prepared by following the procedure of nematode preparation for in vitro endophytic test. Endophytic bacterial consortium was propagated by growing a 100 mL of culture in 100 mL TSB medium and shaken with speed 100 rpm for 24 hours. Secondary metabolites produced by the endophytic consortium bacteria was obtained by centrifugating at a speed of 6500 rpm, the supernatant was filtered with milipore 0.25 mm diameter and pore size of 0.2 µm. The filtrate was used to test the ability of the endophytic bacterial consortium on nematodes mortality. Fifty parasitic nematodes were put in 5 mL of secondary metabolites placed in a petri dish, stored at room temperature and observed after 24 hours. Each treatment was repeated three times.

Chitinolytic activity

Chitinolytic test was conducted following the modified method of Marin *et al.* (2013). Consortium of bacterial isolates were multiplied

in trypsin soybean broth (TSB) media and shaken for 24 hours at a speed of 100 rpm. Sterile filter paper diameter of 0.5 cm was placed on a nutrient agar medium (NA) enriched by 0.4% colloidal chitin in a petridish. A total 4 μ L of culture was inoculated on the filter paper. Cultures were incubated at room temperature for 72–96 hours. Chitinolytic activity was measured based on diameter of clear zone that is formed.

Enzymes activities

Protease activity test was carried out by the modified procedure of Denizci *et al.* (2004). Liquid culture isolates were inoculated onto sterile paper blank disc that had been placed on the skim milk agar media (SMA), with a composition of 900 mL of 100% trypsin soy agar media (TSA) sterile plus 100 mL of sterile skim milk concentration of 10%. Incubation was done at room temperature for 24–72 hours. Proteolytic activity was shown by the formation of a clear zone surrounding the bacterial colonies (Baehaki & Budiman, 2011).

Lipolytic test used rhodamine-B media-agar with 20 mL of liquid medium Hass Bushnell, 0.6 mL (3%) of cooking oil and 0.04 mL rhodamine-B 0.1% in water. Further, the isolates were streaked onto medium and incubated at room temperature for 48 hours. Lipolytic activity was characterized by the appearance of reddish orange luminescence zone under UV light.

Phosphatase test was carried out following the modified method of Gupta *et al.* (2012). Consortium isolates were inoculated on sterile paper disc in selective media Pikovskaya and observed for 48 hours at 30°C. The ability of bacteria live in the phosphate solvent in media was characterized by the formation of a clear zone around the bacteria colony.

N₂ fixation and HCN production

N fixation ability was tested by growing isolates as much as 1 mL in semi-solid NFB (Nitrogen Fixing Bacteria) in 10 mL medium (Yim *et al.*, 2009). Incubation was performed 4–7 days until the color changes from greenish blue media, as well as emerging pellicle/ring bacteria in media.

Production of cyanide was tested following the modified procedures of Wei *et al.* (1991). Bacterial culture medium was 100% nutrient agar (NA) containing 4.4 g glysin. Cyanide production was detected using cyanide detection solution (CDS), which consisted of 200 mL of sterile distilled water containing 2 g of picric acid and 8 g of sodium carbonate. Filter paper was cut to a size of 1 cm x 1 cm and sterilized. Bacterial consortium isolates was streaked in quadrant on NA media enriched with glysin. On the petri dish lid, a sterile filter paper was placed and soaked in a solvent of CDS and dried. Observation was made on day 4 after inoculation. HCN production was characterized by color changing of the filter paper from bright yellow to red orange.

RESULTS AND DISCUSSION

Isolation of endophytic bacteria consortium from coffee plants obtained 27 isolates, where 9 isolates from leaves, 9 isolates from roots, and 9 isolates from stem. Isolates were selected to obtain potential ones as biological agents. One absolute requirement of a bacterium can be used as a biological agent is that the microbe is not a potential pathogen or pathogens to plants. Baker & Cook (1974) stated that antibiotics (secondary metabolites) produced by an antagonist agent may not cause damage to host. Hypersensitivity test is conducted to collect isolates that are non pathogenic. As a result, from 27 isolates,

23 non pathogenic isolates were obtained (Table 1, Figure 1).

Further, a microbe can be grouped as an antagonist agent if it is not pathogenic to human, therefore hemolysis test was conducted. Hemolysis is a destruction process of human erythrocytes (Segel, 2011). Hemolysin enzyme may cause lysis of erythrocytes, that are produced by several pathogenic bacteria, which is gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia* spp., *Proteus* spp. and gram-positive such as *Streptococcus* spp., *Staphylococcus aureus*, *Listeria* spp. (Bauman, 2007). The results showed that there were 9 isolates that are gamma hemolysis, namely K6, K7, K8, K10, K12, K15, K19, K20, and 21 (Table 1, Figure 2)

Nine selected isolates were tested for their ability on the mortality of *P. coffeae* in vitro. The high mortality percentages were shown by K6, K15 and K8 isolates in the range of 43–66% (Table 3). These results are in line with Hami & Khaerati (2013) that 12.3% single endophytic bacteria isolates tested was able to kill 5–80% *P. coffeae*.

The best shoot growth of tomato was shown by K21, K15 and K8 isolates followed, while for root length was shown by K21 followed with K8 and K10 isolates. The

isolates were also significantly different with controls (Table 3). According to Sutariati *et al.* (2006), seed treatment with biological agents can improve seed germination and seedling growth of chili.

The ability of endophytic bacteria to increase the mortality of nematodes was higher than the ability of increasing the tomato shoot growth. Mechanism of endophytic bacteria in suppressing nematodes and plant growth can increase through antibiosis and induced resistance to promote plant growth. This is shown by the ability of the consortium to produce proteolytic, chitinolytic, and lipolytic enzymes, HCN production, dissolving phosphate and fixing N (Table 4 & Figure 3).

Jalgaonwala & Mahajan (2011) mentioned that almost all of endophytic microorganism are able to perform enzymatic activity. The enzymatic activity of all tested isolates were proteolytic amylolytic, cellulolytic, esterolitik, lipolytic and asparaginase, further, that endophytic bacteria had enzymatic activity larger than the endophytic fungi.

The results showed that 100% of isolates could hydrolyze proteins (proteolysis), fat (lipolytic), and produce HCN. Chitinolytic activity was show by 78% isolates meanwhile 67% isolates could fix N₂ and 11.1% of isolates could dissolve phosphates. Three

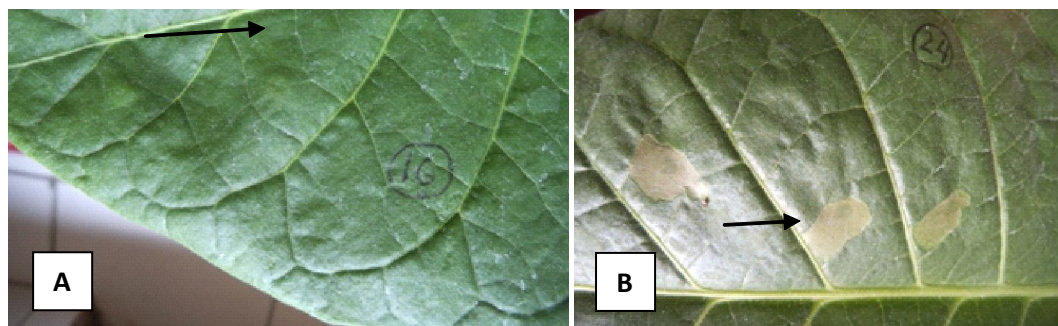


Figure 1. Hypersensitive reaction; negative/nonpathogen (A) and positive/pathogen (B)

Table 1. The origin of isolates consortium endophytic bacteria, hipersensitiv reaction/HR and hemolysis test

Location	Clone	Number of sample	Isolate code	Tissue	HR	Hemolysis
West Java	BP 939	1	K1	Root	+	+
			K2	Shoot	+	+
			K3	Leaves	+	+
		2	K4	Root	+	+
			K5	Shoot	+	+
			K6	Leaves	-	-
		3	K7	Root	-	-
			K8	Shoot	-	-
			K9	Leaves	+	+
East Java	BP 939	1	K10	Root	-	-
			K11	Shoot	+	+
			K12	Leaves	-	-
		2	K13	Root	+	+
			K14	Shoot	+	+
			K15	Leaves	-	-
		3	K16	Root	+	+
			K17	Shoot	+	+
			K18	Leaves	+	+
West Java	BP 308	1	K19	Root	-	-
			K20	Shoot	-	-
			K21	Leaves	-	-
		2	K22	Root	+	+
			K23	Shoot	+	+
			K24	Leaves	+	+
		3	K25	Root	+	+
			K26	Shoot	+	+
			K27	Leaves	+	+
Control	+	+	+	+		

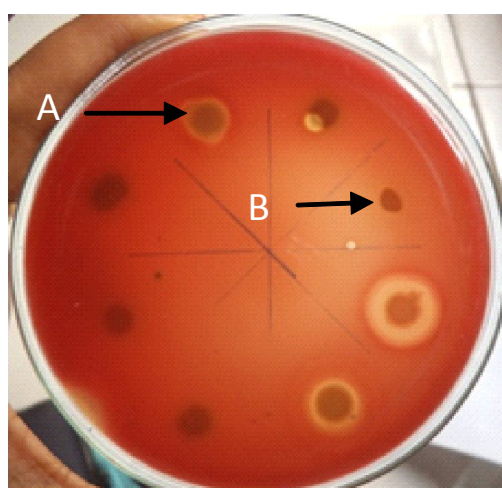


Figure 2. Positive hemolysis (A); Negative hemolysis (B)

Table 2. Effect of endophytic bacteria consortium on shoot and root growth of tomatos

Isolate	Growth	
	Shoot , cm	Root, cm
K6	2,20 ^b	1,58 ^d
K7	3,10 ^{ab}	2,39 ^{dc}
K8	3,40 ^a	4,55 ^{ab}
K10	3,20 ^{ab}	4,36 ^{ab}
K12	2,70 ^{ab}	3,88 ^{abc}
K15	3,60 ^a	372 ^{abc}
K19	2,30 ^b	3,01 ^{bd}
K20	2,90 ^{ab}	3,59 ^{abc}
K21	3,60 ^a	5,13 ^a
Media/medium	-	-
Kontrol/control	2,70 ^{ab}	4,6 ^{ab}

Note: Means in the same column followed by the same letter are not significantly different according to Duncan Test with $\alpha = 5\%$.

Table 3. Effect of endophytic bacteria consortium to mortality of *P. coffeae*

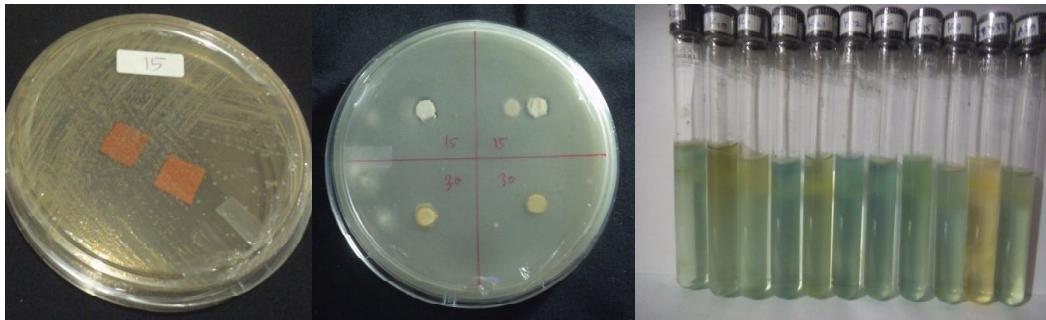
Isolate	Σ live nematode*	Σ killed nematode*	Total*	Mortality, % *
K6	30,33	16,67	47,00	65,80 a
K7	9,00	44,00	53,00	16,67 bdc
K8	21,67	28,33	50,00	43,13 ab
K10	10,67	39,33	50,00	21,21 abc
K12	15,00	32,67	47,67	33,04 abc
K15	22,67	29,67	52,33	43,28 ab
K19	19,33	28,67	48,00	40,26 ab
K20	21,00	28,33	49,33	42,58 ab
K21	16,67	25,67	42,33	37,64 ab
Negative control	0,00	55,33	55,33	0,00 d
Medium	2,67	47,67	50,33	5,23 dc

Note: K: consortium, ^{*)} the value is average from 3 times of treatments; Means in the same column followed by with the same letter are not significantly different according to Duncan Test with $\alpha = 5\%$.

Table 4. Antibiosis mechanism and induce resistance test

Isolate	Proteolytic (mm)	Chitinolytic (mm)	Lipolytic	HCN	P. solubilization (mm)	N ₂ -Fixing
K6	1.05 ^b	3.25 ^c	+	+	0.00 ^b	+
K7	1.00 ^b	2.80 ^c	+	+	0.00 ^b	-
K8	1.93 ^b	2.55 ^c	+	+	0.00 ^b	+
K10	4.50 ^{ab}	1.73	+	+	0.00 ^b	+
K12	5.13 ^b	5.13 ^a	+	+	0.00 ^b	+
K15	2.60 ^b	2.66 ^c	+	+	7.58 ^a	+
K19	7.00 ^a	0.00	+	+	0.00 ^b	-
K20	2.30 ^b	4.00 ^b	+	+	0.00 ^b	+
K21	3.00 ^b	0.00	+	+	0.00 ^b	-

Note: Means in the same column followed by the same letter are not significantly different according to Duncan Test with $\alpha = 5\%$.



Gambar 3. Mechanism test of endophytic bacteria consortium controlling *P. coffeae* nematode, A: positive chitinolysis, B: positive proteolysis, C: positive lipolysis/orange colour of colony under UV light, D: positive produce HCN, E: positively dissolution of phosphate/holozone formed around the colony, F: positively fixation of nitrogen/change of medium colour from green to blue.

isolates (K6, K8, and K15) that showed the high mortality showed a positive reaction to the antibiosis mechanism test, meanwhile K6 and K8 isolates reacted negatively to the P dissolution test (Table 4). This study showed that an endophytic bacteria can control plant pathogens via several mechanisms, including antibiosis and promoting plant growth. Jain *et al.* (2012) mentioned that a consortium of microbes in the rhizosphere can mediate induced systemic resistance (ISR). Chitinolytic and proteolytic bacteria has been widely reported to inhibit the development of pathogenic *Bacillus* spp. (Bressan & Figueiredo, 2010), *Streptomyces tendae* (Abdulkhair, 2012), *Bacillus cereus* QQ308 (Chang *et al.*, 2006). Chitinolytic and proteolytic activity of endophytic bacteria also play a role in antibiosis against nematodes *P. brachyurus* (Harni *et al.*, 2007). Carrim *et al.* (2006) reported that the endophytic bacteria isolated from *Jacaranda decurrens* Cham showed proteolytic (60%) and lipolytic (60%) activities. Proteolytic activity by endophytic bacteria can stimulate immune system of plants.

Bertham & Nusantara (2011) stated that interactions with P inorganic cations (Fe, Al, and Ca) and immobilization into an organic compound by microorganisms cause low availability of P in the soil. Applications of

Pseudomonas synxantha, *Burkholderia gladioli*, *Enterobacter hormaechei* and *Serratia marcescens* bacteria that are phosphate solubilizers can increase the availability of phosphate in soil, the absorption of phosphate in plants and plant growth and Aloe production up to 673% (Gupta *et al.*, 2012). Bacteria of the genus *Azotobacter*, *Bacillus*, *Micrococcus*, *Pseudomonas*, *Staphylococcus*, *Acetobacter*, *Escherichia*, *Flavobacterium*, and *Paracoccus* are main phosphate solubilizers (Marista *et al.*, 2013; Trivedi *et al.*, 2007). Nitrogen is a macronutrient that plants need in large amount, so that N_2 fixation by endophytic bacteria provide more benefits because of endophytic bacteria located in the plant tissue, so the results of N_2 fixation are not easily separated. Adriano-Anaya *et al.* (2011) reported that the application of free-living N_2 -fixing bacteria can suppress nematode populations and *Radopholus similis* attack symptoms.

All consortium isolates test could produce HCN in glycine media. Heydaril *et al.* (2011) found that 37% of the tested fluorescent *Pseudomonas* bacteria were capable of producing hydrogen cyanide gas. *Pseudomonas* bacteria normally can produce secondary metabolites such as cyanide which attack *P. coffeae* through respiratory disorder.

Gallagher & Manoil (2001) found that secondary metabolites such as hydrogen cyanide was a major toxin produced by *P. aeruginosa* and kill *Caenorhabditis elegans*, a cosmopolitan soil nematode.

The consortium endophytic bacteria showed high ability in phosphate dissolution, promoting growth and N₂ fixation (Gupta *et al.*, 2012; Suriaman, 2010). It is thought to be caused by a type of bacteria that are more variable in the consortium than single isolate, allowing colonization was faster in rhizosphere root and dominated the root. Sarma *et al.* (2015) in his review mentioned that biocontrol mechanisms are diverse from each bacteria in a consortium also can increase the suppression of plant diseases.

CONCLUSION

Consortium of endophytic bacteria can control *P. coffeae* nematodes through antibiosis mechanism of proteolytic, lipolytic, HCN production and chitinolytic, and the mechanism of induction of resistance by promoting growth through N fixation and phosphate dissolution. Consortium of endophytic bacteria is more profitable than a single isolate because of its kind that is more varied, so the ability to colonization and dominance rhizosphere and root is more quickly, as well as the mechanism of action of biocontrol agents are more varied can improve the suppression of disease.

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