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BIOCONTROL POTENTIAL OF ENDOPHYTIC BACTERIA ORIGINATED FROM ROOTS OF *RAVENALA MADAGASCARIENSIS* AGAINST *FUSARIUM OXYSPORUM* AND BANANA-PARASITIC NEMATODES

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A B S T R A C T

Ravenala madascascariensis has good resistance to nematode infection. This is thought to be related to endophytic bacteria associated with the plant. This study aimed to isolate endophytic bacteria from the *R. madascascariensis* roots and test its ability as a biocontrol agent against *Fusarium oxysporum* and banana-parasitic nematodes. Endophytic bacteria were isolated using NA and TSA media. Potentially safe bacteria for plants and mammals are tested to inhibit the growth of *F. oxysporum* in vitro. Isolates that can inhibit *F. oxysporum* were tested for their effectiveness in suppressing banana-parasitic nematode populations in vitro. Furthermore, the selected isolates were tested for their ability to increase the growth of Cavendish banana in the greenhouse. The results showed 113 isolates of endophytic bacteria were successfully isolated; 37 of them were safe for plants and mammals. There were five isolates that could inhibit the growth of *F. oxysporum* with inhibition percentage of 47.62% up to 66.55%. The five isolates can also suppress banana-parasitic nematode populations in the treatment periods of 2 h, 6 h, and 12 h. The highest mortality was performed by NAP05 isolate at 12 h treatment duration, with a mortality value of 63.96%. The isolates obtained were known to be able to produce several compounds such as protease enzymes (5 isolates) and chitinase enzymes (2 isolates), also capable of dissolving phosphate (3 isolates). The greenhouse experiment showed that several bacterial isolates were effective in increasing the diameter of banana stems (NAP21 isolate) and fresh roots weight (TAP18, TAP34, and NAP21 isolates). All isolates were reported to be able to suppress nematode populations in the roots of banana.

Keywords: antagonist, lytic enzymes, mortality, phosphate-dissolve, plant growth promoter

INTRODUCTION

Banana (*Musa* sp.) is one of the leading fruit commodities in Indonesia. Banana is planted in almost all regions in Indonesia, both on a small and large scale. The production of banana reported having better performance compared to other fruit commodities in Indonesia. In 2017, banana production in Indonesia was reported to reach 7 million tons, recording a decrease of 4% compared to the previous year (FAOSTAT, 2019).

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Banana cultivation faces several challenges, such as the presence of plant-parasitic nematodes. Gowen *et al.* (2005) reported that plant-parasitic nematodes infection could reduce the production up to 30-80% on the banana. Nematode infection can disrupt the distribution of water and mineral to all parts of the plant leading to disruption of plant growth. At a severe level, nematode infection in banana can cause high yield loss, even plant death. Some important plant-parasitic nematodes on the banana plant are *Radopholus similis, Pratylenchus* sp., *Meloidogyne* sp. and *Helicotylenchus multicinctus* (Swain and Nayak, 2018).

Yield losses due to nematode infections have been reported in various countries. O'Bannon (1976) reported that *Radopholus similis* infection in banana

plants could cause yield losses of up to 12.5 tons per hectare. The banana plant is one of the tropical plants which has a high potential of being infected with nematodes. The banana root structure that spreads and is soft makes it easy for the nematodes to penetrate the roots (Famina *et al*., 2017). Nematode infection is reported to be a predisposing factor for other pathogens (Gowda *et al*., 2019). Wounds caused by nematode infections make plants susceptible to disease by bacteria or pathogenic fungi. Infected root nematodes generally are watery because of the outbreak of the cell that causes the cell fluid out (Fosu-Nyarko and Jones, 2016). One pathogen that is widely reported to be associated with nematodes is the *Fusarium* spp. (Almeida *et al*., 2018).

Fusarium wilt disease commonly found in banana fields. *Fusarium* infection is characterized by yellowing of banana leaves from the lower leaves to the upper leaves (Mostert *et al*., 2017). The leaves of banana plants infected with *Fusarium* generally turn yellow, and eventually, they will dry up and turn brown. Yield losses due to *Fusarium* infection in banana plants vary depending on banana species and environmental conditions (Dita *et al*., 2018). However, *Fusarium* infections generally cause yield losses of between 30% to 70%. In fact, at the severe level of diseases and accompanied by nematode infections, yield losses cause crop failure (Sun *et al*., 2018).

Research on the conventional and modern nematode control has been conducted, but nematode infection is still a severe problem that has not been adequately resolved. Plant-parasitic nematodes infection in banana can be overcome in several ways; one of the efforts to control their infection is the application of endophytic bacteria. Endophytic bacteria are live inside the plant tissue without causing disease symptoms (Liu *et al*., 2017). The use of endophytic bacteria to control plant pathogens has been widely reported. Endophytic bacteria can promote plant growth, optimize yield, suppress plant pathogenic microbes inoculum, dissolve phosphates, and fix free nitrogen (Reinhold-Hurek and Hurek, 2011; Mardhiana *et al*., 2017).

Endophytic bacteria can be isolated from various types of plants, including *Ravenala madagascariensis*. The *R. madagascariensis* is originated from Africa and is grows wildly without humans intervention (Van Wyk, 2015). In general, wild plants have better disease resistance compared to cultivated plants (Boyd *et al*., 2013). The nematodes infection in *R. madagascariensis* in Indonesia has not been reported. This is presumably due to the presence of endophytic bacteria that associate and promote the *R. madascascariensis* to be more resistant to nematode infection (Hoang *et al*., 2019). Therefore, there are great opportunities to explore the endophytic bacteria from the *R. madascascariensis* and observe their biocontrol potential. The effectiveness of the endophytic bacteria from the *R. madascascariensis* as biocontrol agents has not been widely reported. This study was aimed to isolate endophytic bacteria originated from the *R. madascascariensis* roots and to characterize its physiological properties, in vitro nematicidal activity, antagonist activity against *Fusarium oxysporum*, in vivo potential to control plant-parasitic nematodes and to promote the growth of Cavendish banana.

MATERIALS AND METHODS

Isolation of Endophytic Bacteria: Endophytic bacteria were isolated from the roots of the *R. madascascariensis* around IPB University. Isolation of endophytic bacteria was conducted following the modified method described by Munif *et al.* (2012). The *R. madascascariensis* roots were cleaned from the soil particles and were taken as much as 2 g. The roots then sterilized using 1% sodium hypochlorite (NaOCl) solution for 1 minute, 70% ethanol for 1 minute and rinsed with sterile water three times. Sterilized plant roots were placed on 20% Tryptic Soy Agar (TSA) and 20% Nutrient Agar (NA) media to determine the success of surface sterilization. The roots that have passed the surface sterilization process were then macerated by using a sterile mortar with the addition of 1:10 (w/v) sterile distilled water. The obtained suspension then diluted 10-fold using sterile distilled water up to 10^{-4} dilution concentration. A total of 0.1 ml of suspension from each dilution was then flattened on 20% TSA and 20% NA media. The media were then incubated at room temperature for 48 hours. Single colonies with different colony characters were further sub-cultured in respective media (100% TSA and 100% NA), followed by the incubation for 24-48 hours at room temperature.

Hypersensitive Reaction Test: The endophytic bacteria were grown on 100% Tryptic Soya Broth (TSB) media and incubated for 24 hours. A total of 1 ml of endophytic bacterial suspension was infiltrated using a sterile syringe on the leaf blade of the 2-month-old Kemloko variety tobacco. The occurrence of necrosis on leaves of tobacco was carried out 24 hours after injection.

Bacteria that did not show hypersensitive reaction (necrosis) on leaves of tobacco were used for further testing (Klement and Goodman, 1967).

Hemolytic Activity Test: The testing of hemolytic activity was done by grown the bacteria on the blood agar media and then incubated for 48 hours. There are three types of hemolysis in blood agar media, namely α-hemolysis, β-hemolysis, and αβ-hemolysis. Partial or α-hemolysis causes the formation of a greenish or brownish zone around the bacteria colony, complete hemolysis or β-hemolysis causes a clear area around the bacterial colony, and αβ-hemolysis produces clear and a bit dark zone around the colony (Sharma and Gupta, 2014). Bacteria with hemolytic activity were not used in further tests.

In Vitro Test Against *Fusarium oxysporum:* This test was conducted in vitro by a double culture test method. The *F. oxysporum* used was isolated from infected banana plants and obtained from the Laboratory of Plant Mycology, Department of Plant Protection, IPB University, Indonesia. Both *F. oxysporum* and endophytic bacterial isolates were cultured on Potato Dextrose Agar (PDA) media. The endophytic bacteria were grown in the centre of the petri dish, and then the *F. oxysporum* was cultured on a quarter part of the petri dish. Each treatment was repeated three times and incubated for seven days. The relative inhibition rate of endophytic bacteria against *F. oxysporum* was calculated using the percentage inhibition formula (Munif *et al*., 2012).

$$\boldsymbol{H = \frac{R1 - R2}{R1} \times 100\%}$$

which were H = inhibition percentage (%); R1 = radius of the *F. oxysporum* without the presence of bacteria (cm); R2 = radius of the *F. oxysporum* with the tested bacteria (cm).

Endophytic bacteria that were able to inhibit the growth of *F. oxysporum* in vitro were then used in further testing, while bacteria that were not able to inhibit the growth of *F. oxysporum* in vitro were not used in further testing.

In Vitro Test Against Nematodes: This test was conducted to determine the direct effect of endophytic bacteria on plant-parasitic nematodes. The bacteria used in this test were bacteria that had the ability to inhibit *F. oxysporum* in the in vitro test against *F. oxysporum* in this study. Endophytic bacteria were grown on TSA media and then incubated for 48 hours. Endophytic bacteria then suspended by adding 5 ml of sterile water to the petri dish. As much as 1 ml (10^8 CFU ml^{-1}) of endophytic bacteria suspension was inserted into the syracuse dish, then 4 ml of suspension containing 50 banana-parasitic nematodes (20 *R. similis*, 10 *H. multicinctus*, and *10 Hoplolaimus* sp.) were added into the same syracuse dish. The number of dead nematodes was observed every 2 hours, 6 hours, and 12 hours. This study followed a completely randomized design and was repeated three times (Soliman *et al*., 2019). Data were analyzed using analysis of variance, then proceed with Duncan Multiple Range Test (DMRT) analysis with a confidence level of 95%.

Characterization of Physiological Properties of Endophytic Bacteria: *Gram Staining*

One drop of 3% potassium hydroxide (KOH) solution was placed on the preparation glass, then one lup of endophytic bacteria were mixed in the solution. Gram-negative is indicated by the presence of mucus from bacterial colonies, which can be raised as high as 3-5 cm during the testing process (Dash and Payyappilli, 2016).

Proteolytic Activity Test

The test was done using Skim Milk Agar (SMA) media at a pH of 6.5. The media was made by mixing 100 ml of skim milk with 100 ml of 200% TSA media. The bacteria were grown on the media and incubated for 24 hours at room temperature. Proteolytic activity was indicated by a clear zone around the colony (Ahmad *et al*., 2014).

Chitinolytic Activity Test

The test was done using 1% chitin media at a pH of 6.2. The bacteria were grown on the media then incubated for 24 hours. The chitinolytic activity was indicated by a clear zone around the colony (Kuddus and Ahmad, 2013).

Phosphate Dissolving Activity Test

The test was done using Pikovskaya's media. The bacteria were grown on the media and incubated at room temperature for 24 hours. Bacterial ability in dissolving phosphate was indicated by a clear area around the colony (Baliah *et al*., 2016).

HCN Production Test

One loop of bacterial isolates was evenly scratched on 100% TSA media. Then a sterile filter paper that has been previously immersed in a Cyanide Detection Solution (CDS) placed on the lower surface of the lid of the petri dish. The observation was done seven days after the bacteria were scratched on the petri dish. HCN production was indicated by a color change in the filter paper from yellow to orange (Reetha *et al*., 2014).

Effect of Endophytic Bacteria on Banana-Parasitic Nematodes in Greenhouse: The test plants used were the Cavendish cultivar, which obtained through tissue culture techniques at Southeast Asian Regional Centre for Tropical Biology (SEAMEO BIOTROP), Indonesia. The application of endophytic bacteria has followed the method described by Ho *et al*. (2014) that has been modified. The endophytic bacteria used in this test were the same with the isolates used in the in vitro test against nematodes. Banana seedlings were soaked for 6 hours in 100 ml of endophytic bacteria suspension. Banana seedlings then were planted in polybags that contained sterile soil, compost, and husk (1: 1: 1) media. A total of 100 ml of endophytic bacteria suspension were drenched around the roots of banana plants one month after initial treatment. The application of nematodes was conducted two days after the endophytic bacteria application. A total of ±500 mixed J2 banana-parasitic nematodes (±200 *R. similis,* ±100 *H. multicinctus,* and ±100 *Hoplolaimus* sp.) were inoculated around the banana root. Each treatment was repeated four times and arranged in a completely randomized design. Five weeks after the application of nematodes, plants were observed for its plant height, stem diameter, plant fresh weight, root fresh weight, and the population of nematodes per 1 g of the banana root. This study followed a randomized block design and was repeated three times. Data were analyzed using analysis of variance, then proceed with Duncan Multiple Range Test (DMRT) analysis with a confidence level of 95%.

RESULTS AND DISCUSSION

Endophytic Bacterial Isolates: A total of 113 endophytic bacterial isolates were isolated from the roots of the *R. madascascariensis*. Sixty-one isolates were isolated from TSA media, and fifty-two isolates were isolated from the NA media.

Hypersensitive Reaction: The test was conducted to ensure that endophytic bacteria isolated had no potential as a plant pathogen. As biocontrol agents, endophytic bacteria must be safe for plants and not secreting biological compounds that cause physiological disorders in plants. A total of 93 bacteria showed negative reactions or were not pathogenic to plants, and 20 bacteria showed positive reactions. A positive reaction was characterized by the appearance of necrosis in tobacco leaves.

Hemolysis Activity: Bacteria that did not show a positive reaction in the hypersensitive test were then tested for their hemolysis activity. Hemolysis test was performed to ensure the endophytic bacteria isolated are safe to mammals. A total of 37 bacterial isolates did not show hemolysis activities, which indicated that the bacteria were safe for mammals. Furthermore, there were 56 bacteria that showed hemolysis activity. Hemolysis activities are demonstrated by the appearance of clear zones around the colonies (β-hemolysis) and greenish zone (α-hemolysis) around the colonies.

Based on the results of the biosafety test, of the 113 isolates, there were 37 endophytic bacterial isolates that could be used in the antagonistic activity test. The 37 isolates were chosen because they did not cause hypersensitivity reaction to tobacco leaves and did not produce hemolysis toxins.

In-vitro antagonistic activity of endophytic bacteria against *F. oxysporum* and plant-parasitic nematodes

The results showed that five isolates were able to inhibit the growth of *F. oxysporum* in vitro. The five isolates are TAP18, TAP34, NAP05, NAP19, and NAP21. The bacteria that have the highest inhibition capability against *F. oxysporum* were NAP05 with a relative inhibition of 66.55%, followed by TAP18 (59.52%), TAP34 (55.95%), NAP19 (50%), and NAP21 (47.62%). Five endophytic bacteria that effectively inhibit the growth of *F. oxysprum* were tested for their antagonistic activity against banana-parasitic nematodes. The results showed that the mortality of nematodes in each treatment of endophytic bacteria was significantly different from the control in different observation times (2-hours, 6-hours, and 12-hours) (Table 1). The mortality of nematodes increased with time. At the end of the observation (12 h), NAP05 isolates showed the best performance, as indicated by the percentage of nematode mortality of 63.96%, the highest compared to other isolates. Furthermore, TAP34 isolates showed mortality up to 56.44%, followed by NAP21 (45.85%), NAP19 (43.32%), and TAP18 (40.74%). All isolates showed significant differences compared to control (8.94%).

The results showed that the nematodes (*R. similis*, *H. multicinctus*, and *Hoplolaimus* sp.) began to die 2 hours after bacterial treatment. This is evidenced by observations of nematodes by taking them using a fine needle. Nematodes are placed in 20 ml of sterile water for 30 minutes, then categorized as dead nematodes if they show no movement at all within 30 minutes of observation. After 12 hours, the number of dead

nematodes increased and started to form various inactive positions. Nematodes dead positions are known to vary, depending on the genus. The *R. similis* is formed a straight or curved position in the ventral part when dead. Furthermore, nematodes of the genus *Helicotylenchus* formed a spiral (G) or C-shaped when dead. Microscopic observations show that nematodes die because of the degradation of the cuticle. This can be seen from the breakdown of the cuticle in nematodes treated with endophytic bacteria (Fig.ure 1).

Table 1. Effect of endophytic bacteria on mortality of plant-parasitic nematodes of banana in vitro

Isolate	Mortality (%)		
	2 h*	6 h*	12 h*
Control	5.08e	7.34d	8.94d
TAP18	10.40de	32.27c	40.74c
TAP34	33.93b	45.93b	56.44ab
NAP05	48.93a	58.69a	63.96a
NAP19	20.12c	37.01bc	43.31c
NAP21	15.69cd	33.91c	45.85bc

*Values followed different superscript letters are significant at P ≤ 0.05

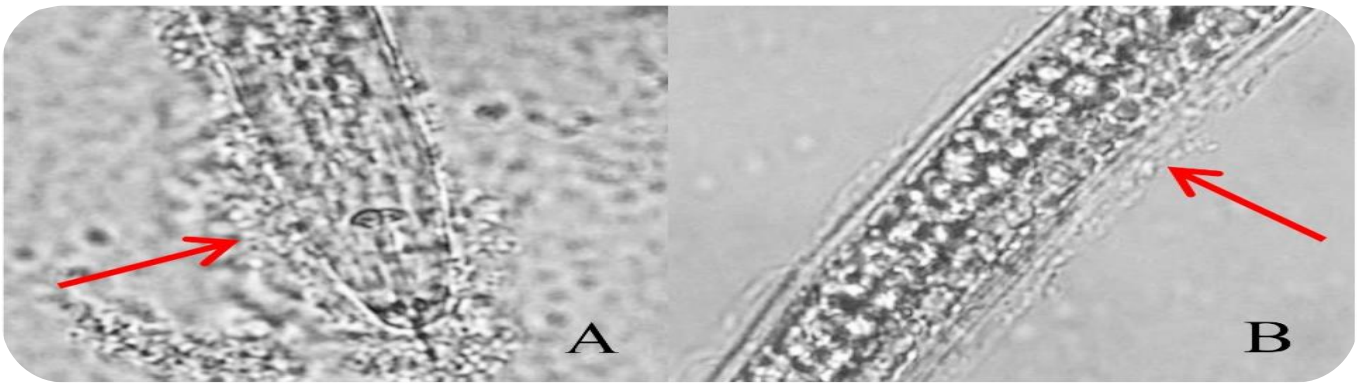

Figure 1. Cuticle degradation in nematodes treated with endophytic bacteria

Physiology Character: The test results showed that all five potentially endophytic bacteria tested were Gram-negative, two bacterial isolates (NAP05 and NAP21) were able to produce chitinase enzymes, three bacterial isolates (TAP18, TAP34, and TAP19) were able to dissolve phosphate, and all bacteria tested were able to produce protease enzymes. However, no isolates of endophytic bacteria were able to produce cyanide acid (HCN) (Table 2).

Table 2. Physiological characteristics of endophytic bacteria originated from roots of the *R. madascascariensis*

Isolate	Physiological Characters				
	Gram	Chitinolytic	Phospate Dissolve	Proteolytic	HCN
TAP18	Negative	-	+	+	-
TAP34	Negative	-	+	+	-
NAP05	Negative	+	-	+	-
NAP19	Negative	-	+	+	-
NAP21	Negative	+	-	+	-

Note: (+) endophytic bacterial isolates had physiological activities tested, (−) endophytic bacterial isolates had no physiological activities tested

Antagonistic Activity of Endophytic Bacteria Against Banana-Parasitic Nematodes in Greenhouse

Banana plants treated with endophytic bacteria had better growth performance (Figure 2). The application of endophytic bacteria can increase plant height, stem diameter, plant fresh weight, and root fresh weight. The NAP19 isolates showed the best performance in increasing plant height, then NAP21 isolates showed the best performance in increasing stem diameter, plant fresh weight, and roots fresh weight (Fig. 3). In this study, the application of endophytic bacteria was able to increase stem diameter by 4.83% to 7.25% compared to control plants. Furthermore, endophytic bacteria were also able to increase root fresh weight by 32.28% to 70.57% higher than control plants.

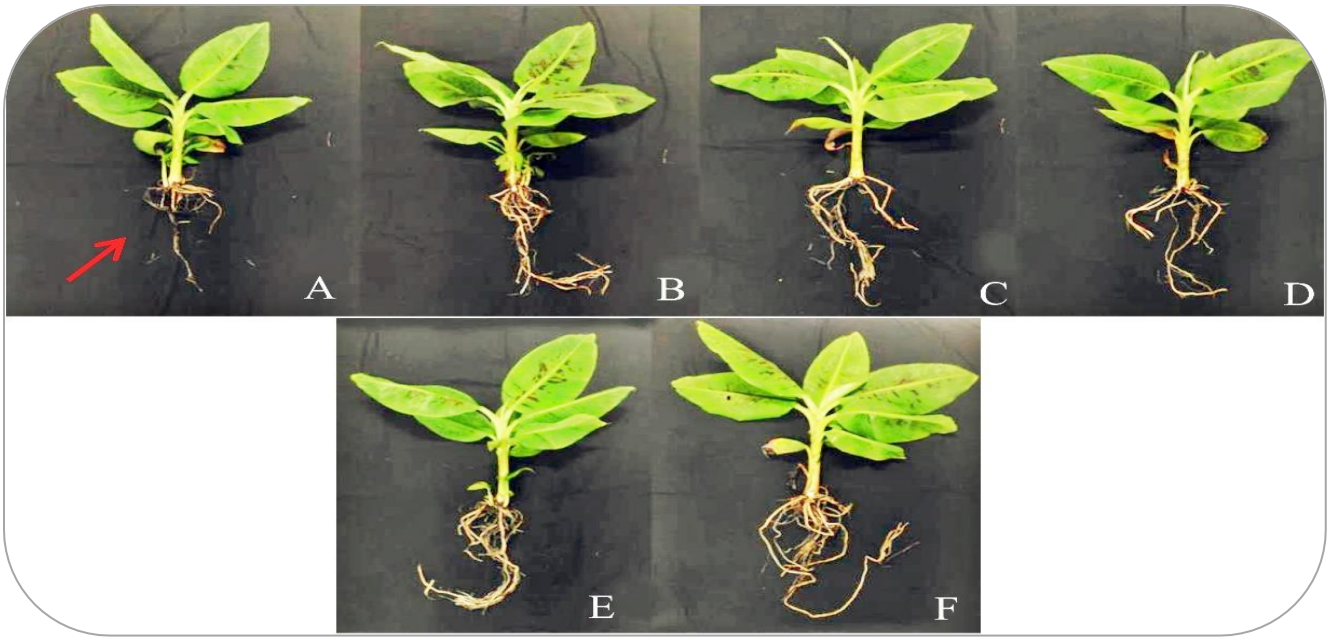

Figure 2. Effect of endophytic bacteria on the growth of banana plants infected with nematodes: (A) control, (B) TAP18, (C) TAP34, (D) NAP05, (E) NAP19, (F) NAP21

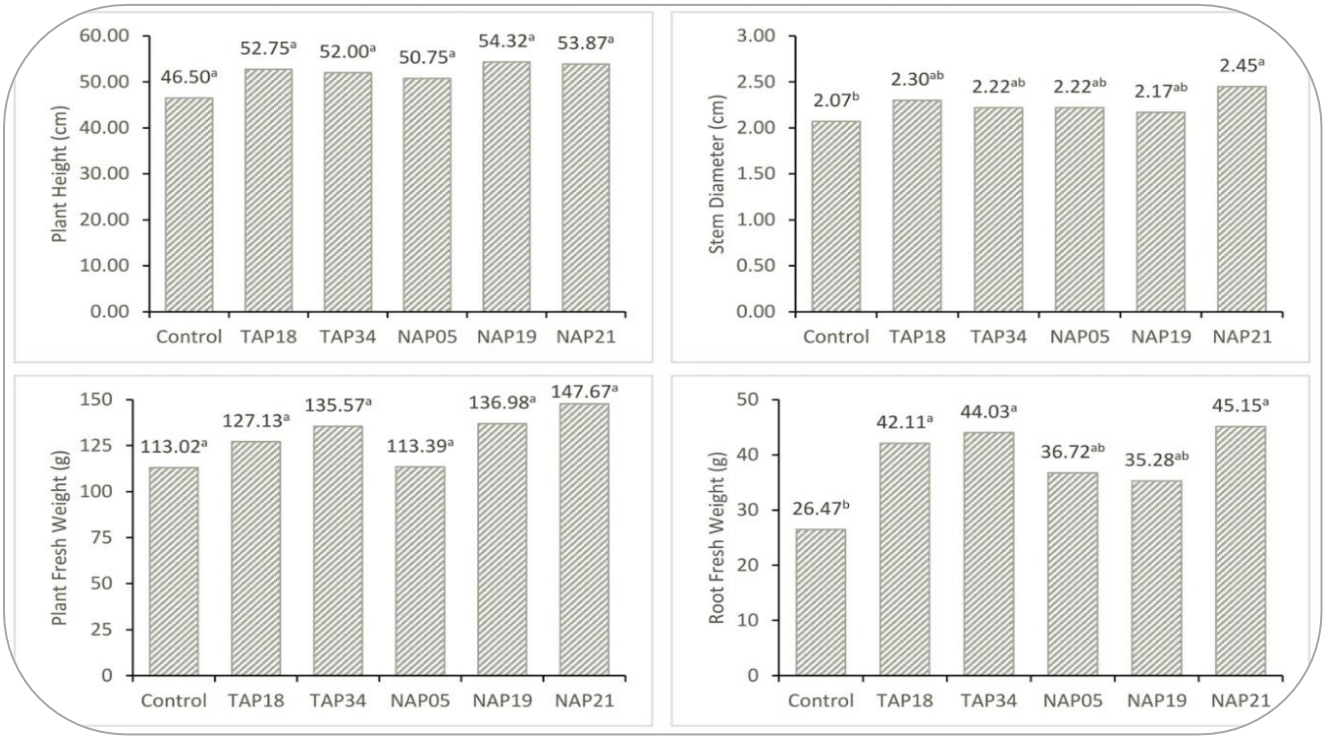

Figure 3. Effect of endophytic bacteria on plant height, stem diameter, plant fresh weight, and roots fresh weight of banana plants infected with nematodes. Note: Values followed different superscript letters are significant at $P \leq 0.05$

Furthermore, banana plants treated with endophytic bacteria showed better levels of resistance against nematode infections. In 1 g of banana plant roots that were not treated with endophytic bacteria, an average of 10.33 nematodes was found. Moreover, the banana plant roots treated with bacteria contained an average of 1.2 nematodes (TAP34), 1.32 nematodes (NAP05), 1.41 nematodes (NAP21), 2.05 nematodes (TAP18), and 4.28 nematodes (NAP19). Furthermore, the number of nematodes in the roots of banana in each treatment is in Table 3.

Table 3. Effect of endophytic bacteria on nematode populations in the roots of banana

Isolate	Nematode Population per 1 g of Root
Control	10.33a
TAP18	2.05b
TAP34	1.20b
NAP05	1.32b
NAP19	4.28b
NAP21	1.41b

*Values followed different superscript letters are significant at P ≤ 0.05

DISCUSSION

Endophytic bacteria are reported can be isolated from the leaves, stems, and roots of various plants. In a previous study, Mardhiana *et al*. (2017) reported successfully isolating endophytic bacteria from the roots of *Cyperus rotundus,* which has the potential as a biocontrol agent against *Meloidogyne incognita* on tomato plants. In another study, Chaves *et al.* (2009) were also successful in using a combination of endophytic bacteria and fungi to control *R. similis* in greenhouse.

As biocontrol agents, endophytic bacteria must be safe for other organisms. The safety of endophytic bacteria on plants can be determined by the hypersensitive reaction test. Hypersensitive reactions are the response of plants to the presence of pathogens. Plants will localize the pathogens in their tissues through a very rapid mechanism of cell death. In addition to localizing the presence of pathogens, cell death also aims to stop water and nutrients that pathogens can use (Wang and Bayles, 2013). Furthermore, to ensure the safety of mammals can be done through a test of hemolysis activity. Hemolysin toxins are reported to cause necrosis in animal and human skin. Furthermore, hemolysin toxins can also cause lysis of animal and human red blood cells (Otto, 2014). The explanation above reinforces the results of this study, which eliminates the isolates that causing a hypersensitive reaction and can produce hemolysin toxins.

Endophytic bacteria have a direct and indirect mechanism in suppressing pathogen populations. Directly, endophytic bacteria can produce secondary metabolites that are toxic to pathogens. Secondary metabolites that play a role in suppressing nematode populations include protease enzymes and chitinase enzymes (Wiratno *et al*., 2019). Both of these enzymes are reported to be able to lyse the nematode epidermis. Previous research by Safni *et al*. (2018) reported *Serattia* sp, *Bacillus* sp., and *Pseudomonas* sp., which are capable of producing protease enzymes and chitinase could effectively suppress nematodes populations by lysing the epidermis of the nematodes. Furthermore, the same study also reported proteolytic and chitinolytic bacteria were able to destroy the nematode stylet. Indirectly, endophytic bacteria can induce plant resistance to pathogen infections through the Induced Systemic Resistance (ISR) mechanism (Mushtaq *et al*., 2017). ISR is a phenomenon of increasing plant resistance that is associated with increasing defense compounds such as Pathogen-Related Protein (PR-Protein) in plants (Chandrasekaran and Chun, 2016).

In a separate study, Vu *et al.* (2006) reported that endophytic microbes are effective in suppressing the population of *R. similis* in banana plants. The use of endophytic bacteria to suppress the population of *R. similis* is not only effective in banana plants, but also effective in pepper plants. Chaves *et al*. (2009) reported that endophytic bacteria have the effectiveness that is commensurate with the efficacy of synthetic chemical nematicides. In the same study, it was reported that endophytic bacteria that were effective in controlling *R. similis* were *Bacillus* B21, *Pseudomonas* P58, *Bacillus* B31, and *Pseudomonas* P52.

As biological agents, endophytic bacteria are reported to have a dual role, namely as biocontrol agents and as plant growth promoters (Munif *et al*., 2019). The potential of endophytic bacteria as a plant growth promoter has been published in previous studies. Endophytic bacteria that are capable of dissolving phosphate are reported to be effective in increasing the growth of various plants (Afzal *et al.*, 2019; Da Silveira *et al.*, 2018). Phosphate is an essential nutrient that is very important for plant growth. Phosphate can be found in the soil in organic compounds and minerals. Nevertheless, its amount of readily available phosphate is meager compared to total phosphate in the soil. Most phosphates are bound to colloidal soils so they cannot be absorbed by plants (Otieno *et al*., 2015). The dissolution

of phosphate in the soil by endophytic bacteria generally occurs through the mechanism of the production of phosphatase enzymes, phytase enzymes, and organic acids by endophytic bacteria (Mohammadi, 2012).

This study concludes that endophytic bacteria from *R. madascascariensis* roots have potential as biocontrol agents against *F. oxysporum* and banana-parasitic nematodes. The application of endophytic bacteria from the *R. madascascariensis* effectively suppressed banana-parasite nematode populations in vitro and in the greenhouse. Endophytic bacteria isolated from *R. madascascariensis* were able to produce secondary metabolites in the form of chitinase enzymes, protease enzymes, and are capable of dissolving phosphate. The application of endophytic bacteria from *R. madascascariensis* roots was also effective in increasing the stem diameter and the roots fresh weight of the cavendish banana infected with the banana-parasite nematode.

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Contribution of Authors:

Abdul Munif	:	Conceived idea and designed experiments
Rafika R. Wulandari	:	Reviewed manuscript and help in conducting research
Ankardiansyah P.Pradana	:	Data analysis and results interpretation