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CONTENTS

CONTENTS

CONTENTS

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THE ENDOPHYTIC BACTERIA ISOLATION AS BIOLOGICAL CONTROL AGENT OF *PRATYLENCHUS COFFEAE*

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Key words : *Pratylenchus coffeae, Endophytic bacteria, Arabica coffee, Coffee plantation*

Abstract- *Pratylenchus coffeae* is the most common nematode and it can endanger the coffee plant. Endophytic bacteria is the ideal candidate for nematode control because of live inside plant without harming the host plant. Isolation of endophytic bacteria from three locations, namely Arabica coffee plantation infected by *P. coffeae*, arabica coffee plantation infected by *Radopholus similis* and Robusta coffee infected by *P. coffeae* was done with technical surface sterilization. The potential of bacterial isolates was determined by the number of nematodes that penetratedto the roots of aged-3-months seedlings arabica. Molecular identification and proteolytic activity testing was done to an isolate that has the ability to suppress the penetration of nematodes. Twenty pure endophytic bacteria isolates were obtained by the isolation process. All endophytic bacteria isolates significantly reduced the nematodes that penetrated the roots. Isolates from Arabica coffee could suppress nematode penetration up to 91.56 % while isolate from robusta coffee only pressed nematode penetration of 54.5%. From molecular identification of three isolates that suppress the penetration of the highest nematode showed that the isolate is *Bacillus subtilis* strain NCIB 3610 and *Antrachis bacillus* strain ATCC 14578. The three isolates showed proteolytic activity. It is can be concluded that endophytic bacteria are potential in controlling *P. coffeae* especially 3 isolates from Arabica coffee root.

INTRODUCTION

Root - lesion nematodes (*Pratylenchus* spp.)is composed of more than 60 species spread all over the world with different types of host plants. *Pratylenchus* spp. is the third most caused economic losses after root knot nematodes and cyst on cultivated plants world (Castillo and Volvas, 2007). The most important *Pratylenchus* spp. species are *P. penetrans*, *P. thornei*, *P. neglectus*, *P. zeae*, *P. vulnus* and *P. coffeae* (Jones *et al*., 2013)

Pratylenchus coffeae (Zimmermann, 1898) is the most common nematodes and endanger the coffee plants. *P. coffeae*, firstly discovered in the roots of *Coffea arabica* L in Indonesia (Whitehead, 1968) and is now a major parasitic nematodes of coffee in Barbados, Brazil, Congo, Costa Rica, El Salvador, Guatemala, India, Jamaica, Madagascar, Malaysia, Martinique, and the Philippines (Campos *et al*., 1990; Kumar and Samuel, 1990; Schieber and Grullon, 1969). The nematodes also became a pathogenic to a various cultivated plants such as banana, citrus, yam, soursop, and potato in tropical and subtropical countries (Silva and Inomoto, 2002). According to Wiryadiputra (1995), *P. coffeae* caused serious damage to the arabica and robusta coffee plantations in Indonesia. In robusta coffee plantations, the yield loss caused by *P. coffeae* can reach 78% with an average of 57%.Meanwhile, in Arabica coffee plantations, the yield losses can reach 100% for the coffee plants were dead at the age of two years.

P. coffeae is semimigratori endoparasitik and reproduce sexually. All stages of juvenile and adult

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are worm-like and mobile and able to infect the roots of the host plant. The nematode has 4 juvenile and mature stages. All stages of life occurred in the cortex of its host (Luc *et al*., 1995; Jones *et al*., 2013). Parasitic nematode management is more difficult than that of other plant pests, because nematodes mostly live in the soil and usually affects the lower part of the plant (Stirling, 1991). The endophytic bacteria colonizing parts of the plant tissue as nematode endoparasitic, this led endophytic bacteria become ideal candidates for controlling nematodes (Hallmann *et al*., 2009). The endophytic bacteria was defined as the bacteria that live inside plant tissues, without causing harm to the host plant (Hallmann *et al*., 1997).

Many researchers have reported the potential of endophytic bacteria in reducing plant parasitic nematodes (Munif *et al*., 2013; Siddiqui and Shaukat, 2003; Vetrivelkalai *et al*., 2010; Hallmann *et al*., 1997; Mekete *et al*., 2009; Aravind *et al*., 2009; Chaves *et al*., 2009; Halimah *et al*., 2015) but the successful application in the field was still inconsistent. Several studies have found that there is a correlation between the plant resistant and the diversity of endophytic bacteria (Sturz *et al*., 1999; Araujo *et al*., 2002; Reiter *et al*., 2002), but the role of endophytic bacteria communities in coffee plants that is resistant to *P. coffeae* has not been reported yet. This research, did an isolation of endophytic bacteria from healthy coffee plants that grow on coffee plantations are attacked by parasitic nematodes and tested their effects on *P. coffeae*.

MATERIALS AND METHODS

Endophytic bacteria isolation

Root samples from healthy coffee plants that grew among the coffee plants affected by parasitic nematodes were taken from three locations in East Javaprovince, Indonesia in June 2015, namely 1) the arabica coffee plantations affected by *P. coffea* in Kalibendo Banyuwangi (KB), 2) the arabica coffee plantations affected by *Radopholus similisin* Sumberwringin Bondowoso (SW), and 3) the robusta coffee plantations affected by *P. coffea* in Kalimalang Banyuwangi (KM).

The isolation of bacteria was carried by surface sterilization technically refers to a method of Hallmann *et al*., (1997). Root samples were washed cleanly, then weighed as much as 1 g fresh weight of roots. Then, the root samples were surface sterilized gradually by soaking it in 70% alcohol for 30 seconds, then they were soaked in a 2% NAOC solution for 1-2 minutes, then rinsed with setrilizedwater 3 times. Then, the samples roots that have been sterilized were crushed with a sterilized mortar until it was fine. Then, it was incubated gradually until it has 10^{-4} dilution. Then, from each dilution, it was taken 0.1 mL and it was grown in 5% TSA media in petri cups, then it was incubated for 24-72 hours at room tempe-rature. As a control, an example of sterilized roots smeared on 5% TSA media. From each petri cup, they were selected and taken bacteria colony then it was cultured or refined by growing it in 100% TSA media.

The effect of Endophytic bacteria on the penetration of *P. coffeae*

The effect of endophytic bacteria against penetrating nematodes was tested using the dipping roots method, refers to a method by Munif *et al*. (2013). Before testing, the isolatas of endophytic bacteria were pre-cultured on tilting tryptic soy agar (TSA) for 48 hours atroom temperature. One loop of bacteria then transferred to 100 mL of media tryptic soy broth (TSB) and incubated in a shaker for 48 hours at a speed of 100 rpm. To obtain a bacterial suspension with a cell density of 10^9 cfu / mL, performed serial dilution of the bacterial suspension then it was cultured on TSA media. A dilution series was chosen based on cfu measuring. Three months old Arabica coffee seedling roots was immersed into the bacterial suspension based on the treatment for 1 hour and then it planted in pots filled with 1.5 kg of a mixture of soil, manure and sand (1:1:1, v/v/v). After one week, arabica coffee seedlings were inoculated with 50 *P. coffeaes* per pot. Each treatment was repeated six times.

The number of nematodes that can penetrate into the roots were counted 10 days after inoculating of nematodes. The calculation is done after the root coloring process with 0.1% lactic acid fuchsin (789 mL lactic acid, 56 mL of glycerol, 1 g acid fuchsin, and 154 mL of distilled water). The number of nematodes in the roots was counted under a microscope. The penetration efficiency of *P. coffeae* into the root system is calculated based on the number of initial nematode (N1) and the number of nematodes in the roots with formula PE (%) = 100 x N2/N1. The penetration reduction (PR) of *P.coffeae* was calculated based on the number of nematodes in the controlingroots (P1) and the

number of nematodes in the roots treated bacteria (P2) with the formula PR (%) = 100 - P2 / P1 x 100.

Protease activity

Proteolytic activity was tested following the procedure of modified Denizci *et al*. (2004). Liquid culture isolates were inoculated on sterile filter paper in the skim milk agarmedia (SMA: 100% sterile of 900 mL media trypsic soy agar (TSA), 10%concentration of 100 mL of sterile skim milk). The incubation was performed at room temperature for 24-72 hours. Proteolytic activity is indicated by the formation of a clear zone around the colony of bacteria (Baehaki and Budiman, 2011). The produced clear zone was calculated from the difference between the diameter of clear zone and the diameter of bacterial colonies (Isnansetyo and Kamei, 2009).

Moleculer analysis

The Identification was carried out using molecular analysis based on 16S rDNA fragments in bacteria. The isolation of bacteria DNA was done used colony PCR method (Packeiser *et al*., 2013). The cell from the single colonies on the solid media surface was taken using sterilized toothpicks and was suspended in 20 µL nuclease-free water. The cell lysis is carried out with a divortex suspension for 10 seconds and was incubated at 98 °C for 5 minutes. Then, the lysate was spined down to separate the supernatant and cell debris. Then, the supernatant was taken and used as a DNA molding in PCR amplification.

And the amplification of 16S rDNA fragments was done using GoTaq (Promega) with 27Fprimer (5'-AGAGTTTGATCCTGGCTCAG-3 ') and the 1492R (5'-GGTTACCTTGTTACGACTT-3') (Zhang *et al*., 2009; Palaniappan *et al*., 2010). The data resulted from sequencing was processed by Bioedit program. The isolates were identified using EzTaxonserver(http://www.ezbiocloud.net/eztaxon; Kim *et al*., 2012) based on 16S rRNA sequence data.

RESULTS AND DISCUSSION

The diversity of endophytic bacteriaon the roots of the coffee

There were 20 pure isolates resulted from the endophytic bacteria isolationof the coffee roots resistant to nematodes, 7 isolates from Kalibendo (KB), 7 isolates from Sumberwringin (SW), and 6 isolates from Kalimalang (KM).The isolates have diversity in colony morphology such as color, shape and edges of colony, cell shape and nature of Gram. According to Liu *et al*. (2012), endophytic bacteria diversity was mainly influenced by the host plant genotype. The observation result can be seen in Table 1.

The effect of Endophytic bacteria on the penetration of *P. coffeae*

The effect of endophytic bacteria against the penetration of nematodes *P.coffeae* determined based on the number of nematodes that successfully penetrated into the seedlings of arabica coffee roots. Table 2 shows that all treatments of endophytic bacteria could reduce the penetration of nematodes *P. coffeae* comparing with the untreated seedlings.The penetration efficiency (PE)of all treated endophytic bacteria isolates is lower and significantly different than that of without bacteria.

All isolates of endophytic bacteria could reduce the penetration of nematodes and the isolat with SWE, SWF and KBFcode, shown in Figure 1, were the endophytic bacteria isolate that successfully reduce the nematode penetration over than 85%. According Hallmann *et al*. (2001), the reduced penetration of nematodes into the roots was the effect of endophytic bacteria that colonized the root epidermis. The process of colonization of the root epidermis is an advantage for the plants because the colonization on epidermis is an initial protection for coffee plants against the *P.coffeae* infection, so that the nematodes can not penetrate to the root. Moreover, bacterial colonization of the roots can stimulate plant resistance. Kimmons *et al*. (1989) reported that the process of endophytic bacteria colonization causing a thickening of the cell walls, thereby reducing the ability of the nematode Pratylenchus and Meloidogyne maryland is cribneri in infecting tall fescue roots. The research of Munif *et al*., (2013) also showed a reductiof the ability to penetrate up to 56% of juvenile Meloidogyne on tomato roots treated with endophytic bacteria.

Protease activity of selected bacteria

The protease activity measurementis only performed on endophytic bacteria which able to reduce the penetration of nematodes up to> 85%. The protease activity was characterized by a form

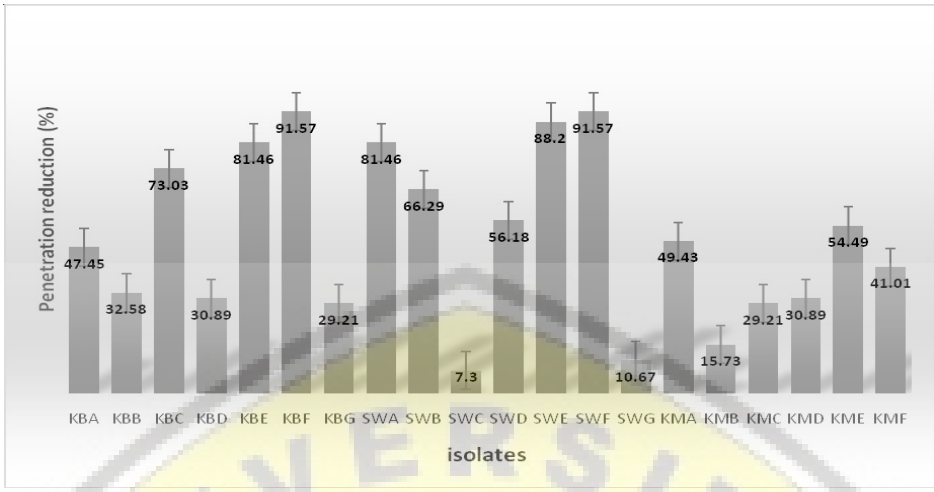

Fig. 1 Penetration reduction value (%) in each treatments

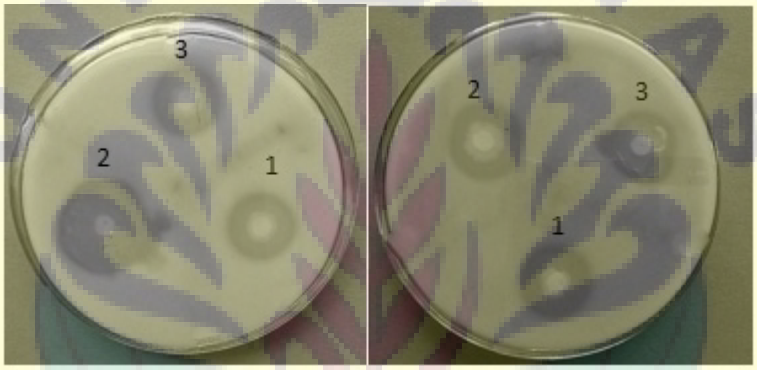

Fig. 2 The zone of inhibition by isolates No.1(KBF), 2 (SWE) and 3 (SWF) on Skimmed milk agar plates

Table 1. Endophytic isolates characteristic obtained from resistant plants among the susceptible plants from three locations

No.	Isolate code	Colony color	Colony shape	Cell shape	Grams (+/-)
1.	KBA	White milk	rounded	Baccil	+
2.	KBB	White	rounded	Baccil	-
3.	KBC	White	irregular round	Baccil	+
4.	KBD	White milk	rounded	Baccil	+
5.	KBE	White milk	rounded	Coccus	+
6.	KBF	White	rounded	Baccil	+
7.	BECs	White	rounded	Baccil	+
8.	SELF	White	irregularity	Baccil	+
9.	SWB	White	Circular	Baccil	+
10.	SWC	White	Circular	Baccil	+
11.	SWD	Slimy white	Cirried	Baccil	+
12.	SWE	transparent white	irregularity	Baccil	+
13.	SWF	Slimy white	Circular	Baccil	+
14.	SWG	Cream	irregularity	Baccil	+
15.	KMA	White	rounded	Baccil	+
16.	KMB	White	rounded	Baccil	+
17.	KMC	White	rounded	Baccil	+
18.	KMD	White	rounded	Coccus	-
19.	KME	White	rounded -amoeboid	Baccil	-
20.	KMF	White	rounded	Baccil	+

of clear zone, as shown in Figure 2.The diameter of the formed clear zone was measured, and the measurement results can be seen in Table 3.

The mechanism of endophytic bacteria in controling parasitic nematodes is not only through the process of root colonization but also through the produce of hydrolysis enzyme. It is well known that the cuticle of nematodes, consists of proteins and chitin, are sticky, especially the outer portion that is protected by a layer of membrane proteins, and it effectively prevents the nematodes from the environmental destruction (Tunlid *et al*., 1994). Therefore, the hydrolysis enzyme such as proteases, collagenase and chitinase becomes the primary choice in controlling nematodes biologically.

Table 2. Penetration of *P. coffeae* into roots of arabica coffee seedling 10 days after inoculation

Treatment	Amount *P.coffeaea*	Penetration efficiency (%)
Without bacteria	19.7778 h	39.56 h
KBA	10.3333 bcdefg	20.66 efgh
KBB	13.3333 defgh	26.66 fgh
KBC	5.3333 dac	10.66 cde
KBD	13.6667 defgh	27.34 fgh
KBE	3.6667 abc	7.34 abc
KBF	1.6667 a	3.34 ab
BECs	14.0000 efgh	28 fgh
SELF	3.6667 abc	7.34 bcd
SWB	6.6667 abcde	13.34 def
SWC	18.3333 gh	36.67 gh
SWD	8.6667 abcdef	17.34 defg
SWE	2.3333 ab	4.66 ab
SWF	1.6667 a	3.34 a
SWG	17.6667 gh	35.34 gh
KMA	10.0000 abcdefg	20 efgh
KMB	16.6667 fgh	33.34 gh
KMC	14.0000 efgh	28 fgh
KMD	13.6667 defgh	27.34 fgh
KME	9.0000 abcdef	18 efgh
KMF	11.6667 cdefgh	23.34 efgh

Each treatment had a six replications. Mean values in the same column Followed by different letter (s) are Significantly different at P>0.05 (Duncan test).

Table 3. Protease activity rate (mm) of three isolates Endophytic bacteria

Isolates code	Protease activity rate (mm)
1(KBF)	1.6550
2(SWE)	0.4925
3(SWF)	0.9945

The results showed that three isolates namely SWE, SWF and KBF could hydrolyze proteins indicated by the diameter of clear zone that was quite high. The ability of these three bacteria indicated the promising potential in controlling nematodes.This is in line with several studies showing that protease bacterial can control the nematodes (Niu *et al*., 2005; Tian *et al*., 2006; Carrim *et al*., 2006; Bonants *et al*., 1995). The protease can degrade the nematode cuticle, it causes these enzymes play an important role in the interaction of bacteria nematode plant - environment because of its nematicidal factors to maintain the balance of nematode populations in the soil (Lian *et al*., 2007).

Moleculer identification of selected bacteria

The results of molecular identification of three isolates that suppress the highest nematode penetration namely SWE, SWF, and KBF indicates that the isolate is *Bacillus subtilis* strain NCIB 3610 (SWE) and *Bacillus antrachis* strain ATCC 14578 (SWF and KBF) with the similarities up to 98-99% as it was shown in Table 4.

It is proven that *B. subtilis* can be a biological control agent of *Meloidogyne* sp. nematode (Mohamedova and Samaliev, 2011; Araujo and Marchesi, 2009; Kumar *et al*., 2013; Khalil *et al*., 2012; Dawar *et al*., 2008; Ruiz *et al*., 2014; Roy *et al*., 2015). *B.subtilis* which was isolated from the rhizosphere was also proved to control *P.coffeae* (Asyiah *et al*., 2015).

Bacillu scereus and *B.subtilis* produce uracil, namely dihydrouracil, that is the promising substance in controlling plant parasitic nematodes, and there was also Dihydrouracil in commercial nematicides carbofuran. *B.cereus* and *B.subtilis* also

Table 4. Homology of b acterial endophytes using partial 16 S rRNA gene sequencing

Isolate	Identification / DNA homology	Homology accession number	Sequence similarity
contigSWE	*Bacillus subtilis* strain NCIB 3610	ABQL0100001	9 8 .4%
SWF_27F	*Bacillus antrachis* strain ATCC 14578	AB190217	99.9%
KBF_27F	*Antrachis bacillus* strain ATCC 14578	AB190217	99.2%

produced phosphoribosyl transferase enzym that is potentially developed to control *Meloidogyne* spp (Ruiz *et al*., 2014). In addition, in the coffee roots, like in this research, the endophytic bacteria *Bacillus anthracis* was also found in *Ceiba pentandra* seeds and *Swietenia macrophylla* stem (Mariza *et al*., 2011), peanut (Wang, 2013), and cassava stem (deMelo *et al*., 2009).

Bacillus anthracis is very closely related to *B.cereus* and *B.Thuringiensis*, and even sometimes it considered to be a single species (Helgason *et al*., 2010; Kolsto *et al*., 2009; Rasko *et al*., 2005). *B.thuringiensis* is already known as a biological control agent of various pest plant organisms, one of the reasons, because it generated Crystal (Cry) proteins, large family of related proteins that kill insects and nematodes (Vilas-Boas *et al*., 2007). Kho *et al*., (2011) proved that the co-culturing of Cry 5B-expressing B. with *B.thuringiensis anthracis* can cause death to the nematode *Caenorhabditis elegans* by *B.anthracis*.

CONCLUSION

There are 20 isolates of endophytic bacteria isolated from the roots of coffee plants resistant to nematodes. Three isolates have the ability to suppress the penetration nematode up to> 85% namely *Bacillus subtilis* strain NCIB 3610 (SWE) and *Bacillu santrachis* strain ATCC 14578 (SWF and KBF). The three isolates produced protease which is stated implicitly promising potential in controlling nematodes.

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