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POTENTIAL USE OF ENDOPHYTIC BACTERIA TO CONTROL *Pratylenchus brachyurus* ON PATCHOULI

Potensi Penggunaan Bakteri Endofit untuk Mengendalikan Pratylenchus brachyurus *pada Tanaman Nilam*

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ABSTRACT

Pratylenchus brachyurus is an important parasitic nematode which significantly decreases quality and quantity of patchouli oil. One potential measure for controlling the nematode is by using endophytic bacteria. These bacteria also induce plant growth. This study aimed to evaluate the potential of endophytic bacteria to control *P. brachyurus*. The experiments were carried out in the Bacteriological Laboratory of the Plant Protection Department, Bogor Agricultural University, and the Laboratory and Greenhouse of the Indonesian Spice and Medicinal Crops Research Institute from April to December 2007. Endophytic bacteria were isolated from the roots of patchouli plants sampled from various locations in West Java. Antagonistic activity of the isolates were selected against *P. brachyurus* and their abilities to induce plant growth of patchouli plants. Isolates having ability to control *P. brachyurus* and promote plant growth were identified by molecular techniques using 16S rRNA universal primers. The results showed that a total of 257 isolates of endophytic bacteria were obtained from patchouli roots and their population density varied from 2.3 x 10^2 to 6.0 x 10^5 cfu g^{-1} fresh root. As many as 60 isolates (23.34%) were antagonistic against *P. brachyurus* causing 70-100% mortality of the namatode, 72 isolates (28.01%) stimulated plant growth, 32 isolates (12.47%) inhibited plant growth, and 93 isolates (36.18%) were neutral. Based on their antagonistic and plant growth enhancer characters, five isolates of the bacteria, namely *Achromobacter xylosoxidans* TT2, *Alcaligenes faecalis* NJ16, *Pseudomonas putida* EH11, *Bacillus cereus* MSK, and *Bacillus subtilis* NJ57 suppressed 74.0-81.6% nematode population and increased 46.97-86.79% plant growth. The study implies that the endophytic bacteria isolated from patchouly roots are good candidates for controlling *P. brachyurus* on patchouly plants.

[***Keywords***: Patchouly, endophytic bacteria, *Pratylenchus brachyurus*, disease control]

ABSTRAK

Pratylenchus brachyurus *adalah nematoda parasit pada tanaman nilam yang dapat menurunkan hasil dan kualitas minyak nilam. Salah satu cara pengendalian yang potensial terhadap nematoda tersebut adalah menggunakan bakteri endofit. Selain dapat membunuh nematoda, bakteri endofit juga dapat menginduksi pertumbuhan tanaman. Penelitian bertujuan untuk mengevaluasi potensi bakteri endofit yang berasal dari tanaman nilam untuk mengendalikan namatoda parasit* P. brachyurus. *Penelitian dilakukan di Laboratorium Bakteriologi, Departemen Proteksi Tanaman, Institut Pertanian Bogor, serta di laboratorium dan rumah kaca Balai Penelitian Tanaman Rempah dan Obat, pada bulan April sampai Desember 2007. Bakteri endofit diisolasi dari sampel akar tanaman nilam dari beberapa lokasi di Jawa Barat. Isolat-isolat bakteri endofit diseleksi kemampuannya untuk membunuh* P. brachyurus *dan menginduksi pertumbuhan tanaman nilam. Isolat bakteri endofit yang potensial selanjutnya diidentifikasi secara molekuler menggunakan primer universal 16S rRNA. Penelitian memperoleh 257 isolat bakteri endofit dengan kerapatan populasi 2,3 x 10^2 sampai 6,0 x 10^5 cfu g^{-1} berat basah akar. Enam puluh isolat (23,34%) di antaranya bersifat antagonis terhadap* P. brachyurus *dengan mortalitas 70-100%, 72 isolat (28,01%) dapat memacu pertumbuhan tanaman nilam, 93 isolat (36,18%) bersifat netral, dan 32 isolat (12,47%) dapat menghambat pertumbuhan tanaman. Berdasarkan hasil pengujian antagonis dan pemacu pertumbuhan tanaman, lima isolat bakteri, yaitu* Achromobacter xylosoxidans *TT2,* Alcaligenes faecalis *NJ16,* Pseudomonas putida *EH11,* Bacillus cereus *MSK, dan* Bacillus subtilis *NJ57 dapat menekan populasi nematoda 74,0-81,6% dan meningkatkan pertumbuhan nilam 46,97-86,79%. Penelitian mengindikasikan bahwa bakteri endofit dari tanaman nilam berpotensi mengendalikan* P. brachyurus *pada tanaman nilam.*

[***Kata kunci***: *Nilam, bakteri endofit,* Pratylenchus brachyurus, *pengendalian penyakit*]

INTRODUCTION

Endophytic bacteria are bacteria that live in the internal tissues of plants, can be isolated from healthy tissues and do not cause a negative effect on the plant itself (Hallmann 2001). The population densities of endophytic bacteria are highly dependent on plant species, tissue types (roots, stems, leaves), plant age, habitat, environmental factors (McInroy and Kloepper 1995; Hallmann 1997; Zinniel *et al*. 2002; Hallmann and Berg 2006), geographic, species, plant genotype and cultivation techniques (Hallmann and Berg 2006). The population density of endophytic bacteria on root is 10^5 cfu g^{-1} fresh root, on stem is 10^4 g^{-1} fresh stem, and on leaf is 10^3 cfu g^{-1} fresh leaf, indicating that the endophytic bacteria are colonized all parts of the plant.

Recently, many endophytic bacteria are used as biological control agent of plant diseases (Antoun and Prevost 2006). Potential use of endophytic bacteria in controlling root lesion nematode on patchouly (*Pratylenchus brachyurus*) need to be evaluated because the namatode causes significant loss in quality and quantity of patchouly oil produced (Harni and Mustika 2000).

In the natural ecosystem, there are various types of endophytic microorganisms associated with plants, such as bacteria, actinomycetes and fungi. The endophytic bacteria can be isolated from various plant tissues, such as roots, stems, leaves, fruits and flowers. The population densities of endophytic bacteria are highly dependent on plant species, tissue types (roots, stems, leaves), plant age, habitat, and environmental factors (McInroy and Kloepper 1995; Hallmann 1997; Zinniel *et al*. 2002; Hallmann and Berg 2006), geographic, species, plant genotype and cultivation techniques (Hallmann and Berg 2006). The population densities of endophytic bacteria on root is 10^5, rod is 10^4, and leave is about 10^3 cfu/g. The study aimed to evaluate the potential use of endophytic bacteria to control the root lesion nematode *P. brachyurus* on patchouly.

MATERIALS AND METHODS

Isolation of Endophytic Bacteria

Endophytic bacteria were isolated in 2007 from the roots of several patchouly varieties grown at different areas in West Java, namely Bogor, Lembang, Sumedang, Tasikmalaya, Garut, and Sukabumi. These areas are the center of patchouli cultivation in West Java, except for Lembang which is an experimental garden of medicinal crops as well as a germplasm collection of patchouly plant of the Indonesian Spice and Medicinal Crops Research Institute.

Endophytic bacteria were isolated from patchouli roots using the method of Hallmann *et al*. (1997). Each root sample was cleaned with water, dried with tissue paper and weighed. As much as 1 g of root tissues was used. The root was then surface sterilized by soaking for one minute in 5% NaOCl solution added with 0.01% Tween 20, and rinsed with sterilized water three times. To ensure the success of surface sterilization, the roots were incubated on 10% Tryptic Soy Agar (TSA) medium in the petri dish and then incubated at room temperature for 48 hours. If microorganisms still grew on the roots, it meant that surface sterilization was failed and should be repeated using new root samples. On the other hand, if microorganisms did not appear, it meant that root surface sterilization was successful and isolation of bacterial endofit can be proceed.

The sterilized roots were then homogenized with a sterile mortar. One ml of the root extract was mixed with 9 ml of sterile water in a test tube (10^{-1} dilution) and diluted until reached 10^{-3}. From the final dilution, 0.1 ml bacterial solution was taken, then spreaded on the 10% TSA medium and incubated at room temperature for 48 hours. Colonies of bacteria grown on the TSA medium were calculated and every single colony was subcultured on the full strength TSA medium. Single cell culture of the endophytic bacteria was kept in sterile distiled water in ependorf tubes and stored at 4°C. Morphological characters, viz. shape, color and edges of the isolates were then identified.

Isolation and Multiplication of Nematode

P. brachyurus was isolated from roots of diseased patchouli plants showing stunted growth and reddish or yellowish leaf symptoms. *P. brachyurus* larvae were reared on sterilized carrot medium using the method of Huettel (1985). Fresh carrot was surface sterilized with 5.25% sodium hypochlorite solution, washed with running water, peeled, cut horizontally into pieces of 3 cm thick, and soaked in 1.5% sodium hypochlorite for 15 minutes. After beeing rinsed with sterile distilled water two times, the carrot cut was placed in a culture bottle or a petridish. *P. brachyurus* larvae were then sterilized with 0.01% $HgCl_2$ and 0.1% streptomycin sulfate for 30 seconds and then rinsed with sterile water. The

nematodes were then aspirated with a sterile pipette and inoculated on the pieces of carrot. The nematode and the carrot were incubated at 27°C for 2 months. The culture was used as the source of nematode inoculum.

Propagation of Plant Material

The plant material used was the one-node cutting of patchouli of Sidikalang variety. This variety is widely grown by farmers because of its high contents of patchouli oil and patchouli alcohol. The cuttings were grown in potting medium in polybags for 4 weeks in the nursery.

Toxicity Test of Endophytic Bacteria to *P. brachyurus*

Isolates of endophytic bacteria were grown on Tryptic Soy Agar (TSA) medium for 48 hours at room temperature. Single colony of the bacteria was transferred into a 100 ml flask containing 250 ml Tryptic Soy Broth (TSB) medium and shake-incubated at 150 rpm at 25°C for 48 hours. The bacterial culture was centrifuged at 7000 rpm for 15 minutes and the supernatant was filtered with a sterile Whatman miliphore filter (0.22 µm diameter). Bacterial filtrate was stored at 4°C until used for toxicity assays to *P. brachyurus*.

The toxicity test was conducted by adding 5 ml fitrate culture into a 10 ml glass and then 50 female larvae of *P. brachyurus* were added into the glass containing the fungal filtrate. The glass was put in a plastic box (30 cm x 20 cm x10 cm) and stored at room temperature for 48 hours. Mortality of the namatode was observed after 24 hours by using a stereocope binocular microscope.

Plant Growth Promoting Activity of Endophytic Bacteria

Plant growth promotion of the isolated endophytic bacteria was tested on cucumber plant as a plant model. Cucumber seeds were soaked in the tested bacterial suspension for 24 hours and grown in the pots containing planting medium. Cucumber seeds soaked in sterile water was used as a control. Seed germination was observed and plant growth parameters, viz. plant height, leaf number, plant weight and root weight were assessed at two weeks after planting.

Greenhouse Selection of Endophytic Bacteria

Fifty isolates of endophytic bacteria showing highly *in vitro* antagonistic response to *P. brachyurus* and stimulated plant growth were tested in the greenhouse. Bacterial isolates were multiplied on TSA medium for 48 hours at room temperature. The cultures were suspended in sterile water and adjusted their population by using a spectrometer to OD600 = 0.1, equal to 10^7 cfu ml^{-1}.

Four-week-old patchouli cuttings grown in polybags were dismantled and the roots were soaked in endophytic bacterial suspension for 60 minutes. The treated cuttings were planted in pots containing a mixture of soil and sand (2:1) sterile medium and mulch (2 kg pot^{-1}). The control plant was soaked in water only.

Nematode inoculation was performed at two weeks after the treatment by pouring nematode suspension (500 adult females and larvae) around the plants at 1 cm depth. At one month after inoculation, the inoculated plants were dismantled and the roots were washed and dried. Antagonistic activity of the nematode was assessed by calculating the reproductive factor of nematodes, i.e. the ratio of the final population over the initial population of nematodes (pf/pi). Nematodes in roots were extracted with funnel spray method, while those in the soil were isolated with Baerman funnel method. The effect of nematode inoculation on plant growth was measured by weighing the shoots and the roots of the inoculated plants.

Potential Use of Endophytic Bacteria to Control *P. brachyurus* on Patchouli

This experiment was conducted in the greenhouse. Ten most potential isolates of endophytic bacteria from the previous experiments were tested in a completely randomized design with five replications. The treatments were 12 isolates of endophytic bacteria, namely TT2, NJ46, NJ16, EH11, MSK, NJ57, CR, BAS, TKU6, NJ2, P24 and B12. Isolates P24 and B12 were originated from the laboratory of Plant Protection Department, Bogor Agricultural University. The inoculation method was similar as that decribed in the previous experiment. At one month after inoculation, the plants were dismantled and the roots were washed and dried. Antagonistic and plant growth promotion activities of the endophytic bacteria were measured following the method previously described.

Identification and Characterization of Endophytic Bacteria

Endophytic bacteria was identified using molecular technique of sequencing method of Klement *et al*. (1990) and Schaad *et al*. (2001). The identification was based on partial sequences of the 16S rRNA. Isolation and purification of DNA was based on the method described by Schaad *et al*. (2001). DNA was extracted with phenol-chloroform and then amplified in a PCR machine using primers 16S-27F: 5'AGAGTTT GATCCT GGCTCAG3 'and 16S-42R: 5'GGTACCTTGT TACGACTT3'. The sequencing data were matched with the NCBI Gene Bank data base using the BLAST program at http://www.ncbi.nlm.nih.gov.

Physiological characteristics of the endophytic bacteria, such as chitinase and protease activity, cyanide production, fluorescence and lipolytic activity were evaluated using standard protocols. Chitinase activity of the bacteria was tested using the method described by Lingappa and Lockwood (1962), protease activity was studied following the method of Hankin and Anagnostakis (1975) *in* Munif (2001), cyanide production of endophytic bacteria was tested by using the procedure described by Wei *et al*. (1991), and lipolytic activity of endophytic bacteria was evaluated using tween 20 following the methods of Hankin and Anagnostakis (1975) *in* Munif (2001).

RESULTS AND DISCUSSION

Exploration of Endophytic Bacteria

Isolation of endophytic bacteria from patchouli roots originated from some areas of West Java (Bogor, Garut, Sumedang, Tasikmalaya, Lembang and Sukabumi) obtained 257 isolates with population density varied from 2.3 x 10^2 to 6.0 x 10^5 cfu g^{-1} wet root weight (Table 1). The highest population density was obtained on Sidikalang variety from Lembang (6.0 x10^5 cfu g^{-1} wet root weight) and the lowest population density was observed on Sidikalang variety from Sukabumi (2.3 x 10^2 cfu g^{-1} wet root weight). Differences in bacterial population densities may be due to the differences in plant origins and environmental conditions. The densities of bacterial population obtained were similar to those previously reported. On coffee, the endophytic bacteria population densites varied from 5.2 x 10^2 to 2.07 x 10^6 cfu g^{-1} wet root (Mekete *et al*. 2009). On sweet corn, the population densities were 10^4-10^6 cfu g^{-1} wet root (McInroy and Kloepper 1995), on cotton were 4.0 x 10^2 to 1.3 x 10^4 cfu g^{-1} roots (Hallmann *et al*. 1997) and on potato was 10^3 cfu g^{-1} root (Krechel *et al*. 2002).

Table 1. Population density of endophytic bacteria on patchouli root isolated from several areas of West Java.

Location	Variety	Population density (cfu g^{-1} of wet root weight)	Number of isolates
Bogor	Sidikalang	12.8 x 10^3	28
Bogor	Tapak tuan	4.4 x 10^4	23
Bogor	Nilam jawa	4.5 x 10^4	17
Leuwiliang	Sidikalang	6.7 x 10^3	18
Garut	Sidikalang	2.8 x 10^2	19
Lembang	Tapak tuan	8.0 x 10^4	15
Lembang	Sidikalang	6.0 x 10^5	25
Lembang	Cirateun	2.7 x 10^3	20
Lembang	Cisaroni	5.0 x 10^3	15
Lembang	Nilam jawa	5.7 x 10^4	24
Lembang	Loksheumawe	6.0 x 10^3	15
Sumedang	Sidikalang	4.2 x 10^3	10
Tasikmalaya	Sidikalang	3.5 x 10^2	13
Sukabumi	Sidikalang	2.3 x 10^2	15
Total			257

The result showed that bacterial population in Lembang (1.14 x 10^5) was higher than those observed in other locations such as Bogor (3.4 x 10^4), Leuwiliang (6.7 x 10^3), Sumedang (4.2 x 10^3) and Garut, Tasikmalaya, and Sukabumi with the average population of 10^2 cfu g^{-1} wet root. The difference of bacterial population densities obtained in this study was due to environmental factors (rainfall, temperature) and cultivation techniques. For example, patchouli plants grown in Lembang and Bogor were rarely treated with synthetic pesticide and chemical fertilizers, whereas in other areas such as in Garut, Tasikmalaya and Sukabumi the plants were intensively fertilized with chemical fertilizers or treated with synthetic pesticides. Other factors affecting endophytic bacterial population density were plant varieties, tissue types (root, stem, leaf), plant age, habitat, biotic and abiotic environmental factors (e.g. temperature and rainfall), cultivation technique and soil amendment (Hallmann *et al*. 1999; Garbeva *et al*. 2004; Berg and Hallmann, 2006; Hallmann 2001; Zinniel *et al*. 2002). Mekete *et al*. (2009) reported that cultivation techniques greatly affect endophytic bacterial population on coffee plants. Endophytic bacterial population densities in semi-forest and forest coffee were higher than those observed in large-scale coffee plantations.

Antagonistic and Plant Growth Promotion Tests of Endophytic Bacteria

Tests of the endophytic bacteria isolates to *P. brachyurus* showed that among the 257 isolates, 60 isolates (23.34%) were able to control nematodes (showing nematicidal effect) causing mortality of 70%-100% (Fig 1). The same result was reported by Mekete *et al.* (2009) on coffee. Of the 201 endophytic bacterial isolates tested, 42 isolates (33%) were able to control *M. incognita* causing mortality of 38-98%, much higher than rhizobacteria activity on *M. incognita* with antagonistic response of only 1% (Becker *et al*. 1988), 7.2% on *Heterodera schachtii* (Oostendorp and Sikora 1989), 9% on *Globodera pallida* (Rache and Sikora 1992), and 12% on *M. incognita* (Siddiqui *et al.* 2001).

The effect of endophytic bacteria on cucumber showed that 72 isolates (28.01%) were able to improve plant growth (Fig. 1). However, 93 isolates (36.18%) of the endophytic bacteria were neutral or not induce plant growth, and 32 isolates (12.47%) inhibited plant growth (Fig 1).

Effects of Endophytic Bacteria in Greenhouse Experiment

Out of 50 isolates of endophytic bacteria tested, 31 isolates (62.0%) demonstrated antagonistic response to *P. brachyurus* (Table 2). *P. brachyurus* population as indicated by the pf/pi values decreased from 0.43 in the control to become 0.06-0.2 in the endophytic bacteria treatment. Nineteen isolates (38.0%) of endophytic bacteria enhanced plant growth (plant and root weight) of patchouli in the greenhouse (Table 2). The highest plant height was obtained on MSK isolate (14.60 g), and the lowest (4.7 g) was on TKU2 isolates in control (without nematodes and endophytic bacteria) 12.2 g. This occurred because endophytic bacteria can be antagonistic to *P. brachyurus* so it can reduce the development of nematodes in roots, also promote plant growth through production of phytohormones and enhancing nutrient availability. Bacon and Hinton (2007) reported that endophytic bacteria promote plant growth by: (1) increasing the availability of plant nutrients such as nitrogen, phosphate, phosphorus and other minerals, (2) stimulating growth by producing growth hormone, such as ethylene, auxin and cytokinin, and (3) reducing the negative effect of the pathogen.

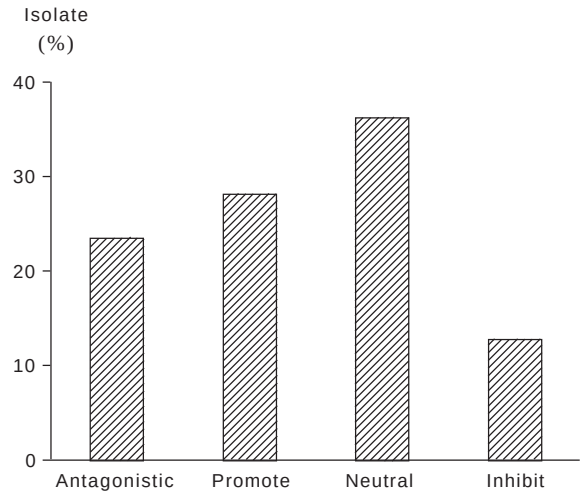

Fig. 1. Percentage of endophytic bacterial isolates from patchouli roots showing antagonistic, promote, neutral and inhibit patchouli growth.

Based on these data (Table 2), ten endophytic bacterial isolates, viz. TT2, NJ16, NJ57, MSK, EH1, CR1, NJ2, TKU6, NJ46 and BAS had nematode reproduction factor (pf/pi) values ranged from 0.06 to 0.09 and plant weights from 13.3 to 14.7 g plant^{-1}. In addition, ten isolates were non-pathogenic bacteria evidenced by negative hypersensitivity test (did not cause necrotic symptoms on tobacco leaves).

Potential Use of Endophytic Bacteria to Control *P. brachyurus* on Patchouli

The results showed that all tested isolates significantly reduced *P. brachyurus* populations compared to the control (Table 3). The highest effects were shown by isolates TT2, NJ16, MSK3, EH11 and NJ57 which were significantly better than other isolates. Nematode populations were highest on control treatment with pf/pi value of 3.7 and the lowest was on TT2 treatment with pf/pi value of 0.68. This is because of the antagonistic effect of bacteria to the nematodes. Several endophytic bacteria were reported to have ability as antagonist agents in suppressing plant disease by colonizing host internal and cartical tissues, occupying ecological niches needed by pathogen, producing metabolites to suppress pathogens and induce plant resistance (Hallmann 2001).

Nematode populations on patchouli plants treated with endophytic bacteria were significantly lower than that on the control. The highest nematode population decrease (81,6%) was shown by TT2 isolates althought it was not different to isolates

Table 2. The effect of 50 endophytic bacterial isolates on *Pratylenchus brachyurus* population and plant growth of patchuoli at 4 weeks after inoculation.

Isolate	*P. brachyurus* population	f/pi[1)]	Antagonistic effect[2)]	Shoot weight (g)	Root weight (g)	Shoot + root weight (g)	Plant growth effect[3)]
TT2	30	0.06	+	10.1	3.5	13.6	+
TT1	90	0.18	+	5.8	1.5	7.3	-
TT35	190	0.38	-	8.8	2.2	11.0	-
TKA4	272	0.54	-	8.4	2.8	11.2	-
TKA7	55	0.11	+	9.2	1.9	11.1	-
TKU5	43	0.09	+	10.8	2.1	12.9	+
TKA1	247	0.49	-	9.1	2.3	11.4	-
EH11	**33**	**0.06**	+	**12.7**	**2.0**	**14.7**	+
E26	270	0.54	-	7.8	2.9	10.7	-
EH9	137	0.27	-	6.2	2.3	8.50	-
EH7	47	0.09	+	12.0	2.0	14.0	+
CR2	175	0.35	-	9.8	1.9	11.7	-
CR1	33	0.06	+	11.5	1.8	13.3	+
AS12	249	0.50	-	5.9	1.1	7.0	-
AS5	100	0.20	+	10.2	2.5	12.7	+
S6	280	0.56	-	8.2	2.8	11.0	-
S16	97	0.19	+	10.3	3.0	13.3	+
B12	43	0.09	+	10.5	2.5	13.0	+
P24	43	0.09	+	10.0	2.5	12.5	+
CS1.2	53	0.12	+	10.8	2.1	12.9	+
CS3.3	248	0.50	-	8.9	1.6	10.5	-
CS3.4	60	0.12	+	8.5	1.8	10.3	-
NJ2	40	0.08	+	10.8	3.6	14.4	+
NJ5	333	0.66	-	7.6	1.7	9.3	-
NJ16	35	0.07	+	10.2	3.5	13.7	+
NJ46	43	0.09	+	11.5	2.0	13.5	+
CR5	257	0.51	-	6.8	2.8	9.6	-
CS1.4	53	0.10	+	11.0	2.5	12.5	+
MSK3.2	65	0.13	+	10.7	2.0	12.7	+
MSK3.3	40	0.08	+	12.1	2.5	14.6	+
MSK3.1	48	0.09	+	10.7	2.0	12.7	+
NJ57	40	0.08	+	10.5	3.5	14.0	+
LS3.6	248	0.50	-	6.8	2.8	9.6	-
TS1.2	80	0.16	+	10.7	2.0	12.8	+
CS5	197	0.40	-	9.1	2.3	11.4	-
MSH1	157	0.34	-	8.4	2.75	11.2	-
BAS S3	40	0.08	+	11.5	2.2	13.7	+
CR1	270	0.54	-	8.2	2.1	10.3	-
TSH4	37	0.07	+	10.2	2.5	12.7	+
CSH3.1	48	0.09	+	9.6	2.9	12.5	+
CSK1.6	243	0.49	-	5.5	1.3	6.8	-
TKU6	40	0.08	+	11.5	2.2	13.7	+
TKU1	33	0.06	+	8.9	2.3	11.1	-
TKU2	81	0.04	+	3.7	1.0	4.7	-
AS12	219	0.44	-	5.9	1.1	7.0	-
AS5	100	0.20	+	10.2	2.5	12.7	+
TKA5	231	0.46	-	9.6	2.9	12.5	+
TKU3	18	0.04	+	5.5	1.3	6.8	-
TSH1.2	80	0.16	+	10.7	2.0	12.8	+
CC5	297	0.60	-	9.1	2.3	11.4	-
K+	215	0.43		9.2	1.6	10.8	-
K-	-	-		10.6	1.6	12.2	

[1)]f/pi = reproduction factor, the ratio of the end population by the initial population of nematodes.

[2)]Antagonistic effects: + = bacteria suppress nematode populations - = bacteria do not suppress nematode populations.

[3)]The effect on plant growth: + = bacteria improve growth, - = bacteria do not improve growth.

Data represent averages of four replications. Isolates in bold are candidates to be tested at a later stage.

Table 3. Effect of endophytic bacterial isolates on *Pratylenchus brachyurus* population on patchouli roots at 8 weeks after inoculation.

Isolate	Nematode population	f/pi	Populations decrease[1)] (%)
TT2	340 ± 46.36d	0.68d	81.6a
NJ16	350 ± 83.36d	0.70d	81.0a
MSK	352 ± 53.57d	0.70d	81.0a
NJ57	430 ± 62.84d	0.86d	76.7a
EH11	480 ± 99.82d	0.96d	74.0a
NJ2	538 ± 67.23c	1.08c	70.8ab
P24	864 ± 106.71bc	1.73bc	53.2ab
NJ46	904 ± 159.48bc	1.81c	51.1ab
TKU6	1002 ± 163.85b	2.00b	46.0b
BAS-S3	1005 ± 146.70b	2.01b	45.6b
CR1	1020 ± 75.82b	2.04b	45.0b
B12	1126 ± 92.29b	2.25b	39.1b
Not use endophytic bacteria	1850 ± 136.90a	3.70a	-

Numbers in the same column followed by the same letter are not significantly different at 5% DMRT.

[1)]Percentage reduction in population is the number of nematodes without endophytic bacteria minus number of nematodes in the treated endophytic bacteria devided by number of nematodes in the control treatment (without bacteria) x 100%.

NJ16, MSK, EH11 NJ57 which were 81.0%, 81.0%, 76.7% and 74.0%, respectively (Table 3) and the lowest was on isolate B12 (39.1%). The similar results were reported by Sikora *et al.* (2007).

The results showed that endophytic bacteria significantly induced plant growth, viz. plant height, canopy weight, root weight and plant dry weight (Table 4). These were also correlated with the effect of endophytic bacteria on reducing nematode infection as shown by better canopy weight. The highest canopy weight was obtained on MSK isolate treatment (86.79% better) though it was not significantly different with other isolates, i.e. NJ57, TT2 and EH11 NJ16, ranged from 46.9% to 82.25%.

Increasing plant growth such as plant height, canopy weight and root weight was due to the nematode population suppression. The endophytic bacteria reduced root damages and stimulated formation of lateral roots and root number thus increasing nutrient absorption by plants (Vasudevan *et al.* 2002).

Plant growth (canopy weight, root weight, plant height and dry weight) of patchouli inoculated with nematodes (K +) was significantly lower than those treated with the endophytic bacteria (Table 4). These low root weight, canopy weight, plant height and plant dry weight of plants inoculated with nematode were caused by damages from the stabbing stilets and secretion of enzymes released by nematode when it was feeding. Agrios (1997) reported that nematodes take root cells thus reduce plants ability to absorb water and nutrients from the soil and cause symptoms such as lack of water and nutrients. It also reduces the concentration of plant growth regulators such as

Table 4. Effect of 12 endophytic bacteria isolates on growth of patchouli plant at 8 weeks after inoculation.

Isolate	Plant height (cm)	Canopy weight (g)	Root weight (g)	Canopy + root dry weight (g)
MSK	40.10 ± 1.43a	18.53 ± 1.10a	5.00 ± 0.10a	2.85 ± 1.10a
NJ57	36.20 ± 1.55ab	18.08 ± 2.29a	4.48 ± 0.96a	2.80 ± 1.20a
TT2	40.40 ± 1.67a	17.26 ± 1.62a	3.20 ± 0.85abc	2.70 ± 0.38a
K-[1)]	39.00 ± 1.00a	16.70 ± 1.36a	3.26 ± 0.49abc	2.53 ± 0.83a
NJ16	36.60 ± 1.67ab	14.78 ± 2.12a	3.56 ± 1.32ab	2.52 ± 0.43a
EH11	35.80 ± 1.90ab	14.58 ± 2.70a	3.08 ± 0.85bc	2.50 ± 0.47a
NJ2	36.40 ± 3.50ab	13.00 ± 0.80ab	3.10 ± 0.81bc	2.00 ± 0.40ab
P24	32.80 ± 2.58ab	12.54 ± 2.62b	3.00 ± 1.26bc	2.00 ± 0.20ab
CR1	36.50 ± 1.79ab	12.40 ± 1.72b	2.62 ± 0.65bc	2.00 ± 0.65ab
NJ46	32.80 ± 2.58b	12.40 ± 3.99b	2.58 ± 0.53bc	1.24 ± 0.56c
TKU6	35.40 ± 2.96ab	11.88 ± 1.91bc	2.58 ± 0.64bc	1.98 ± 0.45b
BasS3	35.20 ± 1.90ab	11.88 ± 3.20bc	2.57 ± 0.30bc	1.97 ± 0.30b
B12	32.40 ± 2.38b	11.80 ± 1.18bc	2.30 ± 0.42c	1.90 ± 0.60b
K+[2)]	29.60 ± 1.67c	9.92 ± 1.23c	1.38 ± 1.18d	1.32 ± 0.51c

Numbers in the same column followed by the same letter are not significantly different at 5% DMRT.

[1)]K- = plants not inoculated with nematodes and endophytic bacteria, [2)]K+ = plants inoculated with nematodes.

auxin, cytokinin and gibberellin on root tip. This is because the nematodes secrete cellulase and pektinase enzymes capable of degrading the cell up to the root tip injuries and rupture, causing auxin inactive and then retarding growth.

Identification of Endophytic Bacteria

Figure 2 shows DNA amplification results based on PCR using a 16S rRNA universal primer on five endophytic bacteria isolates (TT2, NJ16, NJ57, EH11 and MSK). A single 1600-bp DNA band was observed on the five isolates (Fig. 2). Based on the partial squencing using 16S rRNA compared with their similarities values using BLAST program via www.ncbi.nlm.nih.gov (Schaad *et al*. (2001), the endophytic bacteria isolate NJ57 was identified as *Bacillus subtilis* (99% similarity level), isolate NJ16 as *Alcaligenes faecalis* (95% similarity level), isolate MSK as *Bacillus cereus* (93% similarity level), isolate EH11 as *Pseudomonas putida* (similarity level 83-93%), and isolate TT2 as *Achromobacter xylosoxidans* (99% similarity level). These species have been widely used as biocontrol agents to plant parasitic nematodes such as *M. incognita* on coffee (Mekete *et al*. 2009), *B. cereus* on cucumber roots (Hallmann *et al*. 1997), *M. javanica* on cotton (McInroy and Kloepper (1995) and *M . incognita* on tomato (Munif 2001).

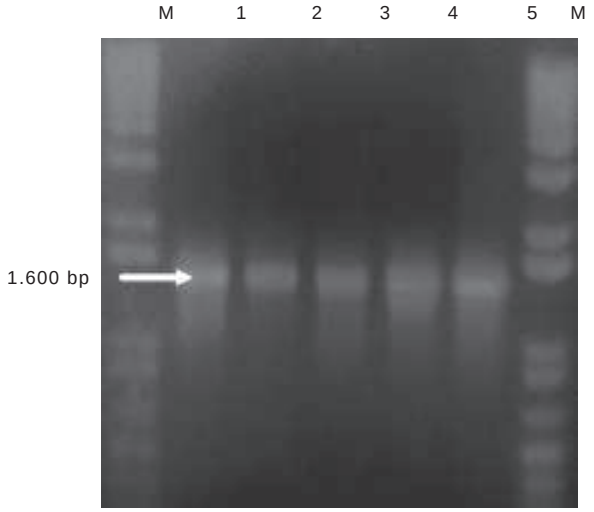

Fig. 2. DNA amplification results of five endophytic bacterial isolates based on PCR using 16S rRNA universal primers. Columns 1-5 were the endophytic bacterial isolates EH11, TT2, NJ16, NJ57, and MSK, respectively. M = marker 1 kb ladder Invitogen.

Characterization of Endophytic Bacteria

Results of physiological characterization of the endophytic bacteria tested *in vitro* are presented in Table 5. Based on the test results, some bacteria were able to produce enzymes, including chitinase (*A. xylosoxidans* TT2), cyanide (*P. putida* EH11), protease (*A. faecalis* NJ16, *A. xylosoxidans* TT2, *B. subtilis* NJ57), lipolytic (*B. subtilis* NJ57, *A. xylosoxidans* TT2, *A. faecalis* NJ16) and fluorescens (*P. putida* EH11). Physiological characters can be related with the role of these bacteria as biocontrol agents.

Chitinase is an enzyme produced by antagonistic bacteria to control pathogens, especially for soil borne pathogens, because the enzyme can degrade pathogen cell walls compiled by chitin compound, such as on fungi, nematodes and insects. Oku (1994) reported that chitinase activity positively correlated with the induction level of systemic resistance. The role of this enzyme in plant resistance to pathogens can be through inhibition of pathogen growth by hydrolyzing cell wall, and releasing endogenous elicitor which then spur a systemic resistance in the plant which decrease or inhibit pathogen invasion.

Protease enzymes produced by endophytic bacteria have a role in degradation of cell wall of pathogens. Siddiqui and Shaukat (2003) reported that filtrate of *P. fluorescens* contains protease which reduces egg hatch of *M. javanica* nematode. In addition to

Table 5. Physiological characters of five isolates of endophytic bacteria on patchouli plants to control *Pratylenchus brachyurus*.

Isolates	Proteolytic activity	Lipolytic activity	HCN	Fluorescense activity	Chitinolytic activity
A. xylosoxidans TT2	+	-	-	-	+
P. putida EH11	+	+	-	-	-
B. subtilis NJ57	-	-	+	+	-
B. cereus MSK	+	+	-	-	-
A. faecalis NJ16	-	+	-	-	-

+ = positive reaction, - = negative reaction

degrade the cell wall, protease can be used by endophytic bacteria to penetrate actively plant tissues. Benhamou *et al*. (1996) reported that pektinase and cellulase enzymes produced by *P. fluorescens* can be used by this bacterium to colonize intercellular area of root cortex tissue.

Hydrogen cyanide (HCN) is a secondary metabolites produced by *Pseudomonas fluorescens* and other *Pseudomonas* species. HCN produced by *Corynebacterium paurometabolu* can kill larvae and inhibit egg hatching on nematodes (Mena and Pimentel 2002) and HCN produced by *P. fluorescens* can control several pathogens, including *Pythium ultimum* on sugar beet (Wiyono 2003).

CONCLUSION

Endophytic bacterial population in roots of patchouli ranged from 2.3 x 10^2 to 6.0 x 10^5 cfu g^{-1} roots. Sixty isolates (23.34%) were potential as biocontrol agents to parasitic nematode *P. brachyurus* on patchouli, 72 isolates (28.01%) stimulated plant growth, and 32 isolates (12.47%) inhibited plant growth. Antagonistic activity of five potential endophytic bacteria (TT2, NJ16, MSK3, EH11 and NJ57) decreased nematode population in root and increased plant growth.

Based on the molecular identification using 16S rRNA universal primers, the five endophytic bacteria isolates were identified as *Achromobacter xylosoxidans* TT2, *Alcaligenes faecalis* NJ16, *Pseudomonas putida* EH11, *Bacillus cereus* MSK3, and *Bacillus subtilis* NJ57. The mechanism of endophytic bacteria in reducing nematode population was attributed with their capability in producing extracellular enzymes such as chitinase, protease and lipase. Further study is justified to test these five potential endophytic bacteria in field scale experiment.

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