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Effects of bacterial endophytes from potato roots and tubers on potato cyst nematode (*Globodera rostochiensis*)

NOOR ISTIFADAH♥, NURHAYATI PRATAMA, SYAHRIZAL TAQWIM, TOTO SUNARTO
Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Padjadjaran. Jl Raya Bandung-Sumedang Km 21, Jatinangor, Sumedang 45363, West Java, Indonesia. Tel./fax.: +62-22-7796316. ♥email: n.istifadah@unpad.ac.id

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Abstract. *Istifadah N, Pratama N, Taqwim S, Sunarto T. 2018. Effects of bacterial endophytes from potato roots and tubers on potato cyst nematode* (Globodera rostochiensis). *Biodiversitas 19: 47-51.* Bacterial endophytes are bacteria that inhabit plant tissues without causing any diseases. The endophytes existence may have negative, neutral, or positive effects on the host plants. This paper discusses the effects of bacterial endophytes isolated from potato roots and tubers on potato growth and their abilities to suppress potato cyst nematode, *Globodera rostochiensis*. The bacterial endophytes were isolated from roots and tubers of potatoes obtained from six plantation areas in West Java. The endophyte isolates were examined for their effects on potato growth. The non-pathogenic isolates were tested for their abilities to suppress *G. rostochiensis* in vitro and in potato plants. The results showed that from 88 bacterial endophyte isolates obtained, 13 isolates caused rot in potato seed pieces, 22 isolates inhibited the potato growth, while, 2 isolates increased the growth, and as many as 51 isolates did not influence the growth. The in vitro test using the isolate culture filtrate revealed that there were seven isolates that caused mortality of *G. rostochiensis* juvenile-2 by 67.5-97.7%. These isolates, however, were not effective in damaging the nematode eggs. In the greenhouse experiment, the bacterial endophyte isolates suppressed the number of cysts by 51.7-65.4% and that of the juvenile-2 of *G. rostochiensis* by 48.6-76.4%.

Keywords: Culture filtrate, eggs, in vitro, juvenile, non-pathogenic isolates

INTRODUCTION

Bacterial endophytes are the bacteria inhabiting plant tissues, for part or all of their life, without causing any apparent disease symptom (Stone et al. 2000; Schulz and Boyle 2008). Based on their life strategy, bacterial endophytes can be divided into three types: obligate endophytes that live inside the plant for their whole life; facultative endophytes that colonize the plant tissues for part of their life cycle; and passive endophytes that enter the plant tissues just by chance, such as via wounded tissues (Gaiero et al. 2013). Endophytes can be found in any plant and reside in any part of the plant organs (Ryan et al. 2008; Schulz and Boyle 2008). Bacterial endophytes may be originated from rhizosphere, phyllosphere or transmitted via seed (Sturz et al. 2000; Ryan et al. 2008).

The occurrence of bacterial endophyte within the plant tissues can exert either harmful, neutral or beneficial effect on the host plants (Rosenblueth and Martínez-Romero 2006; Ryan et al. 2008). In some cases, the bacteria isolated from healthy tissues are latent pathogens that can become pathogenic to the host plant upon favorable condition. Many bacterial endophytes do not pose any effect on the host plants and are therefore considered as neutral. In many cases, bacterial endophytes provide beneficial effects to the plants such as improving plant tolerance to abiotic stresses (Miliute et al. 2015), promoting the plant growth, and suppressing the plant's pests and diseases (Berg and Hallmann 2006; Ryan et al. 2007; Gaiero et al. 2013; Chaturvedi et al. 2016).

The ecological niche similarity between endophytes and plant pathogens, make them suitable for biocontrol agents of plant diseases (Berg et al. 2005; Ryan et al. 2008). The mechanisms of bacterial endophytes in suppressing plant pathogens are similar to that of other antagonistic bacteria, including competition, production of antimicrobial secondary metabolites, and induction of plant resistance (Ryan et al. 2008; Gaiero et al. 2013). The bacteria isolates obtained from healthy plant tissues may include latent pathogens. Thus, the isolates used for biological control agents must be tested for their effects on the host plant. The selected isolates must have neutral or even positive impact on the plant growth.

Potato is one of the important crops for direct consumption and food industries. One of the limiting factors in potato production is potato cyst nematode (*Globodera rostochiensis*). The nematode infection leads to a reduction of potato growth and tuber size. This nematode is difficult to control as it produces cysts that can survive for an extended period in the soil (Brodie et al. 1998). Healthy potato plants may retain bacterial endophytes potential for biological control agents of the cyst nematode disease. The potential of bacterial endophytes for inhibiting fungal and bacterial plant pathogens have been reported (Sturz et al. 1999; Reiter et al. 2002; Sessitsch et al. 2004; Berg et al. 2005). The efficacy of bacterial endophytes to suppress plant-parasitic nematodes have been reviewed (Siddiqui and Shaukat 2003; Tian et al. 2007). This study examined bacterial endophytes isolated from potato roots and tubers and evaluated their effects on potato growth and potato cyst nematode. The endophyte isolates effectively in

suppressing the nematode can be further developed for biological control agents of the disease.

MATERIALS AND METHODS

Isolation of bacterial endophytes

Bacterial endophytes were isolated from potato tubers and roots obtained from several areas in Garut (Samarang, Cikajang, Cisurupan), Bandung (Pangalengan, Ciwidey), and Bandung Barat (Lembang) of West Java, Indonesia. The potato tubers and roots were cleaned up with running tap water. The roots were cut into 1.5-2-cm segments, while the tubers were peeled and the rind was cut into pieces (0.5 x 0.5 cm). The segments were surface sterilized by submersion in 96% ethanol for 1 minute, followed by submersion in a solution containing 2% chlorine for 3-5 minutes and submersion in 96% ethanol for 30 seconds. The potato segments were imprinted on Potato Dextrose Agar (PDA) to ensure that the isolated bacteria were endophytes. The surface sterilization was considered successful if the imprint was free from microbial growth. The segments were then macerated in water 1: 2 (w: v) and the suspension was spread over a Nutrient Agar (NA), and incubated at room temperature for five days. Single bacterial colonies with different characteristic were sub-cultured to obtain pure cultures.

Effects of bacterial endophyte isolates on potato growth

To examine the possibility that the isolated bacteria might be latent pathogens, the effect of each of the endophyte isolates on the growth of potato were examined. Bacterial endophytes were inoculated by soaking potato seed tubers in the bacterial suspension (10^7 cfu.mL^{-1}) for an hour. The inoculated tubers were planted on a growth medium consisting of sterilized soil with 5% charcoal husk. 30 mL bacterial suspension was drenched in the planting hole just before planting the potato tuber. For the control, the potato tuber was soaked in sterile water for an hour.

The potato growth was observed every week by recording the plant height and the numbers of leaves. The isolates that caused disease symptoms on the potato tubers or plants were considered as pathogenic. The bacterial isolates whose inoculated plants exhibited a relative shoot or root fresh weight (as compared to the fresh weight of the control plant) < 0.5, were categorized as inhibitory bacteria. Whereas, the isolates whose inoculated plants showed a relative fresh weight > 1.5 were classified as growth-promoting bacteria. The isolates that caused disease symptoms or inhibited the potato growth were excluded from the further test.

Effects of bacterial endophyte isolates on *Globodera rostochiensis* in vitro

The bacterial endophytes were cultured on nutrient broth. Bacterial cultures (three inoculating loops) were inoculated to the 100 mL liquid media. The inoculated media were homogenized using vortex mixer and incubated on the orbital shaker (180 rpm) for 5 days. The filtrate was collected, centrifuged and filtered (with microfilter 0.2 µm pore size).

Globodera rostochiensis inocula were prepared from the nematode cysts extracted from infested soil using floatation method. In the floatation method, the soil was mixed with sterile water containing 0.05% glucose (1: 2, v/v), stirred and incubated for 5 min. The floated debris was filtered, and the cysts were collected under a microscope. The cyst was then ruptured and soaked in sterile water for 2-3 days to obtain the nematode's second-stage juveniles (J2) and eggs.

The endophyte culture filtrate (2 mL) was mixed with 1 mL of *G. rostochiensis* inoculum suspension (about 40-45 J2 and eggs/mL) and then incubated for 72 hours. For control, the nematode suspension was mixed with nutrient broth medium. The percentage of J2 mortality and damaged eggs were observed and counted under a stereo microscope. The isolates that caused J2 mortality or eggs damage ≥50% were tested again to confirm their effects and for statistical analysis. If the mortality also occurred in control treatment, the mortality data upon treatments were corrected with respect to the control using Abbot's formula (Abbott 1987). The bacterial isolates that were effective in causing mortality of *G. rostochiensis* in vitro were identified based on their biochemical characteristics. This identification was conducted in Microbiology Laboratory, School of Life Sciences and Technology, Bandung Institute of Technology (ITB), Bandung, West Java, Indonesia.

Effects of bacterial endophyte isolates on *Globodera rostochiensis* in potato

The bacterial isolates that caused J2 mortality ≥ 50% in vitro were used for experiments in the potato plant. The experiment was arranged in a randomized complete block design with treatments consisted of bacterial endophytes isolates and control**.** Each treatment was replicated three times. The bacterial endophytes were applied by soaking potato tubers in the bacterial suspension for one hour. The bacteria were also applied in the potato planting holes (30 mL suspension per planting hole). Two-week-old potato plants were taken, and their roots were washed, then transplanted into a polybag containing 2 kg of pasteurized growth medium. These washing and transferring measures were taken to ensure that the tested bacteria acted as endophytes rather than as rhizosphere bacteria. The nematode was then inoculated one week later. The suspension of nematode inocula containing about 4000 J2 and eggs of *G. rostochiensis* was pipetted into the 5 holes surrounding the plants (about 5 cm from the basal stem).

The observation was conducted 7 weeks after the nematode inoculation. The variables observed were numbers of cysts and juvenile-2 of *G. rostochiensis* in 100 g soil. Suppression of *G. rostochiensis* was determined as the number of cyst or J2 observed upon bacterial isolates treatment compared to that in control. The other variables observed were fresh weights (FW) of potato shoot and roots. Relative fresh weight of shoot and root were determined as the fresh weight of the treated plant compared to the fresh weight of the control plant.

Data analysis

Data were subjected to analysis of variance (ANOVA) using SPSS software (SPSS ver. 20). The data were checked for normality and transformed if necessary. Treatments with significant differences were further analyzed using Tukey's honestly significant difference (HSD) test (P≤ 5%).

RESULTS AND DISCUSSION

Effects of bacterial endophyte isolates on potato growth

The isolation of bacterial endophytes from healthy potato roots and tubers resulted in 88 bacterial isolates. In general, the potato roots yielded a higher number of bacterial endophyte isolates than the potato tubers. The number of isolates obtained from the roots were 69 isolates, while that from the tubers were 19 isolates. Istifadah et al. (2016) also found that the number of endophytic fungal isolates obtained from potato roots was higher than that from the potato tubers.

The influence of the bacterial endophyte isolates obtained from potato roots and tubers on the potato growth varied depended on the isolates. Most of the isolates (51 isolates) did not affect the potato growth, and only 2 of them promoted the potato growth (Table 1). The rest of the isolates, however, inhibited the potato growth or even being pathogenic to the plant. Sturz (1995) also found that bacterial endophytes from potato tubers could be either plant-growth-promoting, growth-retarding or neutral endophytes. The variability effects of endophytes on potato growth were also found in fungal endophytes isolated from potato roots and tubers (Istifadah et al. 2017).

In this study, 13 isolates caused potato tuber rot upon their inoculations. These isolates could be latent pathogens that can become pathogenic to the potato plants upon favorable conditions. Reiter et al. (2002) found that some of the bacterial endophytes isolated from potato stem caused disease symptom to potato plant. Berg et al. (2005) also reported that microbiota community in an apparently healthy potato contained some plant pathogenic bacteria such as *Erwinia amylophora*.

Among the bacterial endophyte isolates tested in this study, there were two isolates from potato roots (CKA10 and CISA17) that increased the potato growth. Binod et al. (2014) also found growth-promoting effects of bacterial endophytes from potato roots. The stimulation of plant growth by bacterial endophytes can be due to direct mechanisms such as facilitation in nutrients acquisition, modulation or production phytohormones (Gaiero et al. 2013; Chaturvedi et al. 2016) such as indole acetic acids (Binod et al. 2014).

Effects of bacterial endophytes on *G. rostochiensis* in vitro

Primary in vitro screening experiment was conducted to select bacterial isolates that effectively kill *G. rostochiensis.* The results of in vitro test showed that the culture filtrates of bacterial endophytes had toxic effects to J2 of *G. rostochiensis*. The juveniles incubated in the bacterial culture filtrate were dead, in which the nematode body became stiff, and their cytoplasm was aggregated. In some cases, the nematode cell walls were ruptured or lysed. Bacterial endophytes can produce a range of antimicrobial secondary metabolites (Strobel and Daisy 2003; Brader et al. 2014).The toxicity of the bacterial endophyte culture filtrate was varied depending on the isolates. Most of the bacterial isolates were not effective in causing mortality of *G. rostochiensis* J2. Our primary screening experiment revealed that there were only seven isolates that caused mortality of the nematode ≥50% and just two isolates that resulted in the nematode mortality of more than 70% (Table 2). The isolates that showed mortality of J2 ≥ 50% were used for the second in vitro experiments.

The result of second in vitro experiment confirmed the result of the first experiment. All the bacterial isolates tested caused 69.1-97.7% mortality of *G. rostochiensis* J2 (Table 3). The isolates that resulted in the nematode mortality ≥ 80% were identified as *Bacillus carotarum, Bacillus cereus*, and *Pseudomonas pseudoalcaligenes*. The genera of *Bacillus* and *Pseudomonas* have been known as frequently occurring bacterial endophytes in agricultural crops (Seghers et al. 2004; Miliute et al. 2015). Nematicidal effects of *Bacillus* including those of *B. cereus* on *Meloidogyne javanica* and *M. incognita* have been reported (Moghaddam et al. 2014; Gao et al. 2016). *B. cereus* produced nematicidal compounds such as sphingosine and phytosphingosine (Gao et al. 2016) and cell wall degrading enzyme, protease (Ann 2013). Meanwhile, culture filtrate of *Pseudomonas* spp. has been reported to have nematicidal effects on *M. javanica* (Ali et al. 2002).

Even though the culture filtrates of the bacterial isolates tested were effective causing mortality of *G. rostochiensis* J2, they were not effective in damaging the nematode eggs. The numbers of eggs damaged in all treatments of bacterial endophyte isolates were not significantly different to the control (Table 4). The cell walls of the nematode eggs are thicker than the juvenile's cell walls and therefore it was difficult to be penetrated by toxic metabolites.

Effects of bacterial endophytes on *G. rostochiensis* in potato plant

Seven isolates that showed antagonistic effects to *G. rostochiensis* in vitro were examined for their efficacy in potato plant. The results showed that all seven isolates reduced the number of J2 by 48.6-76.4% and the number of cysts in potato rhizosphere by 51.7-58.6% (Table 5). The isolates whose culture filtrates showed relatively high toxicity to J2 of *G. rostochiensis* in vitro also suppressed the nematode in the soil by more than 70%. This result indicates that toxic metabolites from the bacterial endophytes may involve in suppressing the nematode. Other mechanisms that may contribute to the suppression of *G. rostochiensis* includes the competition on niche occupation and induction of plant resistance (Tian et al. 2007).

Table 1. The effect of fungal endophyte isolates on potato plants

Effect on potato plants	Number of isolates	Percentage of isolates (%)
Pathogenic	13	14.8
Inhibited the growth	22	25.0
Neutral	51	57.9
Increase the growth	2	2.3

Table 2. Primary screening of bacterial endophyte isolates efficacy on viability of *G. rostochiensis* J2

Percentage of dead J2 (%)	Number of Isolates	Percentage of isolates (%)
≤ 30	79	89.8
31-49	3	3.4
50-69	5	6.8
≥ 70	2	1.1

Table 3. In vitro effects of selected bacterial endophyte isolates on *G. rostochiensis* J2 viability

Treatments	J2 mortality (%)	Corrected J2 mortality (%)
Isolate PWA-5 (*B. carotarum*)	92.6 b	91.7
Isolate CISU-2 (*B. carotarum*)	85.1 b	83.2
Isolate CISA-3	78.2 b	75.5
Isolate CISA-4 (*B. cereus*)	88.3 b	86.8
Isolate CISA-15	74.6 b	71.4
Isolate CISA-18 (*P. pseudoalcaligenes*)	97.9 b	97.6
Isolate LBA-10	70.3 b	66.6
Control	11.1 a	-

Note: Values are mean of three replicates. Values in each column followed by different letters are significantly different based on Tukey's HSD test ($P < 0,05$).

Table 4. In vitro effects of selected bacterial endophyte isolates on *G. rostochiensis* eggs survival

Treatments	Percentage of damaged eggs (%)	Corrected percentage of damaged eggs (%)
Isolate PWA-5 (*B. carotarum*)	25.2 a	20.3
Isolate CISU-2 (*B. carotarum*)	36.0 a	31.8
Isolate CISA-3	36.5 a	32.4
Isolate CISA-4 (*B. cereus*)	33.1 a	28.8
Isolate CISA-15	19.8 a	14.6
Isolate CISA-18 (*P. pseudoalcaligenes*)	21.5 a	16.4
Isolate LBA-10	19.7 a	14.5
Control	6.1 a	-

Note: Values are mean of three replicates. Values in each column followed by different letters are significantly different based on Tukey's HSD test ($P < 0.05$).

Table 5. Effects of the bacterial endophytes on *G. rostochiensis* in potato rhizosphere (7 weeks after nematode inoculation)

Treatments	Juvenile-2		Cysts	
	Number /100 g soil	Suppression rate (%)	Number /100 g soil	Supression rate (%)
Isolate PWA-5 (*B. carotarum*)	22.3 b	73.4	8.0 b	58.6
Isolate CISU-2 (*B. carotarum*)	20.2 b	75.8	6.7 b	65.4
Isolate CISA-3	40.7 c	51.3	9.3 b	51.7
Isolate CISA-4 (*B. cereus*)	23.7 b	71.7	8.3 b	56.9
Isolate CISA-15	43.0 c	48.6	8.3 b	56.9
Isolate CISA-18 (*P. pseudoalcaligenes*	19.7 b	76.4	8.7 b	55.1
Isolate LBA-10	27.7 bc	66.9	8.0 b	58.6
Control	83.7 d	0.0	19.3 c	0.0
Nematicide (carbofuran)	0.3 a	99.6	0.0 a	100.0

Note: Values are mean of three replicates. Values in each column followed by different letters are significantly different based on Tukey's HSD test ($P < 0.05$).

Table 6. Effects of bacterial endophytes on the potato growth infected with *G. rostochiensis* (7 weeks after inoculation)

Isolates	Shoot weight (g FW)	Relative shoot weight (times)	Root weight (g FW)	Relative root weight (times)
Isolate PWA-5 (*B. carotarum*)	8.26 a	1.2	15.26 ab	1.4
Isolate CISU-2 (*B. carotarum*)	9.13 a	1.3	17.60 ab	1.6
Isolate CISA-3	8.56 a	1.3	17.86 ab	1.6
Isolate CISA-4 (*B. cereus*)	8.20 a	1.2	15.96 ab	1.4
Isolate CISA-15	8.86 a	1.3	17.13 ab	1.6
Isolate CISA-18 (*P. pseudoalcaligenes*)	9.00 a	1.3	17.20 ab	1.6
Isolate LBA-10	8.53 a	1.3	20.80 b	1.9
Control	6.76 a	-	11.00 ab	-
Nematicide (carbofuran)	5.90 a	0.9	9.06 a	0.8

Note: Values are mean of three replicates. Values in each column followed by different letters are significantly different based on Tukey's HSD test ($P < 0.05$).

In this experiment, the bacterial endophytes did not significantly improve the potato growth. However, the relative fresh shoot weight of the potato, treated with the bacterial endophytes were 1.2-1.3 times higher compared to the control plants. The potatoes treated with the bacterial isolates also exhibited a higher relative root weight (1.4-1.9 times) compared with the control (Table 6). The isolates used in this experiment were not the ones that promoting the growth in the selection step. The tendency of growth improvement in the endophyte-treated plants may be related to the plant's health, in which the treated plants were healthier than the control plants. Backman and Sikora

(2008) stated that root endophytic community is an important regulator of root health.

The overall results of this study showed that the effects of bacterial endophytes from potato roots and tubers were varied. Some isolates showed antagonistic effects on *G. rostochiensis* in vitro and *in vivo*, and therefore, they are potential agents for biological control of potato's parasitic nematodes. Their abilities to reduce the number of juvenile-2 and cysts of *G. rostochiensis* in the soil can provide short-and long-term improvements in soil health. Bacterial endophytes can be utilized as biological control agents because they can suppress the nematode after penetration. Their existences within plant tissues ensure the availability of nutrients resources and protection from unfavorable conditions and fast-growing competitors. The bacterial isolates can be combined with other antagonistic microbes in the rhizosphere to improve the biological control effects. The development of biological control for nematodes is part of, integrated and eco-friendly disease management that supports sustainable agriculture.

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