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Use of endophytic bacteria from roots of *Cyperus rotundus* for biocontrol of *Meloidogyne incognita*

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Abstract. *Mardhiana, Pradana AP, Adiwena M, Santoso D, Wijaya R, Murtilaksono A. 2017. Use of endophytic bacteria from roots of* Cyperus rotundus *for biocontrol of Meloidogyne incognita. Biodiversitas 18: 1308-1315.* Yield loss due to *M. incognita* infection in tomato plants cultivation can reach 60%. The problem is able to be solved through the application of endophytic bacteria. In this study, endophytic bacteria from root *Cyperus rotundus* were isolated using Tryptic Soy Agar media. The bacteria isolates were then tested their safety against plants and mammals. The phenotypic and physiological properties of selected isolates were characterized and tested to know their resistance to antibiotics, and their ability in suppressing the infection rate of *M. incognita* on tomato. Eighteen bacterial isolates were obtained and 8 of them are categorized as safe bacteria for plants and mammals, which could be used in further tests. A result of the physiological test showed that bacterial isolates were able to produce protease enzyme (87.5%), chitinase enzyme (62.5%), and HCN (37.5%), having urease activity (75%) and could dissolve phosphate (87.5%). Based on the test results, all endophytic bacteria effectively increased tomato growth and suppressed the severity of *M. incognita* infection with the most stable isolate as a biocontrol agent of *M. incognita* was CRS16.

Keywords: Antibiotic resistance, biosafety, lytic enzymes, plant growth

INTRODUCTION

Root-knot nematodes (RKN, *Meloidogyne* spp.) are one of plant pest, which has significant negative impact on the tropical area. They can have a wide host range and interact with other pathogens causing the yield loss in some agricultural commodities. Infestation of *Meloidogyne* spp. can reduce the production up to 40-60% on tomato, 35% on potato, and 20% on bean. Symptoms caused by infestation with *M. incognita* are indicated by gall at the root. The existence of gall can disrupt water and minerals distribution systems from the soil through the roots to all parts of the plant leading to the disruption of new root growth, plant wilting, slow plant growth, stunted growth, and chlorosis (Moens et al. 2009). *Meloidogyne* spp. Infestations can also increase the severity of the wilt disease caused by *Fusarium oxysporum* and *Ralstonia solanacearum* in tomato (Muthamia and Ravichandra 2012).

Researches on chemical, physical and mechanical control of nematodes had been conducted, but the nematodes infestation is still a major problem, which could not be fully resolved. One of efforts to control nematodes is using endophytic bacteria as biocontrol agents. According to Hardoim et al. (2015), endophytic bacteria are bacteria living in plant tissues without causing disease symptoms within the plant and can be isolated from sterilized surface of plant tissue. Action mechanisms of endophytic bacteria as biocontrol agents include the production of antimicrobial compounds, spatial and nutrients competition, micronutrient competition, the production of siderophores, and the induction of plant resistance against pathogen infestation.

Biological control using endophytic bacteria is one of the alternative controls, which can be expected to tackle the issue. The endophytic bacteria were reported to effectively control nematodes in several crops. Endophytic bacteria isolated from potato such as *Pseudomonas* and *Streptomyces* species were reported to be able to reduce the population of *Meloidogyne incognita* (Krechel et al. 2002). Application of endophytic bacteria through seed treatment could reduce 30-50% galls of *M. incognita* on tomato (Munif et al. 2013). Treatment of *P. chlororaphis* strain Sm3 in strawberries could reduce population of root-lesion nematode (*Pratylenchus penetrans*) by 41-61% and was able to enhance plant growth (Hackenberg et al. 2000).

The advantages of endophytic bacteria as biocontrol agents are their ability to increase the growth of plants, known as Plant Growth Promoting Bacteria (PGPB), by increasing the availability of nutrients, and also their ability to produce the growth hormone for inducing plant resistance, known as induced systemic resistance (ISR). ISR is an alternative mechanism to control the broad-spectrum plant diseases (Pieterse et al. 2014, Chebotar et al. 2015).

The endophytic bacteria were reported to have a broad host specificity. Mekete et al. (2009) have successfully isolated endophytic bacteria from coffee plant to control the root-knot nematodes on tomato. In addition, Mohanty et al. (2017) have also isolated endophytic bacteria from *Jatropha curcas* to promote the growth of maize.

Therefore, there are great opportunities to explore the endophytic bacteria from various plants. The species of plant that their endophytic bacterial effectiveness as a biocontrol agent has not been widely reported is *Cyperus rotundus*. Therefore, this study was aimed to isolate and characterize the most effective endophytic bacteria originated from *C. rotundus* roots as a biocontrol agent of *Meloidogyne incognita* on tomato.

MATERIALS AND METHODS

Study area

This research has been done in the laboratory and greenhouse experiment at Laboratory of Plant Nematology (Department of Plant Protection), and green house Faculty of Agriculture, Bogor Agricultural University, Bogor, Indonesia.

Isolation of endophytic bacteria

The endophytic bacteria were isolated from the roots of *C. rotundus*. Samples were taken around Bogor Agricultural University campus, Indonesia. Roots of plant were taken as much as 1 g, which was then washed using tap running water to remove soil particles. Roots surface were further sterilized using 1% sodium hypochlorite (NaOCl) solution for 1 minute, 70% alcohol for 1 minute, and rinsed with sterile distilled water 3 times. Subsequently, the roots were dried using a sterile tissue paper. Samples were further placed on the Tryptic Soy Agar (TSA) media to determine the success of surface sterilization.

Sterile root samples were then macerated using a sterile mortar until smooth with the addition of 1: 10 (w/v) distilled water. The suspension was diluted 10-fold using sterile distilled water. A total of 0.1 mL 10^{-3} dilution suspension was further spread on 20% TSA media using sterile microbiology glass beads then incubated at 28°C for 72 hours. Single colonies with different colony shape, size, texture, and color were further sub-cultured in respective media (100% TSA), followed by the incubation for 48 hours.

Hypersensitivity reactions test

The endophytic bacteria were grown on TSA media using 9 cm diameter petri dish and incubated for 24 hours. The growing bacteria were then harvested using 2 mL of sterile distilled water. The suspension was further infiltrated on the leaf blade of tobacco (Kemloko 3 variety) at the bottom and incubated for 48 hours. The occurrence of necrosis on leaves of tobacco plants was observed. Bacteria that did not show necrosis on leaves of tobacco were used for further testing (Klement and Goodman 1967).

Hemolysis activity test

Endophytic bacteria with no indication of hypersensitivity reaction were used in this test. The bacteria were grown on blood agar media, incubated for 24 hours. The formation of hemolysis zones was observed. α-hemolysis toxin produced by endophytic bacteria would form a dark zone, while β-hemolytic toxin would form a light zone, and αβ-hemolysis toxin will form a zone of light followed by a bit dark around the colony. Bacteria with hemolytic activity were not used in the further test (Tille 2015).

Characterization of phenotypic and physiological properties of endophytic bacteria

Phenotypic characters of endophytic bacteria colonies

The phenotypic performance of Endophytic bacteria colony was observed based on the shape of the colony, size, texture, color, and elevation.

Gram staining

Endophytic bacteria Gram staining was conducted using Himedia Gram-stain kit. 24-hour-old bacterial colonies were taken using a needle loop and placed on a slide surface contained a drop of sterile water. The suspension of bacteria on the slide was flattened and passed through the bunsen burner.

Crystal violet was dripped over the preparation of bacteria that had been fixed, left to stand for 1 minute and then washed with distilled water and left for a while. Iodine solution was dripped on the dried preparations, left to stand for 1 minute and washed with 96% alcohol until the solution flow was not colored. Preparations were washed with distilled water and left for a moment. The next step, dried preparations were dripped with safranin, left to stand for 1 minute and washed again using distilled water. Observations were conducted after preparations were dried, using a light microscope with a magnification of 1000x. Gram-positive bacteria bound the crystal violet after washing with alcohol so that preparations would be dark violet after staining and Gram-negative bacteria did not bind the crystal violet so they would be red after staining (Beveridge 2001).

Proteolytic activity

Proteolytic activity was tested using skim milk media at pH 6.5. Media composition consisted of 10 g of skim milk, 15 g of TSB, 7.5 g of agar and 500 mL of distilled water. Agar, TSB and distilled water were sterilized by autoclaving at 121°C for 15 minutes. After sterilization was finished, skim milk was further added when the media was still hot. Bacterial cultures were streaked on the media added to skim milk. Proteolytic activity was indicated by a clear zone around the colonies of bacteria, 48 hours after treatment (Sokol et al. 1979).

Chitinolytic activity

Test medium used was 1% chitin, consisted of 15 g of agar, 5 g of glucose, 2 g of peptone, 10 g of colloidal chitin, 0.5 g of K_2HPO_4, 0.5 g of MgSO4, 0.5 g of NaCl in 1 L of distilled water with a pH of 6.2. Bacterial cultures were streaked onto the surface of the medium and incubated at room temperature for 4 days. Chitinolytic activity was observed with the clear zone around the bacterial streak (Kuddus and Ahmad 2013).

HCN production

The medium used to grow the bacteria consisted of 4.4 g of glycine, 30 g of TSB, 15 g of agar in 1 L of distilled water. The medium was poured into a 9 cm diameter petri dish. Preparation of cyanide detection solution (CDS) paper was performed using 2 g of picric acid and 8 g of sodium carbonate, then dissolved in 200 mL of sterile distilled water. The solution was used to soak the 1x1 cm sterilized filter paper. The filter paper was soaked up to bright yellow; then the filter paper was dried in a laminar air flow.

The endophytic bacteria were streaked at test medium. Filter paper which had been dipped in a solution of CDS was attached to the inside of the petri dish lid. The bacteria were incubated for 7 days at room temperature, and then on the 7th day of treatment, the color filter paper was observed. Bacteria that can produce cyanide was detected by a color change on filter paper from yellow to orange-brown (Lorck 1948).

Urease activity

The test was performed on NFB semi-solid medium with pH of 6.8. The compositions of this medium were 0.5 g of malic acid, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of NaCl, 0.02 g of $CaCl_2.2H_2O$, 2 mL of the micronutrient, 2 mL of Bromthymol blue, 4 mL of Fe (III) EDTA (1.64%), 1 mL of vitamins solution, and 0.5 g of agar. The micro-nutrient solution used in this medium were conducted with the composition of 0.4 g of $CuSO_4.5H_2O$, 0.12 g of $ZnSO_4.7H_2O$, 1.4 g of H_3BO_3, 1 g of $Na_2MoO_4.2H_2O$, 1.5 g of $MnSO_4.H_2O$, in 1 L of distilled water. Vitamins solution was conducted with the composition of 10 mg of biotin, 20 mg of Pyridoxol HCL in 1 L of distilled water.

The test was performed by growing 1 mL of the bacterial suspension with the density of 10^8 cfu mL^{-1} in 9 mL of NFB semi-solid media and incubated for 48 hours. Bacterial ability in fixing nitrogen was indicated by a color change, turned blue or dark blue, and mucus or pellicle layers on the surface of the media appeared (Baldani et al. 1986).

Phosphate dissolving activity

The test was performed using Pikovskayas Agar medium (Himedia, India). Bacterial cultures were streaked onto the surface of the medium and incubated at room temperature for 48 hours. Bacterial ability in dissolving phosphate was indicated by the clear zone around the bacterial streak (Sharma et al. 2011).

Antibiotic resistance test

24-hours-old endophytic bacteria isolates were grown in TSB medium containing (25, 50, 75, and 100) µg mL^{-1} of chloramphenicol, amoxicillin and rifampicin antibiotics. If the media color turned murky then the isolates were resistant to antibiotics with the tested doses. The test was repeated 3 times (Vibhaw et al. 2017).

Propagation of *Meloidogyne incognita* inoculum

M. incognita used as materials was taken from the collection of Plant Nematology Laboratory, Department of Plant Protection, Bogor Agricultural University. The nematode was reproduced on Tantyna F1 variety of tomato plants.

Effect of filtrate culture of endophytic bacteria on nematode egg hatching

The endophytic bacteria were grown in 100 mL of TSB medium and shaken for 24 hours at room temperature. After 24 hours, the suspension of the endophytic bacterial was centrifugated at 12000 rpm for 15 minutes. Furthermore, the supernatant was filtered using 0.22 µm and diameter of 25 mm sterile syringe filter. The filtered supernatant was used to test the ability of endophytic bacteria on egg hatching of *M. incognita*.

M. incognita eggs isolated from extract of the roots were sterilized by soaking in a solution containing 600 ppm of Streptomycin sulfate for 20 minutes then washed and soaked in sterile water for 30 seconds. About 0.5 mL (containing 50 eggs) of suspension of *M. incognita* eggs was added into 5 mL of endophytic bacterial filtrate and incubated at room temperature for 48 hours. As a control, the eggs were added into 5 mL of sterile distilled water. After incubation, the eggs were washed with sterile water and incubated at room temperature for 14 days.

This experiment used a completely randomized design (CRD) with bacterial culture filtrate as fixed factor and each isolate was repeated 3 times. The percentage of eggs hatching indicating nematodes was out of the cuticle (the egg protectors) was recorded. Data were then analyzed using DSAASTAT program version 1.021 (Siddiqui and Shaukat 2003).

Effect of filtrate culture of endophytic bacteria on mortality of *M. incognita* J2

The test of the effect of bacterial filtrate on *M. incognita* was conducted by taking a 5 mL of bacterial culture filtrate and put into a small petri dish (diameter of 6 cm) then 0.5 mL of the suspension of *M. incognita* juvenile (containing 100 juveniles) was added and stored at room temperature for 24 hours. This test used a completely randomized design (CRD) with bacterial culture filtrate as fixed factor and repeated 3 times. As a control, sterile water and TSB were used to prove that *M. incognita* juveniles died because of bacterial filtrate. Observations were conducted on the dead and living *M. incognita*. The dead *M. incognita* were characterized by straight shape and did not move after 2 hours in sterile water. The data were then analyzed using the DSAASTAT program version 1.021 (Siddiqui and Shaukat 2003).

Selection of endophytic bacterial as RKN controlling agent and plant growth promoter

The endophytic bacteria were grown in TSB medium and incubated for 48 hours. F1 Tantyna tomato variety seeds sterilized using NaOCl 1% for 1 minute and sterile distilled water were soaked in the suspension of endophytic bacteria for 12 hours. Then, tomato seeds were sown in sterile soil and after four main leaves appeared, the seeds were moved and planted in pots with a diameter of 15 cm. Planting was conducted in a completely randomized design

(CRD) with three replications, each test consisted of two test plants. Afterward, tomato plants were watered with 100 mL of the bacterial suspension with a density of 10^8 cfu mL^{-1} at the first, 2nd, and 5th week. Watering was conducted around the base of the stem. Watering was applied in the afternoon at 16.30.

M. incognita infestation was performed 1 month after tomato plants were moved. Total *M. incognita* infested to the plants was 500 nematodes in the infective stage (J2). Measurement of observation variables was conducted at 40 days after inoculation. Agronomic variables included height, root length, both fresh and dry weight of plants. Pathological variables included the number of nematodes per 5 g of roots and the number of gall at the root. Data analysis was performed at the end of the observation using analysis of variance at the 5% level confidence. If they are significantly different then Duncan Multiple Range Test (DMRT) at 95% level confidence was applied. The analysis was performed using DSAASTAT program version 1.021 (Munif et al. 2013).

RESULTS AND DISCUSSION

Biosafety of endophytic bacteria

Eighteen isolates of endophytic bacteria were isolated from the roots of *C. rotundus*. Among those bacteria isolates, 33.33% (6 isolates) of them caused necrosis based on hypersensitivity reaction test. Therefore, those isolates were not used in the next test. The remained isolates (12 isolates) were tested for their safety using hemolysis test. The hemolysis test showed that 16.66% of endophytic bacteria were able to produce β-hemolysis toxins, and other 5.55% were able to produce α-hemolysis toxins. Bacteria with the indication of both negative hypersensitivity test and negative toxic hemolysis test were used in the further test. The biosafety test showed that eight isolates from plant roots of *C. rotundus* could be used in the advance test (Table 1).

Phenotypic characters and endophytic bacterial Gram

Among 8 isolates of endophytic bacteria, 100% of colonies were circular of shape. Colony size of endophytic bacteria is varied; the observations showed that 12.5% of punctiform, 37.5% of small, 37.5% of moderate and 12.5% of large size. The texture of endophytic bacterial colonies consisted of 2 types, smooth and rough. 25% were rough, and 75% were smooth. There were five colors of endophytic bacterial colonies, white (50%), yellow (12.5%), brown (12.5%), red (12.5%), and green (12.5%). Furthermore, elevation of endophytic bacteria colonies consisted of 4 types; those were flat (25%), raised (50%), convex (12.5%), umbonate (12.5%). Based on the observations, 37.5% of endophytic bacteria were Gram-positive, and 62.5% were Gram-negative (Table 2).

Physiological characteristics of endophytic bacteria

Five physiological activities were tested included proteolytic, chitinolytic, production of HCN, urease activity and phosphate dissolving activity. The tests showed that 87.5% of bacteria had proteolytic activity, 62.5% of bacteria had activity chitinolytic, 37.5% of bacteria were able to produce HCN, 75% of bacteria had urease activity, and 87.5% of bacteria were able to dissolve phosphate (Table 3).

Table 1. Hypersensitive reaction and hemolysis activity of endophytic bacterial isolates originated from roots of *C. rotundus*

Isolate code	Hypersensitive reaction	Hemolysis activity
CRS01	-	β
CRS02	-	-
CRS03	-	-
CRS04	+	x
CRS05	-	β
CRS06	+	x
CRS07	+	x
CRS08	+	x
CRS09	-	-
CRS10	+	x
CRS11	-	-
CRS12	-	-
CRS13	-	α
CRS14	-	-
CRS15	-	β
CRS16	-	-
CRS17	-	-
CRS18	+	x

Note: (+) endophytic bacterial isolates tested caused necrosis on tobacco leaf in hypersensitivity test, (−) endophytic bacterial isolates tested did not cause necrosis on tobacco leaf in hypersensitivity test dan had no hemolysis activity, (α) endophytic bacterial isolates tested could produce α-hemolysis toxins in blood agar media, (β) endophytic bacterial isolates tested could produce β-hemolysis toxins in blood agar media, (x) endophytic bacterial isolates were not tested in hemolysis test because they were detected to cause necrosis in hypersensitivity test on tobacco plant.

Table 2. Phenotypic and Gram characteristics of endophytic bacterial isolates originated from roots of *C. rotundus*

Isolate code	Shape	Size	Texture	Color	Elevation	Gram
CRS02	Circular	Small	Smooth	White	Flat	Negative
CRS03	Circular	Moderate	Smooth	White	Raised	Positive
CRS09	Circular	Small	Rough	Brown	Flat	Negative
CRS11	Circular	Small	Smooth	White	Raised	Negative
CRS12	Circular	Moderate	Smooth	Red	Umbonate	Positive
CRS14	Circular	Moderate	Rough	Yellow	Raised	Negative
CRS16	Circular	Large	Smooth	White	Convex	Positive
CRS17	Circular	Punctiform	Smooth	Green	Raised	Negative

Endophytic bacterial resistance to antibiotics

In general, endophytic bacteria tested for resistance varied to three types of antibiotics. Most of the endophytic bacteria had antibiotic resistance in the concentration of 25 µg mL^{-1} up to 50 µg mL^{-1}. Results of test on the antibiotic chloramphenicol with concentration of 25 µg mL^{-1}, 50 µg mL^{-1}, 75 µg mL^{-1}, and 100 µg mL^{-1} were 100%, 100%, 37.5% and 0%, respectively. Furthermore, the results of the test on amoxicillin antibiotic with the concentration of 25 µg mL^{-1}, 50 µg mL^{-1}, 75 µg mL^{-1}, and 100 µg mL^{-1}, respectively were 100%, 62.5%, 25% and 0%. Another antibiotic tested was rifampicin, with the results in the concentration of 25 µg mL^{-1}, 50 µg mL^{-1}, 75 µg mL^{-1}, and 100 µg mL^{-1} were 100%, 100%, 50%, and 0%, respectively (Table 4).

Effectiveness of endophytic bacteria as biocontrol agents against *M. incognita* on tomato plants

Filtrate cultures of endophytic bacterial were able to inhibit eggs hatching of *M. incognita* up to 87.33% (CRS09), 85.33% (CRS12), 78.66% (CRS11), 69.33% (CRS03), 67.33% (CRS17), 61.33% (CRS14), 52% (CRS16), 49.00% (CRS02). Furthermore, the same filtrate cultures were also able to kill *M. incognita* J2 by 85% (CRS09), 81.67% (CRS12), 76.66% (CRS11), 74.33% (CRS14), 74% (CRS17), 72% (CRS03), 65% (CRS02), and 60% (CRS16) (Table 5).

Table 3. Physiological characteristics of endophytic bacterial isolates originated from roots of *C. rotundus*

Isolate code	Proteolytic activity	Chitinolytic activity	Production of HCN	Urease activity	Phosphate dissolving activity
CRS02	+	-	+	+	+
CRS03	+	-	+	+	+
CRS09	+	+	-	+	+
CRS11	+	+	-	+	-
CRS12	+	+	-	+	+
CRS14	-	+	-	-	+
CRS16	+	-	-	-	+
CRS17	+	+	+	+	+

Note: (+) endophytic bacterial isolates had physiological activities and were able to produce compounds tested, (−) endophytic bacterial isolates had no physiological activities and were not able to produce compounds tested

Table 4. Resistance of endophytic bacteria originated from roots of *C. rotundus* on chloramphenicol, amoxicillin, and rifampicin antibiotics on various concentrations.

Isolate code	Chloramphenicol (µg mL^{-1})				Amoxicillin (µg mL^{-1})				Rifampicin (µg mL^{-1})			
	25	50	75	100	25	50	75	100	25	50	75	100
CRS02	+	+	+	-	+	+	+	-	+	+	+	-
CRS03	+	+	-	-	+	+	-	-	+	+	-	-
CRS09	+	+	+	-	+	-	-	-	+	+	+	-
CRS11	+	+	+	-	+	-	-	-	+	+	-	-
CRS12	+	+	-	-	+	+	-	-	+	+	-	-
CRS14	+	+	-	-	+	-	-	-	+	+	+	-
CRS16	+	+	-	-	+	+	+	-	+	+	+	-
CRS17	+	+	-	-	+	+	-	-	+	+	-	-

Table 5. Effectiveness of filtrate culture of endophytic bacterial originated from roots of *C. rotundus* in inhibiting eggs hatching and killing *M. incognita* J2 in vitro.

Isolate code	Inhibition of eggs hatching of *M. incognita* (%)	Mortality of *M. incognita* J2 (%)
CRS02	$49.00^{e} \pm 6.24$	$65.00^{de} \pm 2.65$
CRS03	$69.33^{c} \pm 1.15$	$72.00^{cd} \pm 7.94$
CRS09	$87.33^{a} \pm 4.93$	$85.00^{a} \pm 4.36$
CRS11	$78.66^{b} \pm 1.53$	$76.66^{bc} \pm 2.08$
CRS12	$85.33^{a} \pm 2.08$	$81.67^{ab} \pm 2.08$
CRS14	$61.33^{d} \pm 1.53$	$74.33^{bc} \pm 3.79$
CRS16	$52.00^{e} \pm 3.00$	$60.00^{e} \pm 6.24$
CRS17	$67.33^{c} \pm 3.79$	$74.00^{bc} \pm 2.65$
Control	0^{f}	0^{f}

Note: Values followed by different superscript letters are significant at $P \leq 0.05$ over control

In general, applications of endophytic bacteria in tomato plants infested with *M. incognita* had positive effects. Plants treated with endophytic bacteria had higher plant height compared with plants untreated with the endophytic bacteria. Differences in height on the plant treated and control were 32.38% (CRS03), 32.41% (CRS02), 39.75% (CRS17), 44.78% (CRS09), 46.30% (CRS11), 46.54% (CRS14), 48.70% (CRS12), and 59.78% (CRS16). In addition to plant height, all of endophytic bacteria also had a positive effect on the growth of roots. The roots of plants treated with endophytic bacteria were longer by 39.07% (CRS09), 41.16% (CRS17), 43.26% (CRS16), 43.54% (CRS02), 46.90% (CRS14), 47.03% (CRS11), 49.20% (CRS03), and 51.31% (CRS12). On the other hand, all isolates were able to increase the fresh weight of the plant. The bacteria that were able to increase the fresh weight of the plants were CRS03 (10.47%), CRS09 (12.19%), CRS16 (20.25%), CRS12 (22.41%), CRS11 (23.17%), CRS02 (25.19%), CRS14 (29.28%), and CRS17 (30.11%). Furthermore, endophytic bacteria were also able to increase plant dry weight. The isolates, which effectively increased plant dry weight were CRS02 (24.83%), CRS12 (24.95%), CRS16 (25.92%), CRS17 (29.70%), and CRS14 (31.45%).

All endophytic bacteria tested were also able to effectively reduce the number of *M. incognita* in the roots of tomato plants and reduce the number of gall caused by the infestation of *M. incognita* (Figure 1). Reduction of *M. incognita* in the roots occurred at 25.30% (CRS12), 26.51% (CRS11), 35.54% (CRS17), 39.16% (CRS03), 42.77% (CRS02), 45.18% (CRS09), 49.40% (CRS14), and 58.43% (CRS16). Endophytic bacteria isolates were also able to reduce the number of gall formed by 24.38% (CRS12), 29.80% (CRS11), 34.44% (CRS17), 37.58% (CRS03), 38.76% (CRS09), 38.76% (CRS09), 39.67% (CRS02), 41.70% (CRS14), and 56.67% (CRS16).

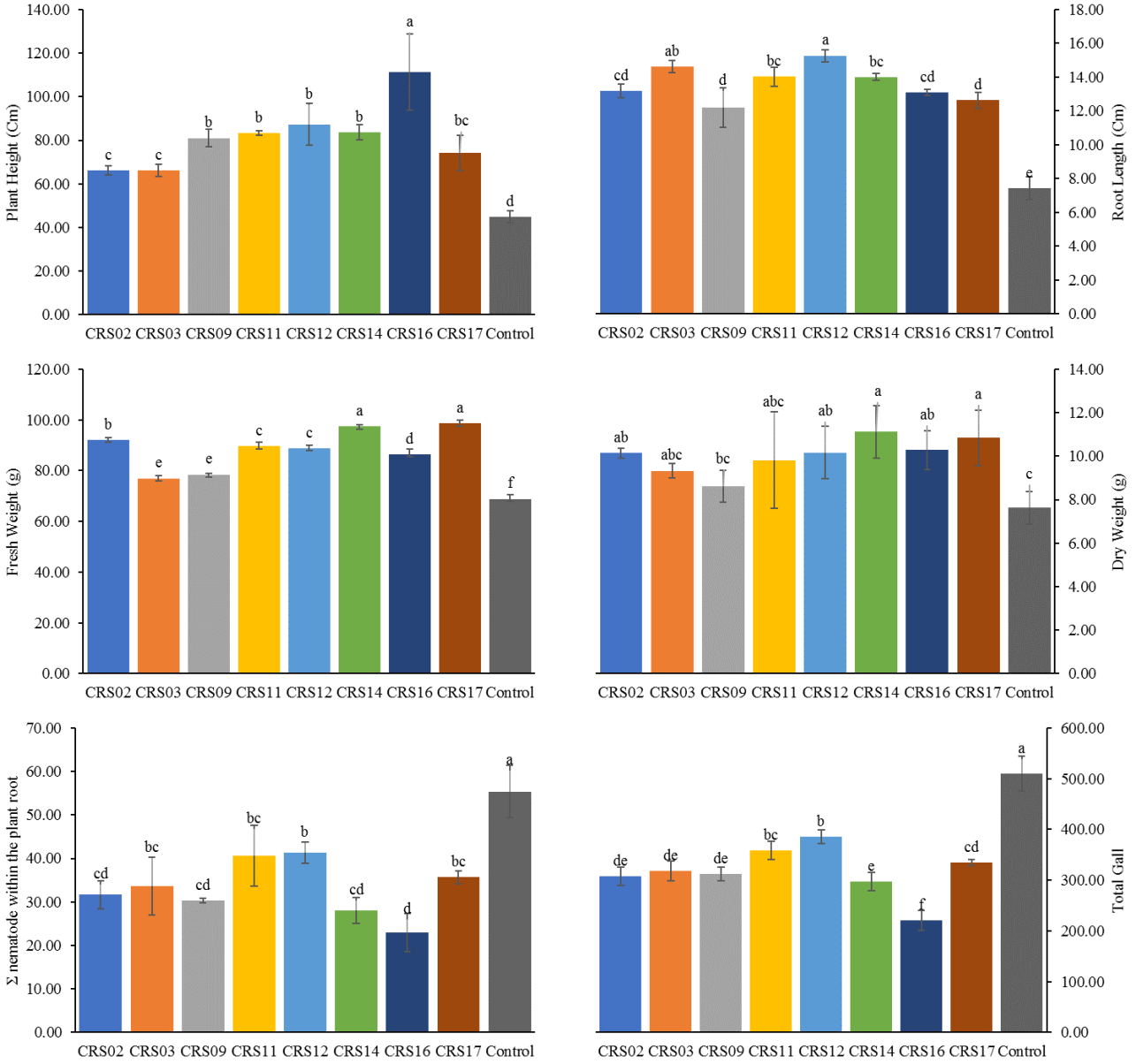

Figure 1. Growth of tomato plant infested with *M. incognita* and treated using endophytic bacteria from roots of *C. rotundus* and number of *M. incognita* and gall on each test plant. Note: values followed by different letters are significantly different from control

Discussion

The endophytic bacteria are known to be associated with almost all part of the plants, including the roots, stems, leaves, and seeds. In terms of the life cycle of endophytic bacteria, all or part of the life cycle was in the plant tissue. Not all the bacteria living within the plant tissues are beneficial to the host plant. Some endophytic bacteria are neutral, and some are pathogenic (Hardoim et al. 2015). The bacteria caused necrosis of hypersensitivity test show that these bacteria are pathogenic to plants. Hypersensitivity reactions are response of plants to pathogens infection in plant tissue that is an attempt to inhibit the growth of pathogens. Hypersensitivity reactions are influenced by *hrp* gene commonly found in plant pathogenic Gram-negative bacteria, such as *Xanthomonas* sp. groups (Zhu et al. 2000). Hypersensitivity reactions are part of the cell death program that occurs very fast and localized. The cellular membrane on the leaves of tobacco plants that have contact with pathogenic bacteria will be destroyed, undergoing drying and necrosis (Klement and Goodman 1967). Safety of bacteria as biocontrol agents is also determined from their effects on mammals. The bacteria producing hemolysis toxin cannot be used as a biocontrol agent. Hemolysis toxin is highly cytotoxic to humans granulocytes, monocytes, and lymphocytes (Herlax and Bakas 2002).

Each bacteria can have the same or different characters from other bacteria. Phenotypic character is expression of the interaction between genes and the environment. The same bacteria grown in different environments can show different phenotypic characters. Pathogenic bacteria *Ralstonia solanacearum* , for example, is white turbid in Nutrient Agar media, but the colony will turn red when it is grown on media containing tetrazolium chloride antibiotics (Williamson et al. 2002). The diversity of phenotypic characters of endophytic bacteria colonies observed in this study is the result of the interaction between genes and the environment.

As biocontrol agents, endophytic bacteria can act directly or indirectly. Endophytic bacteria can directly control pathogens by producing antagonistic enzymes against pathogens. The protease and chitinase enzymes are reported to have a nematicidal effect on root-knot nematodes. HCN produced by endophytic bacteria is also reported to be able to kill nematodes. Endophytic bacteria can indirectly increase plant resistance through induced systemic resistance (ISR) mechanism. Some studies have also stated that the endophytic bacteria were capable of fixing nitrogen from the environment, and dissolving phosphorus. The abilities are closely related to the mechanisms of plant growth promoting on endophytic bacteria (Brader et al. 2014, Chebotar et al. 2015, Ma et al. 2016).

As biocontrol agents, endophytic bacteria can be applied in a vast environment. Such capabilities are indicated from how bacteria survive on the stress of antibiotics. Agricultural environment, especially in developing countries has been contaminated by a wide variety of active compounds of pesticides (De Bon et al. 2010). The bacteria which are able to survive in media containing antibiotics is expected to survive well in the environment (Myresiotis et al. 2012).

Extracellular protease enzyme produced by the endophytic bacteria has an important role in controlling some species of plant pathogens. Tian et al. (2007) reported that the production of extracellular protease by bacteria is one of the mechanisms of bacteria as control agents of root-knot nematodes *Meloidogyne* spp. In addition to the protease, other enzymes such as chitinase, lipase, catalase, also have a role in the antagonistic activity of endophytic bacteria against nematodes. Chitinase has an important role in nematode control because this enzyme is able to degrade the middle layer of *M. Incognita* eggs (Van Nguyen et al. 2007). Cronin et al. (1997) described that chitinase can inhibit eggs hatching of *Globodera rostochiensis* up to 90%. This explains why the hatching of nematode eggs in this study may be delayed, and the cause of death of *M. incognita* J2 treated with filtrate cultures of endophytic bacteria. This also explains why the number of *M. incognita* in the roots and the number of gall on tomato plant roots are less than the control plants.

Several growth variables of tomato plants treated with endophytic bacterial showed better results than the control due to the activities of endophytic bacteria that are able to fix nitrogen and dissolve phosphorus. Some species of endophytic bacteria in symbiosis with plants are known to be able to fix nitrogen from the environment. Elbeltagy et al. (2001) successfully isolated the endophytic bacteria of wild rice, which the bacteria showed the ability to fix nitrogen. The bacteria are then inoculated on rice seedlings. Rice seedlings treated with endophytic nitrogen-fixing bacteria showed better growth than the control. Phosphate solvent bacteria are able to produce organic acids, such as citric, glutamate, succinate, lactate, oxalic, glyoxylic, malate, fumarate, tartrate, and α-ketobutyric. Such organic acids have an important role in the process of dissolving phosphate that is difficult to dissolve in the medium and in the soil (Mohammadi 2012). Manzoor et al. (2017) reported that some phosphate solvent bacteria are able to promote the growth of corn plants.

This study provides new information that endophytic bacteria originated from roots of *C. rotundus* have potential as a biocontrol agent. Endophytic bacteria are able to reduce the number of *M. incognita* in the roots of plants and are able to reduce the number of gall on the roots of tomato plants infested with *M. incognita*. Each isolate had activities of proteolytic, chitinolytic, and was able to produce HCN, fix nitrogen and dissolve phosphate. In general, CRS16 isolate has the best performance and stable as a biocontrol agent of *M. incognita* on tomato plants.

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