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CHARACTERIZATION OF ENDOPHYTIC *Bacillus* ISOLATED FROM SHALLOT ROOT AS BIOCONTROL OF BACTERIAL LEAF BLIGHT DISEASE

Zurai Resti[1], Trimurti Habazar[1], Deddi Prima Putra[2], & Nasrun[3]

[1]Department of Plant Protection, Faculty of Agriculture, Andalas University, Indonesia
[2]Faculty of Pharmacy, Andalas University, Indonesia
Kampus Unand Limau Manis Kec. Pauh Kota Padang 25163
[3]Research Institute for Medicinal and Aromatic Plants, Laing, Solok, Indonesia
Kel. Laing Kec. Tanjung Harapan Kota Solok
E-mail: zurairesti@agr.unand.ac.id

ABSTRACT

Characterization of endophytic* Bacillus *isolated from shallot root as biocontrol of bacterial leaf blight disease. Endophytic *Bacillus* isolated from the roots of healthy shallots has potential as biological control agent of bacterial leaf blight (BLB) disease. Based on the *in planta* screening, four endophytic bacteria that capable of controlling BLB diseases from the group of *Bacillus* were obtained, such as *Bacillus cereus* P14, *Bacillus cereus* Se07, *Bacillus* sp. HI, and *Bacillus* sp. SJI. The aims of this study were to investigate the characteristics of endophytic *Bacillus* that capable of controlling BLB disease and to determine the characteristic that most responsible in the disease control. This study method was descriptive. The characteristics observed were production of antibiotics by paper disc method, production of salicylic acid by capillary electrophoresis, and root colonization by *in planta* method. Linear regression analysis was used to determine the characteristic most responsible in the disease control. The results showed that four endophytic *Bacillus* were capable of producing salicylic acid and colonizing the root, and three of them were also capable of producing antibiotic. Salicylic acid production varied ranging from 13.96 to 14.72 ppm mL^{-1}. Three endophytic *Bacillus* were capable of producing antibiotic with inhibition zone of 16.25 to 20.25 mm. Endophytic *Bacillus* was able to colonize the shallot roots with a population of bacteria ranged from $3.20 \times 10^{5-6}$, 20×10^5 CFU g^{-1} root. Based on the correlation coefficient of linear regression analysis, the root colonization of endophytic bacteria played the role in decreasing BLB disease.

Key words: antibiotics, *Bacillus*, endophytic bacteria, root colonization, salicylic acid

ABSTRAK

Karakteristik bakteri endofit* Bacillus *yang diisolasi dari akar bawang merah sebagai agen pengendali hayati penyakit hawar daun bakteri. Bakteri endofit *Bacillus* yang diisolasi dari akar bawang merah sehat, memiliki potensi sebagai agens pengendali hayati penyakit hawar daun bakteri (HDB). Berdasarkan hasil skrining *in planta* didapatkan empat bakteri endofit unggul yang mampu mengendalikan penyakit hawar daun bakteri dari kelompok *Bacillus*, yaitu *Bacillus cereus* P14, *Bacillus cereus* Se07, *Bacillus* sp. HI, dan *Bacillus* sp. SJI. Penelitian ini bertujuan untuk mengetahui karakteristik dari bakteri endofit *Bacillus* yang mampu mengendalikan penyakit HDB serta menentukan karakter yang paling berperan dalam pengendalian penyakit HDB. Penelitian ini bersifat deskriptif dan karakter yang diamati meliputi produksi antibiotik dengan metode kertas cakram, produksi asam salisilat dengan elektroforesis kapiler, dan kolonisasi akar dengan metode*in planta*. Analisis regresi linear digunakan untuk menentukan karakter yang paling berperan dalam pengendalian penyakit HDB. Hasil penelitian menunjukkan empat bakteri endofit *Bacillus* mampu memproduksi asam salisilat dan mengkolonisasi akar, serta tiga diantaranya juga mampu menghasilkan antibiotik. Produksi asam salisilat bervariasi, berkisar antara 13,96-14,72 ppm mL^{-1}. Tiga bakteri endofit *Bacillus* mampu memproduksi antibiotik dengan zona hambatan antara 16,25-20,25 mm. Bakteri endofit *Bacillus* mampu mengkolonisasi akar bawang merah dengan jumlah populasi bakteri berkisar antara $3,20\ 10^5$-$6,20 \times 10^5$ CFU g^{-1} akar. Berdasarkan nilai koefisien korelasi dari analisis regresi linear, kolonisasi akar paling berperan dalam interaksi bakteri endofit dengan penekanan penyakit HDB pada bawang merah.

Kata kunci: antibiotik, asam salisilat, *Bacillus*, bakteri endofit, kolonisasi akar

INTRODUCTION

Endophytic bacteria are bacteria that colonize the internal tissues of plants and do not cause infection or negative effects on host plants (Schulz & Boyle, 2006) Endophytic bacteria from the genus *Bacillus* are reported to be isolated from various plant species such as potatoes, bananas, pears, beans (Lian *et al*., 2008), and gingseng (Vendan *et al*., 2010). To colonize host tissue, endophytic bacteria require only one cell within a few days after inoculation. The ability of colonization is influenced by host differences, age and origin of host plants, tissue types, and environmental conditions. The characteristics of endophytic bacteria that can act as biocontrol agents include the ability to suppress the development of pathogens directly by producing antimicrobial compounds (Wang *et al*., 2010), siderophores, and lytic enzymes (Lugtenberg & Kamilova, 2009), ability to compete in obtaining iron, nutrients, and space, as well as parasitism ability indirectly through the mechanism of systemic resistance induction to host plants.

As a biocontrol agent, endophytic bacteria have their advantages due to their presence in plant tissue, so as to survive in biotic and abiotic stress (Hallman *et al*., 1997). Endophytic bacteria from the genus *Bacillus* can induce cotton resistance to damping off disease caused by *Rhizoctonia solani* through enhancement of plant defense enzymes (Rajendran & Samiyappan, 2008). *Bacillus lentimorbus* (Dutky) and *B. Cereus* (Frank & Frank) effectively control rust disease of coffee leaves (Shiomi *et al*., 2006). Endophytic *Bacillus* spp. isolated from different types of vegetables can reduce the severity of fruit rot disease of cocoa through the mechanism of systemic resistance induction (Melnick *et al*., 2008).

Endophytic *Bacillus* isolated from healthy shallot roots has potential as biocontrol agent of bacterial leaf blight (BLB) disease. Based on *in planta* screening results, four superior endophytic bacteria from genus *Bacillus* were able to control BLB disease of shallot, i.e. *Bacillus cereus* P14, *Bacillus cereus* Se07, *Bacillus* sp. HI, and *Bacillus* sp. SJI (Resti *et al*., 2013). Characteristics of endophytic *Bacillus* associated with their role as biocontrol agents need to be studied further. The aims of this study were to determine the characteristics of endophytic *Bacillus* capable of controlling BLB disease as well as to determine the characteristics most responsible in the disease control.

MATERIALS AND METHODS

Research Site. The research was conducted in Bacteriology Laboratory of Plant Protection Department, Natural Product Chemistry Laboratory of Faculty of Pharmacy, and Nursery house of Faculty of Agriculture, Universitas Andalas Padang. The study was carried out from April to October 2015.

Isolation of Endophytic Bacteria. Endophytic bacteria were isolated from healthy shallot roots from endemic areas of BLB disease in West Sumatra. Isolation was performed by serial dilution method (Klement *et al*., 1990). Sterilization of the surface was done by soaking the roots in a solution of ethanol 70% for 5 minutes as many as three times, then by rinsing them with sterile distilled water. A gram of roots tissues was crushed with sterile mortar added with 1 ml of sterile distilled water and diluted to 10^{-4}. The bacterial suspension at 10^{-3} and 10^{-4} dilution was cultured on Nutrient Agar (Merck NA) medium and incubated for 48 hours. Single colony with different morphological features was taken with sterilized toothpicks, grown on the same medium, and incubated for 24 hours at 30 °C. The growing bacteria isolates were transferred into microtube (1 mL volume) containing sterile distilled water, labeled, and stored at 5 °C. All isolates were morphologically characterized (colony color, colony shape, and colony surface). Gram reaction and hypersensitivity reaction were tested by using 3% KOH solution (Schaad *et al*., 2001) and Klement *et al*. (1990) method, respectively.

Production of Salicylic Acid. Endophytic bacterial isolates were cultured in a Nutrient Broth liquid medium (NB Merck) and incubated in a shaker at 210 rpm for 48 hours at room temperature. Liquid culture was centrifuged at 7000 g for 10 minutes. The supernatant was analyzed by capillary electrophoresis using pure salicylic acid as the standard. Capillary electrophoresis was applied to a voltage of 10 KV with a 210 nm wave length and a temperature of 20 °C (Resti *et al*., 2016).

Observation of salicylic acid analysis results was done by comparing the peak of salicylic acid solution electrophoregram with supernatant solution of selected endophytic isolate. The concentration of salicylic acid produced was calibrated by using standard salicylic acid curve (0-50 ppm mL^{-1}).

Production of Antibiotics. Selected indigenous endophytic bacterial isolates were cultured in Nutrient Broth medium (NB Merck) and incubated in shaker for 2 × 24 hours at 200 rpm and room temperature. The bacterial culture was centrifuged at 7000 g for 10 minutes. The supernatant was further separated from the pellet. Sterilized filter papers with 0.5 cm diameter were soaked in supernatant for 5 minutes and dried. The filter paper was prepared on Potato Dextrose Agar (PDA Merck) medium which had been inoculated with *Xaa* bacteria and incubated for 2 × 24 hours (Balouiri *et al*., 2006).

The ability to produce antibiotics is characterized by inhibition zone (clear zone) around the disc paper. The determination of the difference in the ability to produce antibiotics was done by measuring the diameter of the inhibition zone formed.

Colonization of Shallot Roots Tissues. Colonization of shallot roots by endophytic bacteria was performed using bacterial mutant isolates (*B. cereus* P14, B. *cereus* Se07, *Bacillus* sp HI and *Bacillus* sp SJI) resistant to Rifampicin. Mutant strains were used to distinguish the bacteria we applied from other bacteria which may be present. Mutants were obtained by growing endophytic bacterial isolates on NA medium in which Rifampicin antibiotics were added with concentrations continuously increased from 10, 20, 50, to 110 ppm. The mutants of endophytic bacteria were subsequently cultured on Nutrient Broth (NB Merck) medium (Habazar *et al*., 2007).

Colonization of shallot roots tissues was determined by introducing the selected indigenous mutant of endophytic bacteria in shallot seeds cv. Medan. Introduction was done by soaking the shallot seeds in endophytic bacteria suspension (population density of 10^8 cells mL^{-1}). The seeds that had been soaked were dried and then grown on sterile soil media. The ability of endophytic bacterial colonization on root tissue was observed by re-isolating bacteria from plant roots grown on sterile soil. Re-isolation was carried out by harvesting the shallot roots as much as 1 g and crushing them with sterile porcelain mortal, and they were diluted until 10^{-6}. Isolated bacteria suspension was grown on NA medium (Merck) which had been added with Rifampicin antibiotics with concentrations corresponding to the antibiotics treatment at the time of mutant development. Bacterial isolates that are able to grow are mutants of endophytic bacterial isolates introduced in previous seeds (Nasrun, 2005).

Observation of bacterial populations colonizing shallot roots tissues was done by re-isolation of endophytic bacterial from shallot roots tissues harvested at 7-day intervals and observations were performed 5 times until the plants were 35 days old. Endophytic bacterial re-isolation was performed by serial dilution method. Endophytic bacteria were cultured in NA (Merck) media in which Rifampicin antibiotics had been added. The calculation of endophytic bacterial populations was using the Klement *et al*. (1990) formula as follows.

$$JP = A \times B$$

Notes:
JP = number of bacterial population (cfu/ml)
A = number of bacterial colony
B = dilution factor

Data Analysis. For morphological and physiological characteristics, the average data of the observed results are shown in the form of tables and figures. To determine the correlation between the observed variables of endophytic bacteria characteristic, linear regression analysis was performed. The value of the correlation coefficient (r) will determine the relationship between observed variables and determine the characteristics that correlate with the suppression ability against BLB disease.

RESULTS AND DISCUSSION

Morphological Characteristics, Physiological Characteristics, and Hypersensitivity Reactions. Based on the results of *in planta* screening (Resti *et al*., 2013) four superior endophytic bacteria from the genus *Bacillus* were obtained. After identification based on the 16rDNA sequence, two isolates of *Bacillus cereus* type and two other types of *Bacillus* sp. with different strains were obtained. The four endophytic bacteria had similar morphological characters, Gram reactions, and hypersensitivity reactions (Table 1), as well as indicated the morphological character of the genus *Bacillus*. Pereira *et al*. (2011), isolated endophytic bacteria from corn roots and acquired that bacteria from the genus *Bacillus* were more dominant in association with corn roots than other genus.

Production of Salicylic Acid. The four endophytic bacteria of the genus *Bacillus* were able to produce salicylic acid with varying concentrations (Table 2), the highest was in *B.cereus* P14 (14.72 ppm mL^{-1}), followed by *Bacillus* sp. SJI (14.67 ppm mL^{-1}), *Bacillus* sp. HI

(14.40 ppm mL^{-1}), and the lowest was in *B. cereus* Se07 (14.40 ppm mL^{-1}). Though originating from the same genus, these four bacteria have the different ability to produce salicylic acid. Even of the same species, *B.cereus*, with different isolates, have different capabilities in producing salicylic acid (Figure 1). Ran *et al*. (2005) found that different *Pseudomonas fluorescens* isolate (*P. fluorescens* WCS417 and WCS374), produced salicylic acid in different amounts, of 5 fg $cell^{-1}$ for WCS417 and > 25 fg $cell^{-1}$ for WCS374.

The ability of *B. cereus* P14 to produce salicylic acid was positively correlated with its ability to suppress the severity of high BLB disease (64.30%). It is in accordance with Maurhofer *et al*. (1994) stating that certain endophytic isolates were capable of producing salicylic acid and playing roles in mechanisms of Induced systemic Resistance (ISR) in plants. Kloepper & Ryu (2006) reported that endophytic *B. pumilus* SE34 in *Arabidopsis* was able to induce plant systemic resistance to *Pseudomonas syringae* pv. *maculicola* attack associated with its ability to produce salicylic acid. Salicylic acid has an important role in the signal path of systemic resistance induction and is associated with the accumulation of PR proteins (pathogenesis-related), such as PR1 (Lyon, 2007).

Table 1. Colonial morphology, Gram reaction, and hypersensitivity reaction of screened endophytic bacteria

Endophytic bacteria	Origin	Shape	Color	Elevation	Gram	HR
Bacillus cereus P14	Agam	Round	White, dull	Flat	Positive	-
Bacillus sp. SJI	Solok	Round	White, dull	Flat	Positive	-
Bacillus sp. HI	Solok	Round	White, dull	Flat	Positive	-
Bacillus cereus Se07	Solok	Round	White, dull	Flat	Positive	-

HR = hypersensitivity reaction.

Table 2. Physiological characteristics of endophytic *Bacillus* capable of controlling BLB disease of shallot

Endophytic bacterial isolate	Physiological characteristics			
	BLB severity suppressing rate (%)	Salicylic acid production (ppm/ml)	Inhibition zone (mm)	Root colonization (CFU/g)
Bacillus cereus P14	64.30	14.72	0	4.41×10^5
Bacillus sp.SJI	61.06	14.67	1.25	4.84×10^5
Bacillus sp.HI	59.01	14.40	20.25	3.20×10^5
Bacillus cereus Se07	28.32	13.96	16.25	6.20×10^5

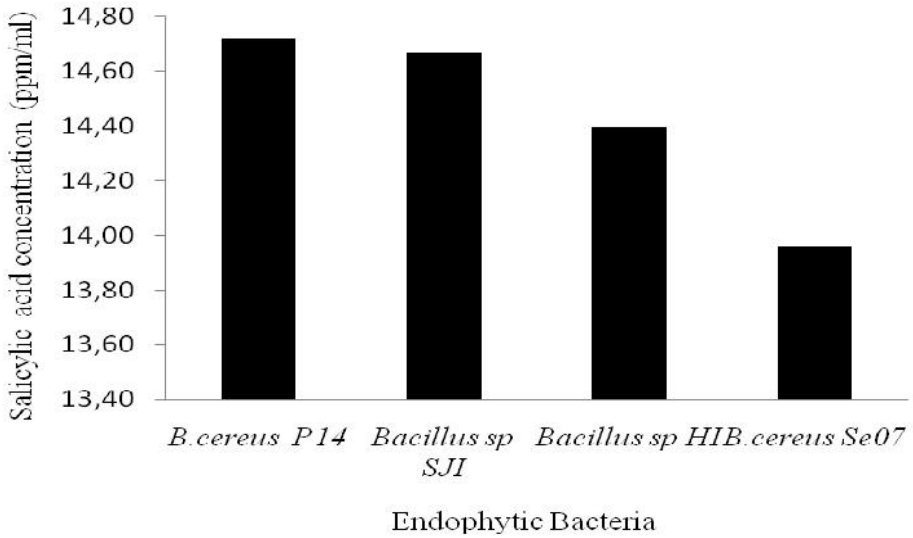

Figure 1. Salicylic acid production of endophytic bacteria from genus Bacillus capable of controlling BLB disease of shallot.

Production of Antibiotics. Not all endophytic bacteria from the genus *Bacillus* tested were able to produce antibiotics.

The production of endophytic bacterial antibiotics varied, as indicated by the inhibition zone ranging from 0 to 20.25 mm (Table 2). The largest inhibition zone was produced by *Bacillus* sp. HI, while *B. cereus* P14 did not produce antibiotics but had the highest ability to suppress the severity of BLB disease. The results of this study differ from previous studies that reported that bacteria from the genus *Bacillus* were capable of producing antifungal and antibacterial metabolites (Dragana *et al*., 2011). Similarly, Pal *et al*. (2012) had evaluated the antimicrobial ability of 20 isolates of endophytic bacteria isolated from *Paederia foetida* L. (Rubiaceae), revealing that most endophytic bacteria exhibited significant activism of *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumonia*. Munif *et al*. (2012) showed that 41 isolates of endophytic bacteria were antibiotic to *Ralstonia solanacearum* on Potato Dextrose Agar (PDA) medium, 14 bacterial isolates showed antibiotic reaction on Trypticase Soy Agar (TSA) medium, 25 isolates showed antibiotic reaction to *Pseudomonas grisea* on PDA medium, and 31 isolates showed their inhibition zone on TSA medium. Based on the results of in vitro testing, the antibacterial activity of some endophytic bacterial isolates from potato plants was antibiotic to *Streptomyces* sp. and *Xanthomonas* sp. (Sessitsch *et al*., 2004).

This phenomenon is different from the results of previous studies. *B. cereus* P14 was able to suppress the severity of BLB disease, although it did not produce antibiotics, due to its ability as a driver of plant growth. Resti *et al*. (2017) measured the ability of *B. cereus* P14 which produced IAA of 93.16 ppm mL^{-1} and dissolved phosphate with solubility index 2. Furthermore, according to Resti *et al*. (2013), *B. cereus* P14 was able to increase the fresh and dry weight of shallot bulb by 42.65% and 50.65%, consecutively.

Colonization of Roots Tissue. The four endophytic bacteria of the genus *Bacillus* can be re-isolated from the root tissue after a week (7 days) of inoculation. This indicates that the four endophytic bacterial isolates were able to survive in the roots tissues (able to colonize the roots tissues of shallot) with the total population reaching 6.20×10^5 CFU g^{-1} roots (*B. cereus* Se07) during the first week of observation (Table 2). *Bacillus cereus* P14 which can suppress BLB severity the most can also colonize the roots tissues of the shallot with a population of 4.41×10^5 CFU g^{-1}. The number of bacterial population in roots tissues tends to be stable on all bacteria in each observation week. Colonization of endophytic bacteria could last up to 5 weeks (35 days) after inoculation (Figure 2) and there was no deterioration due to its presence in roots tissues of shallot crops.

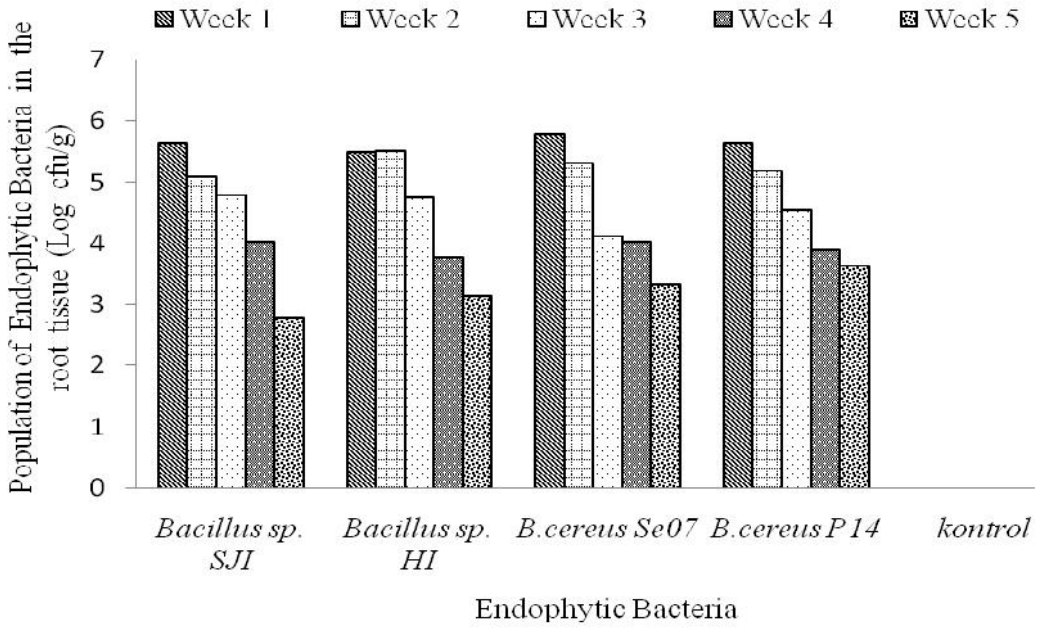

Figure 2. Population of endophytic bacteria in shallot roots tissues during 5 weeks of observation (CFU / g root).

Colonization of shallot root tissues by endophytic bacteria occurring from the first week until the fifth week after introduction (Figure 2) showed that endophytic bacteria were able to colonize the tissues within 35 days after being introduced to shallot bulbs. The Durham (2013) study reported that endophytic *B. firmus* isolate GB123 can colonize corn, soybeans, and cotton up to 12 weeks after inoculation.

The colonization ability of endophytic bacteria on shallot roots tissues is because the bacteria can adapt quickly and are compatible with shallot roots tissues which are their original habitat. According to Rosenbleuth & Martinez-Romero (2006), endophytic bacteria must be compatible with host plants and able to colonize the host without damaging host tissue. The highest shallot roots tissues colonization occurred on *B. cereus* Se07 i.e. about 6.20×10^5 CFU g^{-1} roots. This capability is higher than in those conducted by Hung & Annapurna (2004) which stated that endophytic bacteria population in soybean roots was about 2.3×10^5 CFU g^{-1} and soybean stems was about 3.6×10^5 CFU g^{-1}.

Endophytic bacterial colonization could be detected from the first week (7 days after inoculation) to the fifth week (35 days after inoculation) and the population tended to decrease from the third week to the fifth week after inoculation (Figure 2). The population decline is probably because the bacteria have been transferred to other tissues. According to Ji *et al*. (2010), *B. cepacia* isolate Lu 10-1 was detected in roots tissues up to 7 days after inoculation, whereas in leaf tissues and other parts were detected 14 to 21 days after introduction.

Correlation Regression. Colonization of roots tissues by endophytic bacteria played more important role in the interaction between bacteria and the suppression of BLB disease severity (Figure 3) compared with the ability to produce salicylic acid and antibiotics. Based on regression analysis, there was correlation between roots tissues colonization by endophytic bacteria and decreased severity of BLB disease with correlation coefficient value of r = 0.729. This value is higher than the regression analysis between salicylic acid and the severity of BLB disease (r = 0.529) as well as than the regression analysis between ability to produce antibiotic and disease severity (r = 0.265). The endophytic bacteria in the shallot tissues will produce signals for plant defense. According to Kloepper & Ryu (2006), when the bacteria are in the roots tissues, the bacteria can generate signals for plant defenses that immediately alter the expression of *avr* genes in plants. This process is known as systemic resistance induction. The results of this study indicate a difference in physiological characteristics of endophytic bacteria from the genus *Bacillus*. Although originating from the same genus, four endophytic bacteria capable of controlling BLB disease of shallot have diverse physiological characteristics.

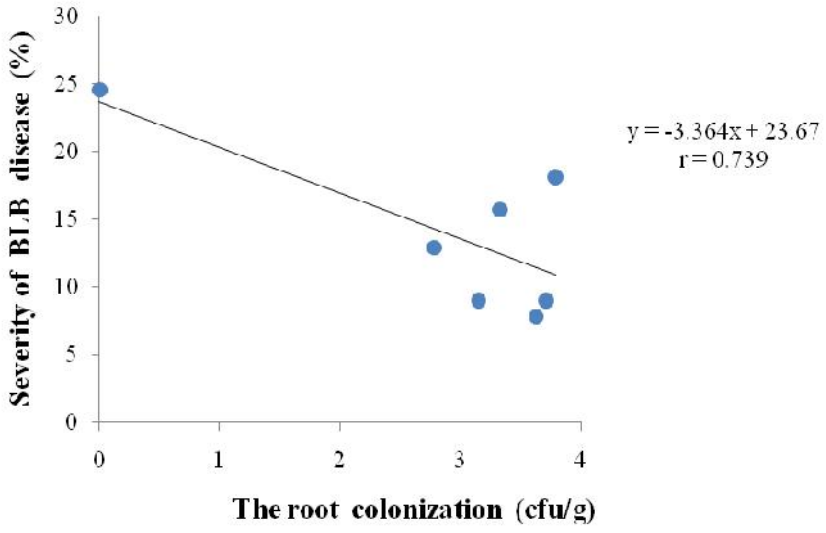

Figure 3. Correlation analysis between endophytic bacterial colonization and the severity of BLB disease of shallot.

CONCLUSION

Four endophytic bacteria from the genus *Bacillus* were capable of producing salicylic acid and colonizing the roots, three of which were also capable of producing antibiotics. Salicylic acid production varied, ranging from 13.96 to 14.72 ppm mL^{-1}. Three endophytic *Bacillus* were capable of producing antibiotics with a zone of resistance between 16.25 and 20.25 mm. Endophytic *Bacillus* were capable of colonizing the shallot roots with bacterial populations ranged from 3.20×10^5 to 6.20×10^5 CFU g^{-1} roots. Based on the correlation coefficient value of linear regression analysis, root colonization plays a major role in endophytic bacterial interactions with suppression of BLB disease of shallot.

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