

## Indigenous *Bacillus* spp. ability to growth promoting activities and control bacterial wilt disease (*Ralstonia solanacearum*)

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**Abstract.** Yanti Y, Habazar T, Reflinaldon, Nasution CR, Felia S. 2017. Indigenous *Bacillus* spp. ability to growth promoting activities and control bacterial wilt disease (*Ralstonia solanacearum*). *Biodiversitas* 18: 1562-1567. Among the species of Plant Growth Promoting Rhizobacteria, *Bacillus* spp. has been found to provide beneficial effects to different plant species. Based on our previous research from in planta screening's method, we found six indigenous strains of *Bacillus* spp., which had the ability to control bacterial wilt and increased growth and yields of chili. Those were *Bacillus subtilis* BSn5,q *Bacillus* sp. RD-AZPVI-03, *Bacillus* sp. Y81-1, *B.cereus* strain C38/15, *B.cereus* strain APSB-03, and *B. subtilis* strain CIFT-MFB-4158A. This research aimed to characterize the mechanism of selected *Bacillus* spp. as biocontrol agents of *R. solanacearum* and as biofertilizer of chili *in vitro*. We have observed the biocontrol characters (production of HCN, siderophore, biosurfactant, protease, root colonization, and antibiotic), and biofertilizer's characters (production of Indole Acetic Acid (IAA), ammonia and phosphate solubilization) of *Bacillus* spp. All strains had ability to produce variable concentrations of IAA, *B.subtilis* strain CIFT-MFB-4158A can produce siderophore, all isolates cannot produce HCN and biosurfactant, 4 isolates can produce protease. Based on the ability to colonize roots, *B.subtilis* strain CIFT-MFB-4158A had the best ability to colonize root surface ( $4.17 \times 10^5$  CFU/g root), while *B. subtilis* BSn5 had the best ability to colonize internal roots ( $2.08 \times 10^5$  CFU/g), and 2 *Bacillus* strains can suppress *R. solanacearum* *in vitro*.

**Keywords:** *Bacillus*, in vitro, Indole Acetic Acid, siderophore, protease

**Abbreviations:** IAA = Indole Acetic Acid, IRB = Indigenous Rhizobacteria, ISR = Induce systemic resistance, NA = Nutrient agar, PGPR = Plant Growth Promoting Rhizobacteria, R = *Ralstonia*, B= *Bacillus*

### INTRODUCTION

*Ralstonia solanacearum*, the causal agent of bacterial wilt disease, is one of the important serious vascular diseases of chili crop which cause losses between 15% to 55% around the world (Basu 2014; El-Argawy and Adss 2016). Control is difficult due to high variability of the pathogen, limited possibility for chemical control, high capacity of the pathogen to survive in diverse environments and its extremely wide host range. Controls using biological agents are more desirable because of the availability of variable results from another methods (Nguyen and Ranamukhaarachchi 2010). Since biological control is a key component of integrated disease management, it is important to search for plant growth promoting rhizobacteria (PGPR) active against specific pathogens and evaluates the antagonists for wider application (Nakkeeran et al. 2006).

Between the species of PGPR, *Bacillus* spp. is well-known as powerful genera. Several *Bacillus* species known to promote the health and control diseases by plant pathogens suppressions or by nutrients competitions like iron and phosphate or indirectly fixing nitrogen (McSpaden Gardener 2004). Many *Bacillus* has the ability to serve as biofertilizers and biopesticides but they are not recognized as pathogenic which makes them easier to handle. *Bacillus*

spp. has ability to move freely, and a good competence on rhizosphere and facultative anaerobes, and those adds its survival ability in soil at various environmental conditions. These bacteria can sporulate in unfavorable conditions and make them more resistant to harsh conditions. This ability is very useful for commercial applications because it will provide long shelf life (Niazi et al. 2014). *Bacillus*-based biocontrol agents are quite important to control bacterial plant pathogens, such as *Pseudomonas syringae* on *Arabidopsis* (Bais et al. 2004), *Xanthomonas campestris* pv. *campestris* on Brassica (Wulff et al. 2002), *X. axonopodis* pv. *glycines* on soybean (Salerno and Sagardoy 2003), *Xanthomonas euvesicatoria* and *Xanthomonas perforans* on tomato (Roberts et al. 2008), *Ralstonia solanacearum* on mulberry (Ji et al. 2008) tobacco (Maketon et al. 2008) and chili (Yanti et al. 2017).

Our previous research, based from in planta technique we have found six selected isolates of *Bacillus* spp. to control *R. solanacearum* and increase growth rate of chili plants (Yanti et al. 2017). From previous 16S rRNA sequencing and identification using BLAST with genebank (<http://ncbi.nlm.nih.gov>), all six isolates were identified that RZ.2.1.AP3 as *Bacillus subtilis* BSn5, RZ.2.1.AP1 as *Bacillus* sp. RD-AZPVI-03, RZ.2.2.AG2 as *Bacillus* sp. Y81-1, RZ.2.1.AG.1 as *Bacillus cereus* strain C38/15,

RZ.1.2.AP1 as *Bacillus cereus* strain APSB-03, and RZ.1.1.AP1 as *Bacillus subtilis* strain CIFT MFB 4158A (Yanti et al. 2017 unpublished). In this research, we want to characterize *in vitro* the selected *Bacillus* spp. as biocontrol agents of *R. solanacearum* (production of HCN, siderophore, biosurfactant, antibiotic) and as biofertilizer (production of Indole Acetic Acid (IAA), ammonia, protease, phosphate solubilization) and in planta (root colonization).

## MATERIALS AND METHODS

### Study area

This research has been done as an experiment at Laboratory of Microbiology, Department of Plant Protection, and greenhouse, Faculty of Agriculture, University of Andalas, Padang, Indonesia from April to August 2016.

### Bacterial strains and culture conditions

Pathogenic bacteria *R. solanacearum* and six-selected *Bacillus* spp. (Table 1) from our culture collections in 1.5 mL microtube contain sterilized aquadest stored in room temperature (27°C) were used for this study (Yanti et al. 2017). All *Bacillus* isolates were streaked on Nutrient Agar (NA) (Himedia® composition per litre, peptone 5g, NaCl, 5g, beef extract 1.5g, yeast extract 1.5g, agar 15 g, pH 7.4±0.2) culture medium and incubated at room temperature for 48 h. *R. solanacearum* isolate was streaked on Tetrazolium Triphenyl Tetrachloride (TZC) culture medium (composition per litre dextrose 10g, peptone 10g, casamino acid 1g, agar 18g, TZC 0.005%) and incubated with the same methods. These inoculants were prepared in order for them to be used *in vitro* tests of the *Bacillus* isolates and also their colonization on chili roots in planta.

### *In vitro* characterization of selected *Bacillus* spp. mechanisms

#### Biological agents

**Hydrogen cyanide production.** Cyanide (HCN) production was determined with cyanide detection agar containing TSA (Himedia®) amended with 0.44% of glycine and detected using 1cm<sup>2</sup> of filter paper dipped in CDS solutions (2g picric acid, 8g of Na<sub>2</sub>CO<sub>3</sub> in 200mL of aquadest) based on method of Lorck (1948). Color shifting from yellow to brownish orange on filter paper was sign of HCN productions.

**Siderophore production.** Siderophore production was determined with a method by putting spotted strains into the center of a Chrome Azurol S agar ((compositions are 60.5 mg of Chrome azurol S dissolved in 50mL of aquadest, and mixed with 10mL of FeCl<sub>3</sub>.6H<sub>2</sub>O solutions (1mM of FeCl<sub>3</sub>.6H<sub>2</sub>O+10mM of HCl) then added by 72.9mg of HDTMA dissolved in 40mL of aquadest, the solutions then was added to 900mL King's B agar (Himedia®, per litre contains of protease peptone 20g, K<sub>2</sub>HPO<sub>4</sub> 1.5g, MgSO<sub>4</sub> 1.5g, agar 15g, glycerol 10mL pH 7.2)) and incubated at 28°C for 5 days. The medium color shifting from blue to orange was a sign of siderophore activity (Alexander and Zuberer 1991).

**Table 1.** List of bacterial strains used in this experiment

Bacterial strain	Species
RZ.2.1.AP3	<i>Bacillus subtilis</i> BSn5
RZ2.1.AP1	<i>Bacillus</i> sp. RD-AZPVI-03
RZ2.2.AG2	<i>Bacillus</i> sp. Y81-1
RZ2.1.AG1	<i>Bacillus cereus</i> strain C38/15
RZ1.2.AP1	<i>Bacillus cereus</i> strain APSB-03
RZ1.1.AP1	<i>Bacillus subtilis</i> strain CIFT MFB 4158A

**Hemolytic assay** Hemolytic activity was determined using agar diffusion technique by Monteiro et al. (2005) namely by using Blood Agar (TSA (Himedia®) enriched with 5% of sheep blood pH of 7.3) where halo zone (hemolysis) around the colony observed as hemolytic activity.

**Biosurfactant production.** Biosurfactant production was assayed using Nutrient Broth (NB) Medium (Himedia®, which was per litre contains of peptone 5g, NaCl 5g, beef extract 1.5g, yeast extract 1.5g pH 7.2). *Bacillus* spp. was cultured in culture bottle containing 20 mL of NB and was incubated for 48h without shaking. Biofilm formations in the surface of the medium was sign of biosurfactant production.

**Production of antibiotic against *R. solanacearum*.** *R. solanacearum* suspension (10<sup>8</sup> CFU/ml adjusted with McFarland solution scale 8) was spread on NA plates and on three holes of 5 mm diameter punched into the agar. In these holes, 20 µl of the culture filtrate of each *Bacillus* was added and the plates were incubated at 28°C for 48 h. Inhibition of *R. solanacearum* growth with halo clear zone is the indicator.

#### Biofertilizer

**Indole Acetic Acid (IAA) production.** *Bacillus* spp. was inoculated in Nutrient Broth (HiMedia®) containing 500mg L<sup>-1</sup> L-tryptophan and incubated for for 48h on shaker (110rpm). After incubation, the culture was centrifuged and added by 1 ml of supernatant mixed with 4ml of salkowsky reagent (1ml of FeCl<sub>3</sub> 0.5M, 50ml of HClO<sub>4</sub> 35%), and incubated for 20 minutes in temperature room. IAA Production quantitatively was analyzed with methods used in Patten and Glick (2002) using spectrophotometer (Thermo Spectronic, Merck, SA), absorbance at 535 nm.

**Ammonia production.** Isolates assayed separately in 1% peptone was dissolved in sterilized water, *Bacillus* spp. was inoculated in 10 mL peptone water and incubated for 48-72 h at 28±2 °C. 0.5 mL of Nessler's reagent (Sigma-Aldrich® - HgI<sub>4</sub>K<sub>2</sub> 0.09m/L) was added after incubations and the color change to brown yellow color which was a positive result (Cappuccino and Sherman 1992).

**Phosphate solubilization.** The isolates' ability to solubilize tri-calcium phosphate was assayed using methods of Wahyudi et al. (2011). The isolates was inoculated to Pikovskaya's Agar (Compositons per litre glucose 10g, Ca<sub>3</sub> (PO<sub>4</sub>)<sub>2</sub> 5g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5g, KCl 0.2g,

MgSO<sub>4</sub>.7H<sub>2</sub>O 0.1g, MnSO<sub>4</sub>.H<sub>2</sub>O 0.01g, Yeast extract 0.5g, FeCl<sub>3</sub>.6H<sub>2</sub>O 0.01 g, Agar 15g, pH 7) separately and was incubated at room temperature for five days with formation of halo indicating positive results.

**Protease activity.** Protease activity was assayed on Luria Bertani Broth medium (compositions per litre contains Casein enzymic hydrolysate 10g, Yeast extract 5g, NaCl 10g, pH 7.5(Himedia®)), and was amended with 2% of skim milk powder (Nestle) and 15g of agar based on methods of Bakker and Schippers (1987). Clearing zones appear around isolates expressed activity of protease.

#### Root colonization of rifampicin-resistant (Rif) mutants of selected *Bacillus*

Root colonization ability of *Bacillus* strains was assayed with firstly mutated strains accompanied with Rifampicin which was gradually streaked on TSA (Himedia®) (compositions Tryptone 15 g/L, soya peptone 5g/L, NaCl 5 g/L and agar 15g/L) with concentration of 0, 10, 20, 50 and 100 ppm rifampicin each for 24-36 h. Culture streaked on the last concentration (100ppm) was used for assaying Chili seedlings and then was dipped into mutated *Bacillus* spp. ( $10^6$  CFU/mL) for 5 min and was planted in sterilized soil. Roots of chili then were harvested after 9 days. *Bacillus* spp. then was re-isolated from external root (rhizospheric) with dipping methods to 10 mL of sterilized aquadest and vortexed for 2 minutes, and from internal root (endophytic), first, the surface was sterilized sequentially for 1 minute with aquadest, for 1 minutes with NaOCl 1%, and for 3 times 1 minutes with aquadest, then was macerated and both (external and internal suspension) was diluted to  $10^{-4}$ . 0.1 mL of each was homogenized with TSA (when medium was still in liquid state before pouring, and temperature was 45-50°C) containing 100 ppm rifampicin and was plated to petri dishes for 48 h. Colonies growth with the same morphologies then counted.

## RESULTS AND DISCUSSION

#### Biocontrol mechanism

*In vitro* production of HCN by the six-selected *Bacillus* spp. was carried out using the picric acid assay. None of these isolates produced HCN (Table 1). Siderophores provide a competitive advantage to organism producer over plant pathogens for the absorption of available iron. Only *B. subtilis* CIFT MFB 4158A produced siderophore on CAS agar medium. while the others were negative. The hemolytic activity showed by lipopeptides can be used for selecting lipopeptide-producing microorganisms. Therefore, hemolytic activity tests were performed to investigate the possible role of these compounds in the antimicrobial activity of the *Bacillus* isolates. None of these isolates showed hemolytic activity. None of these isolates produced biosurfactant. In agar well diffusion assays for antibacterial activity, two *Bacillus* strains inhibited the growth of *R. solanacearum*, namely *B. cereus* C38/15 and *B. subtilis* CIFT-MFB-4158A.

#### Biofertilizer

Production of IAA by all the selected *Bacillus* isolates was detected by the production of pink color by all of them. All the *Bacillus* isolates produced indole acetic acid when grown in media containing tryptophan and it is obvious by the production of pink color by all isolates (Table 2). Using spectrophotometer (Thermo Spectronic, Merck, SA), absorbance at 535 nm revealed that *Bacillus subtilis* BSn5 had the highest IAA production (29.25 ppm) while *Bacillus subtilis* CIFT-MFB-4158A had the lowest IAA production (8.3 ppm). Bacterial isolates were tested for the production of ammonia in peptone water. A total five selected *Bacillus* isolates produced ammonia and only *Bacillus* sp. Y81-1 did not produce ammonia. Qualitatively phosphate solubilization was detected on Pikovskaya agar plate, indicated by halo around the inoculated spot. Only *Bacillus subtilis* CIFT-MFB-4158A showed positive results. All the others were negative without any clear zones. In the study of protease detection, it was found that four-selected *Bacillus* spp. secreted protease.

#### Root colonization

All selected *Bacillus* spp. have colonized the chili's rhizosphere until 21 days after introduction or after planting, but their population densities have fluctuated from  $1.1 \times 10^4$  to  $8.48 \times 10^5$  cfu g<sup>-1</sup> (Figure 1). All *Bacillus* spp. had ability to colonize and have a good persistence in both rhizospheres and endophytic root.

Colonization of external and internal roots of chili by *Bacillus* spp. shown various amounts (Figure 1 and 2). All strains showed its ability to colonize both on external and internal (endophyte) of chili seedlings roots. All strains shown decreasing trends based on days after introduction. Although, some strains still showed an increase of populations from time to time, which shown its ability to maintain its populations, others showed inversely.

#### Discussion

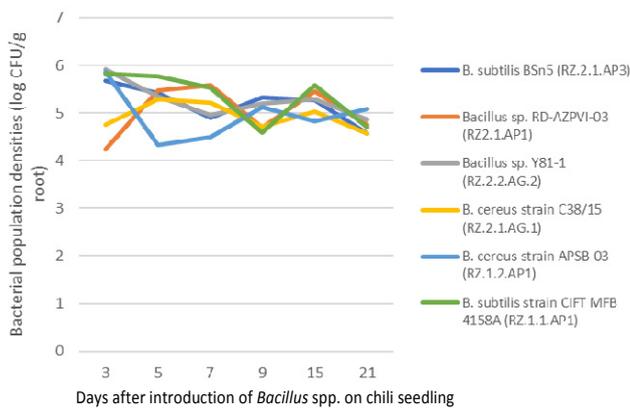
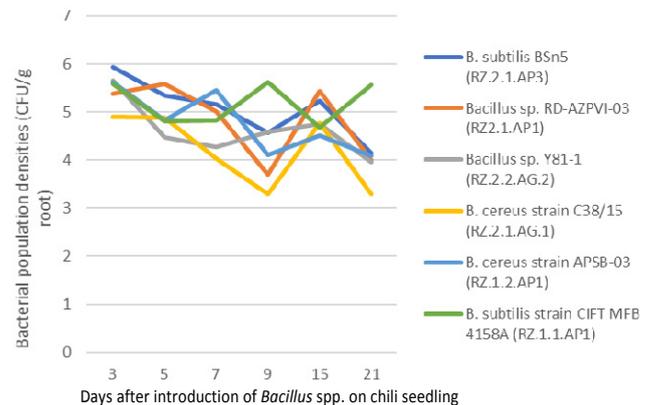
Our previous study has demonstrated that six-selected *Bacillus* spp. controlled 100 % of *R. solanacearum* on chili with no Wilt symptom and improved the growth of *R. solanacearum*-inoculated chili plants (Yanti et al. 2017). These may have been happened by direct antagonism of pathogens, production of antibiotic, or competition with pathogens for nutrients (Gamliel and Katan 1993). The present study demonstrates that biocontrol characters of the selected *Bacillus* spp. were presented in Table 1. As shown in Table 1, only *B. subtilis* CIFT-MFB-4158A produced siderophore and antibiotic for growth inhibition of *R. solanacearum*. The other *Bacillus* spp. which also produced antibiotic was *Bacillus cereus* C38/15. All selected *Bacillus* spp. showed negative for the other characters, such as: HCN, siderophore, hemolytic activity and biosurfactant. This study showed that direct mechanism's characters of *Bacillus* spp. have not played an important role to control *R. solanacearum* on chili.

**Table 1.** Characters of selected *Bacillus* spp. as a biocontrol agent.

Bacterial strains	<i>Bacillus</i> species	Characteristics as biocontrol agent				
		HCN production	Siderophore production	Hemolytic activity	Biosurfactant production	Antibiotic production
RZ2.1.AP3	<i>Bacillus subtilis</i> BSn5	-	-	-	-	-
RZ2.1.AP1	<i>Bacillus</i> sp. RD-AZPVI-03	-	-	-	-	-
RZ2.2.AG2	<i>Bacillus</i> sp. Y81-1	-	-	-	-	-
RZ2.1.AG1	<i>Bacillus cereus</i> C38/15	-	-	-	-	+
RZ1.2.AP1	<i>Bacillus cereus</i> APSB-03	-	-	-	-	-
RZ1.1.AP1	<i>Bacillus subtilis</i> CIFT MFB 4158A	-	+	-	-	+

**Table 2.** Characters of selected *Bacillus* spp. as biofertilizer

Bacterial strains	<i>Bacillus</i> species	Characteristics as biofertilizer			
		IAA Production (ppm)	Ammonia production	Phosphate solubilization	Protease activity
RZ2.1.AP3	<i>Bacillus subtilis</i> BSn5	29.25	+	-	-
RZ2.1.AP1	<i>Bacillus</i> sp. RD-AZPVI-03	24.05	+	-	+
RZ2.2.AG2	<i>Bacillus</i> sp. Y81-1	23.05	-	-	+
RZ2.1.AG1	<i>Bacillus cereus</i> C38/15	15.75	+	-	+
RZ1.2.AP1	<i>Bacillus cereus</i> APSB-03	22.05	+	-	-
RZ1.1.AP1	<i>Bacillus subtilis</i> CIFT-MFB-4158A	8.30	+	+	+

**Figure 1.** Population densities of selected *Bacillus* spp. in the rhizospheres of chili at 3 until 21 days after planting (DAP)**Figure 2.** Population densities of *Bacillus* spp. as an endophyte in the chili root tissue from 3 until 21 days after planting (DAP)

The biofertilizer characters of the selected *Bacillus* spp. were as shown in Table 2. All selected *Bacillus* spp., could induced IAA productions. The IAA productions of selected *Bacillus* spp. were varied from 8.30 to 29.25 ppm. *Bacillus subtilis* BSn5 produced higher IAA than the other *Bacillus* spp.. IAA production by PGPR had been reported to be varied among different species and was influenced by culture condition, growth stage and substrate ability (Mirza et al. 2001; Mishra et al. 2010).

The IAA produced by *Bacillus* played a major role in roots elongation and could directly promote roots growth by cells elongation and cells division stimulation or could indirectly influence ACC-deaminase activity in rhizobacteria (Rathaur et al. 2012). Patten and Glick (2002)

also reported that various IAA concentrations could induce different stimulations to plants. Low levels of IAA could stimulate roots elongation, while high levels of IAA would stimulate the lateral and adventitious roots formation. IAA was part of many secondary metabolites produced by PGPR in abundance during the stationary phase (Wahyudi, et al. 2011). This explained that the ability of *B. subtilis* CIFT MFB 4158A with low level of IAA production and its combination with phosphate solvent could also increase growth rate. *B. amyloliquefaciens* strain FZB42 was also found producing IAA and stimulating the growth of plant (Idris et al. 2007).

*Bacillus subtilis* strain produced a variety of powerful antibiotic metabolites (Peyopux et al. 1999) and

lipopeptides belonging to surfactin, iturin and fengycin families (Rahman et al. 2002). Hemolysis assay is the sole method widely used to screen biosurfactant production (Yonebayashi et al. 2000). In this study, all strains did not show any ability to produce biosurfactants. However, all strains showed a good ability to colonize roots, and it is showed that there is any other ability that helps the strains to colonize rhizosphere and endophyte of roots. Bacterial endophytes are well known for its beneficial effects for host plants (Puente et al. 2009). The ecological niches that are similar with phytopathogens, makes endophytic bacteria more suitable as biocontrol agents (Berg et al. 2005) and more stable to control pathogens. The growth reductions of *R. solanacearum* by *Bacillus* spp. and its in vitro inhibition zones are probably due to its ability to produce lytic enzymes like protease and a synthesis of antibiotics, which are most common character associated in PGPR (Mazurier et al. 2009).

Based on the properties of strains, only little direct activities showed relation to biocontrol activity, whereas from previous research (Yanti et al. 2017), all isolates have shown best biocontrol activity with no *R. solanacearum* symptoms appears. Based on these results, it can be concluded that most of all strains' ability in controlling *R. solanacearum* are not via direct mechanisms. According to Kloepper et al. (1999), ISR could be one of the most important mechanisms against systemic pathogens such as *R. solanacearum*. Other studies had reported that ISR can be triggered by inoculation of bacteria (Van Peer et al. 1990; Benhamou et al. 1996; Wilhelm et al. 1998). A further research is needed to be done to characterize all the bacterium activity in inducing systemic resistance of chili plants.

Our research concluded that these *Bacillus* strains had good traits characters to both promote plant growth and control pathogens. All strains also had ability to colonize internal (endophyte) and rhizosphere of chili roots. These *Bacillus* strains have potential to be used as biofertilizer and biocontrol agent of bacterial wilt of chili and further research for the commercial production is needed.

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