

Characterizations of endophytic *Bacillus* strains from tomato roots as growth promoter and biocontrol of *Ralstonia solanacearum*

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Abstract. Yanti Y, Warnita, Reflin, Nasution CR. 2018. Characterizations of endophytic *Bacillus* strains from tomato roots as growth promoter and biocontrol of *Ralstonia solanacearum*. *Biodiversitas* 19: 906-911. Bacterial wilt caused by *Ralstonia solanacearum* is the most damaging vascular pathogens in tomato and many other crops in tropical, subtropical and warm temperate areas of the world limiting its production. Biological agents such as Plant growth Promoting Rhizobacteria (PGPR) is considered as a potential biological control agent for the suppression of plant diseases such as bacterial wilt. *Bacillus* spp. are one of the most potential genera of PGPR group used for controlling pathogens and promoting plant growth because of their spore-forming ability which increases their adaptation to the environment. The aims of the research were to isolate Endophytic *Bacillus* isolates, to characterize its ability as plant growth promoter and pathogen controller, and to identify its molecular genetic using 16S rRNA. *Bacillus* strains were isolated from healthy tomato roots. All *Bacillus* spp. strains acquired from isolation were then screened directly on plants in completely randomized design experiments with 3 replications. All potential strains were screened and identified using 16S rRNA with 27F and 1492R primers. Results showed that out of 15 obtained isolates, 6 of them showed a good ability to both promote growth and control *R. solanacearum*. All isolates were identified as *B. Pseudomycooides* strain NBRC 101232, *B. cereus* strain CCM 2010, *B. toyonensis* strain BCT-7112, *B. anthracis* strain ATCC 14578, *B. cereus* strain JCM 2152 and *B. cereus* ATCC 14579.

Keywords: *Bacillus*, biocontrol, PGPR, *Ralstonia solanacearum*

INTRODUCTION

Bacterial wilt disease is one of the important serious vascular diseases in tomato crop caused by *Ralstonia solanacearum*. This disease affected the crop losses around 15% to 55% worldwide (Basu 2014; El-Argawy and Adss 2016). Several efforts to control this disease are difficult due to the high variability of the pathogen. In addition, the use of chemical agents to control this disease is limited because this pathogen has a high capacity to survive in diverse environments and has an extremely wide host range. Therefore, another effort to control this disease with environment-friendly is important. Biological agents are desirable environmental friendly methods to control pathogen diseases (Nguyen and Ranamukhaarachchi 2010). Since biological control is a key component of integrated disease management, it is important to find another plant growth promoting rhizobacteria (PGPR) used to against specific pathogens and evaluated its antagonistic activity to other pathogens for wider applications (Nakkeeran et al. 2006).

Endophytic bacteria are one of candidates of PGPR group. They inhabit plant tissues, for part or all of their life without causing any apparent disease symptom (Schulz and Boyle 2008). Endophytes can be found in any plants and reside in any part of plant organs such as roots (Ryan et al. 2008). Bacterial endophytes had no effect on host plants and are considered as neutral bacteria. In many cases,

bacterial endophytes provide beneficial effects to the host such as improving plant tolerance to abiotic stresses (Miliute et al. 2015). Thus the same ecological niche between endophytes and pathogens are suitable environment for bacteria to be used as biocontrol agents of plant diseases (Berg et al. 2005). Endophytes also had similar antagonistic effects as other bacteria such as competitors, producers of secondary antimicrobial metabolites, and inductors of plant resistance (Gairo et al. 2013).

Amongst the species of PGPR group, *Bacillus* spp. is well known as powerful genus. Varieties of *Bacillus* can promote the health and control diseases by plant pathogens suppressions, or by nutrients competitions like iron and phosphate or indirectly fixing nitrogen (Mc Spaden Gardener 2004). In addition, *Bacillus* is also powerful biofertilizers, biopesticides, non-pathogenic to plants. It can also move freely and has a good competence and survivability on rhizosphere and facultative anaerobes, so that it can adapt living in soil at various environmental conditions. *Bacillus* spp. can also sporulate in unfavorable conditions; so that they become more resistant to harsh conditions. Those abilities are 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), *Xanthomonas euvesicatoria* and *Xanthomonas perforans* on tomato (Roberts et al. 2008), *X. campestris* (now *axonopodis*) pv. *glycines* on soybean (Salerno and Sagardoy 2003), *Ralstonia solanacearum* on chili (Yanti et al. 2017), mulberry (Ji et al. 2008), and tobacco (Maketon et al. 2008).

The present study was undertaken to acquire potential *Bacillus* spp. for biocontrol agents of bacterial wilt disease caused by *R. solanacearum*. The aims of the research were to isolate Endophytic *Bacillus* isolates from tomato roots, characterize its ability to promote growth and control pathogens, and identify its molecular characteristics using 16S rRNA.

MATERIALS AND METHODS

Study area

This research has been done in Microbiology Laboratory, Department of Plant Protection, and screen house, Faculty of Agriculture, Universitas Andalas, Padang, Indonesia.

Bacillus spp. isolation

Samples of tomato roots collected from healthy tomato plants in tomato fields diseased by *R. solanacearum* were obtained in the Tanah Datar and Solok Districts, West Sumatra, Indonesia. *Bacillus* spp. was isolated from roots by firstly surface sterilization with sequentially 1-minute aquadest. Subsequently, roots were soaked in NaOCl 1% for 1 minutes followed by washing with 3 times aquadest for 1 minutes each. Roots were then macerated and both (external and internal suspension) diluted to 10^{-4} , followed by heating in water bath at 80°C for 10 minutes. 0.1 mL of each suspension were cultured into medium containing Tryptic Soy Agar (TSA, HiMedia®). This medium was made by pouring into petri dishes when it was still in liquid state with temperature between 45 to 50°C for 48 h. Each Different colonies growth in the medium were then re-isolated and cultured in TSA to acquire the pure colony.

Hypersensitive (HR) reaction

The Hypersensitive reactions were done by infiltrating the *Mirabilis jalapa* leaves with 10^8 CFU/mL, which was acquired by comparing *McFarland* solutions in the scale of 8. The infiltrated leaves were then incubated for 48 hours (Yanti et al. 2017). The occurrence of necrosis on the *M. jalapa* leaves indicated the positive HR reactions of *Bacillus*, which was suspected as pathogens so that it could not be used further.

Multiplication of isolates

1 pure isolated colony was streaked and cultured in 25mL of liquid medium containing Tryptic Soy Broth (TSB, HiMedia®), which was then incubated in rotary shaker with the speed of 110 rpm for 24 hours. 1 mL of those liquid-incubated preculture bacteria were then transferred into 150 mL of sterile coconut water in Erlenmeyer flask for main culture and incubated for 2x24 hours (Yanti and Resti 2010). The suspension of *Bacillus* spp. isolates from main culture were then diluted to obtain

the estimated density of 10^8 CFU/mL by comparing with *McFarland* with scale of 8 (Habazar et al. 2007).

Bacterial infiltrated seed procedure and seed germination

Tomato seeds were infiltrated with each *Bacillus* spp. strains by dipping into the bacterial suspension culture with the density of 10^8 CFU/mL (Compared with *McFarland* Scale 8). The bacteria infiltrated seeds were germinated into seed trays for 21 days.

Planting

All tomato seedlings infiltrated with each *Bacillus* spp. strains growth in seedtrays were then removed into polybag (30 x 30 x 40 cm³) containing with sterilized soil and organic manure (2: 1 v/v) (Yanti et al. 2013). Seedling samples were set in triplications, which were then introduced with the same *Bacillus* spp. isolates using dipping methods. *R. solanacearum* was inoculated into seedlings at 2 weeks after planting.

Growth observation and data analysis

The growth parameters, i.e., seedlings' height, incubation time, incidence, severity, plant height, number of leaves, first flowering time and yields have been observed. Data were then analyzed using analysis of variance (ANOVA) with statistic 8 software. Effectivity of data also calculated to know its effectiveness from control using formula by Sivan and Chet (1986) that is $E = \{ (\text{treatment-control})/\text{control} \} \times 100\%$. Treatments with significant differences were further analyzed using Duncan's Multiple Range Test (DMRT) test with 5% significance.

16S rRNA bacteria identifications

Bacillus spp. isolates were identified based on its 16S rRNA. Bacterial Genomic DNA was extracted by following manufactures of PureLink Genomic DNA mini Kit (Invitrogen, Thermo Scientific Inc. USA). The extracted DNA was then amplified using PCR with universal primers (27F and 1492R). PCR conditions were done by following Xiong et al. (2014) consisting of denaturation at 94°C for 1 min, annealing at 54°C for 30 s and extension at 72°C for 1 min for 30 cycles and final extension for 30 minutes. The 16S fragment was then sequenced in Macrogen Inc. (Korea).

RESULTS AND DISCUSSION

Effects of PGPR derived from *Bacillus* spp. on tomato seedlings

The isolation of *Bacillus* spp. endophytes from healthy tomato roots resulted in 15 bacterial isolates. Those isolates are B.1.1.1, B.1.1.2, B.1.2.1, B.1.3.5, B.2.3.4, B.5.2.6, B.3.4.1, B.3.3.2, B.3.3.4, B.4.2.2, B.4.1.2, B.4.3.6, B.4.4.2, B.1.3.1 and B.2.2.1 Of all sampling locations, the obtained *Bacillus* spp. Isolates was differed (Fig. 1a). Thus, isolates were then assayed for its ability to be used as PGPR for promoting growth rate of tomato and increasing yields in

plant conditions. The ability of bacterial isolates used as PGPR had been assayed in seeding stage and planting stage. Tomato seeds infiltrated by 12 out of 15 *Bacillus* spp. isolates showed a significant difference in seedling height and leaves (Table 1). In this study, 12 of 15 isolates could increase plant germinability and seedling height. In addition, indigenous *Bacillus* spp isolates could increase seedling height with the effectivity between 20.23 to 61.13%. Isolates affecting on the higher seedling height and leaves number than control were B.1.1.1, B.1.1.2, B.1.2.1, B.1.3.5, B.2.3.4, B.5.2.6, B.3.4.1, B.3.3.2, B.3.3.4, B.4.2.2, B.4.1.2 and B.4.3.6.

Besides their ability to promote growth of tomato seeding stage, the ability of *Bacillus* spp. isolates to control *R. solanacearum* in plant had been observed. The ability of *Bacillus* spp. isolates introduced to plant showed the decrease trends in terms of incubation time, incidence and severity of diseases (Table 3). Interestingly, 6 isolates of *Bacillus* had decreased in the incidence level-up to 100% without infectious symptoms until the end of observations (Fig. 1b). It could also be seen that *Bacillus* spp. Infiltration could promote plant growth after inoculation of pathogen into plant. Isolates B.1.1.1, B.1.1.2, B.1.2.1, B.1.3.5, B.2.3.4, and B.5.2.6 had the highest ability to promote growth rate of tomato and also had zero incidences without showing symptoms of diseases.

The height of plants after *Bacillus* spp. isolates introductions increased compared to control (Table 3). Only 10 isolates introduced to plant showed the lower plant height than that in control (Table 3). Meanwhile, plant height of another isolates introduced to plant, i.e., B.1.1.1, B.1.1.2, B.1.2.1, B.1.3.5, B.2.3.4, and B.5.2.6 had the higher plant height compared to that in control. B.1.1.2 isolate was the best isolates for promoting plant height with reaching to 127.20 cm and the effectivity level of 56.52% compared to those of control. The total number of tomato leaves introduced with IEB also increased where the highest one was obtained from plant introduced by B.1.1.1 isolate with total number of leaves was 54.00.

The infiltration of *Bacillus* spp. isolates to plant also showed the higher promotion rate of generative stage on tomato (Table 4). The used isolates i.e. B.1.1.1, B.1.1.2, B.1.2.1, B.1.3.5, B.2.3.4 and B.5.2.6 could also promote flowering time and increase yields of tomato plants. *Bacillus* spp. isolates introduced to tomato plants could reduce the disease development and increase plant growth as well as increase flowering time and yields. B.1.1.1 isolate introduced to plant showed the fastest flowering time (22.00 days after planting (DAP) followed by B.1.1.2 isolate (24 DAP). Those isolates also showed the highest yields 299.00 with the effectivity of 115.73% and 260.00g with the effectivity of 87.59% compared to control (138.60 g).

Table 1. The growth of tomatoes introduced with Indigenous *Bacillus* spp. isolates

Isolates of <i>Bacillus</i> spp	Seedlings' height (cm)	Effectivity (%)	Seedlings' leaves	Effectivity (%)
B.1.1.1	9.023 a	61.13	4.10 a	105.00
B.1.1.2	8.650 ab	54.46	4.00 ab	100.00
B.1.2.1	8.070 bc	44.11	4.00 ab	100.00
B.1.3.5	7.933 bc	41.66	4.00 ab	100.00
B.2.3.4	7.867 bc	40.48	3.90 ab	95.00
B.5.2.6	7.567 bcd	35.13	3.90 ab	95.00
B.3.4.1	7.567 bcd	35.13	3.90 ab	95.00
B.3.3.2	7.500 cd	33.93	3.90 ab	95.00
B.3.3.4	7.500 cd	33.93	3.90 ab	95.00
B.4.2.2	7.300 cd	30.36	3.80 ab	90.00
B.4.1.2	6.833 d	22.02	3.80 ab	90.00
B.4.3.6	6.733 d	20.23	3.80 ab	90.00
Control	5.600 e	-	3.20 ab	-
B.4.4.2	4.333 f	-22.63	3.20 ab	0.00
B.1.3.1	4.120 f	-26.43	2.00 c	-37.50
B.2.2.1	4.115 f	-26.52	2.00 c	-37.50

Note: Values followed by the same letters in the same column do not differ significantly at 5% probability level according to Least Significance Different test

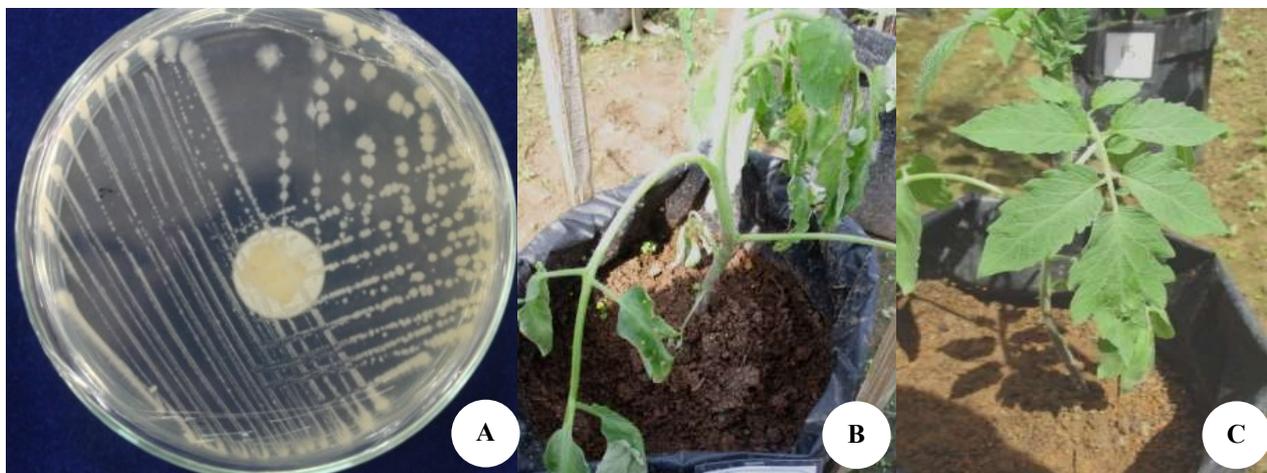


Figure 1. A. *Bacillus* spp. isolated from tomato roots; B. Tomato plants inoculated with *R. solanacearum* showed symptoms; C. Healthy tomato (control)

Table 2. Disease development of *R. solanacearum* to tomato plants introduced with Indigenous *Bacillus* spp. isolates

Isolates	Incubation		Incidence		Severity	
	DAI	Effectivity (%)	%	Effectivity (%)	Severity	Effectivity (%)
B.1.1.1	42.00*	46.55	0	100.00	0	100.00
B.1.1.2	42.00*	46.55	0	100.00	0	100.00
B.1.2.1	42.00*	46.55	0	100.00	0	100.00
B.1.3.5	42.00*	46.55	0	100.00	0	100.00
B.2.3.4	42.00*	46.55	0	100.00	0	100.00
B.5.2.6	42.00*	46.55	0	-100.00	0	-100.00
B.3.4.1	37.20	29.80	20	-66.67	1	-50.00
B.3.3.2	34.60	20.73	20	-66.67	2	0.00
B.3.3.4	32.20	12.35	40	-33.33	2	0.00
B.4.2.2	30.40	6.07	40	-33.33	2	0.00
Control	28.66		60		2	
B.4.1.2	25.00	-12.77	40	-33.33	1.5	-25.00
B.4.3.6	27.00	-5.79	60	0.00	2	0.00
B.4.4.2	23.60	-17.66	60	0.00	3	50.00
B.1.3.1	20.00	-30.22	60	0.00	4	100.00
B.2.2.1	20.00	-30.22	60	0.00	4	100.00

Note: * = no symptoms appear until last day observations. Values followed by the same letters in the same column do not differ significantly at 5% probability level according to Least Significance Different test

Out of 15 isolates, 6 isolates showed the best ability both to promote plant growth and to control *R. solanacearum*. According to 16S rRNA sequencing analysis (Table 4), sample isolates were identified as *B. Pseudomycooides* strain NBRC 101232, *B. cereus* strain CCM 2010, *B. toyonensis* strain BCT-7112, *B. anthracis* strain ATCC 14578, *B. cereus* strain JCM 2152 and *B. cereus* ATCC 14579.

Discussions

The biological control of plant diseases is one of viable alternatives to control chemical agents in sustainable agriculture (Killani et al. 2011). The application of PGPR is one of the best potential approaches to disease management and crop productivity improvement in sustainable agriculture (Rammamoorthy et al. 2001). The present study carried out to screen indigenous *Bacillus* isolates to promote growth yields and to control *R. solanacearum* in plant and to identify the selected isolates. The results show that *Bacillus* spp. introduced to tomato plant significantly reduced the severity of bacterial wilt disease caused by *R. solanacearum* and also promoted plant growth as well as increased yields of tomato.

Utilization of indigenous endophyte bacteria isolates in decreasing pathogen diseases had been developed such as the increase of plant defense using P12Rz2.1 isolate and P14Rz1 isolate to control bacterial pustule disease (Yanti et al. 2013). Another isolate, i.e., ST26c could control *Phytophthora capsici* on chili (Khaeruni et al. 2011), RB.2.4 isolate could control incidence and severity of *Fusarium* wilt on tomato (Khaeruni et al. 2013), PKLK5 and P11a isolates could decrease *Xanthomonas oryzae* pv. *oryzae* on rice (Khaeruni et al. 2014), CRb-26, CRb-39, CRb-17, CRb-9 dan CRb-14 isolates could increase seedlings, growth and control blight disease on cotton (Mondal et al. 1999) and S188, s215, s288 isolates could control *R. solanacearum* on tomato (Ramadasappa et al. 2012).

Bacillus strain produces a variety of powerful antibiotic metabolites (Peypoux et al. 1999) and lipopeptides belonging to surfactin, iturin and fengycin families (Rahman et al. 2007). Bacterial endophytes are well known for its beneficial effects on host plants (Puente et al. 2009). The ecological niches which are similar to phytopathogens, makes endophytic bacteria are 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 their in vitro inhibition zones might be due to the activity of lytic enzymes like protease and the synthesis of antibiotics, which are the most common characteristics associated with PGPR (Mazurier et al. 2009).

Table 3. Height and total of leaves of tomato introduced with Indigenous *Bacillus* spp. isolates

Isolates	Height		Total of leaves	
	(cm)	Effectivity (%)	Leave (s)	Effectivity (%)
B.1.1.1	126.60 a	105.52	54.00 a	175.51
B.1.1.2	127.20 a	106.49	52.00 a	165.31
B.1.2.1	115.00 b	86.69	44.80 a	128.57
B.1.3.5	109.00 c	76.95	43.80 b	123.47
B.2.3.4	95.60d	55.19	35.60 c	81.63
B.5.2.6	92.60 d	50.32	36.00 c	83.67
B.3.4.1	86.80 e	40.91	32.80 d	67.35
B.3.3.2	83.60 ef	35.71	27.60 e	40.82
B.3.3.4	80.80 fg	31.17	24.00 f	22.45
B.4.2.2	80.80 fg	31.17	19.60 g	0.00
Control	61.60 j		19.60 g	
B.4.1.2	74.00 h	20.13	16.00 h	-18.37
B.4.3.6	81.80 f	32.79	14.80 h	-24.49
B.4.4.2	80.00 fg	29.87	16.40 h	-16.33
B.1.3.1	68.00 i	10.39	16.20 h	-17.35
B.2.2.1	62.40 j	1.30	17.20 gh	-12.24

Note: Values followed by the same letters in the same column do not differ significantly at 5% probability level according to Least Significance Different test

Table 4. The first flowering time and yields of tomato introduced with indigenous *Bacillus* spp. isolates

Isolates	First flowering time		Yields	
	DAP	Effectivity (%)	(g)	Effectivity (%)
B.1.1.1	22.00 a	48.60	299.00 a	115.73
B.1.1.2	24.00 ab	43.93	260.00 b	87.59
B.1.2.1	25.60 b	40.19	248.00 bc	78.93
B.1.3.5	26.00 b	39.25	227.60 c	64.21
B.2.3.4	29.20 c	31.78	192.00 d	38.53
B.5.2.6	32.80 d	23.36	190.60 d	37.52
B.3.4.1	34.40 d	19.63	152.00 e	9.67
B.3.3.2	38.40 e	10.28	152.00 e	9.67
B.3.3.4	41.20 f	3.74	150.00 e	8.23
B.4.2.2	42.00 fg	1.87	143.00 e	3.17
Control	42.80 fgh	0.00	138.60 ef	0.00
B.4.1.2	43.20 fgh	-0.93	136.00 ef	-1.88
B.4.3.6	43.60 gh	-1.87	113.00 f	-18.47
B.4.4.2	44.00 ghi	-2.80	69.600 g	-49.78
B.1.3.1	44.80 hi	-4.67	68.400 g	-50.65
B.2.2.1	46.00 i	-7.48	22.800 h	-83.55

Note: Values followed by the same letters in the same column do not differ significantly at 5% probability level according to Least Significance Different test

Table 4. The nucleotide similarity of indigenous *Bacillus* spp. isolates based on 16S rRNA identifications using BLAST-N

Isolates	Sequence analysis results	% of Similarity
B.1.1.1	<i>B. pseudomycooides</i> strain NBRC 101232	94
B.1.1.2	<i>B. cereus</i> strain CCM 2010,	94
B.1.2.1	<i>B. toyonensis</i> strain BCT-7112,	95
B.1.3.5	<i>B. anthracis</i> strain ATCC 14578	97
B.2.3.4	<i>B. cereus</i> strain JCM 2152	95
B.5.2.6	<i>B. cereus</i> ATCC 14579.	96

Based on properties of strains, only little direct activities showed being related to biocontrol activity, whereas from previous research (Yanti et al. 2017), all isolates showed the best biocontrol activity with no *R. solanacearum* symptoms appears. Based on these results, it could be concluded that most of all strains had ability in controlling *R. solanacearum* via indirectly mechanisms such as Induced Systemic Resistance (ISR). 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 also reported that ISR could be triggered by bacteria inoculation (Van Peer et al. 1991; Benhamou et al. 1996; Wilhelm et al. 1998). However, Need further research to characterize all the *Bacillus* spp. activity in inducing systemic resistance of tomato plants should be conducted.

Strains of *Bacillus* have been widely studied for enhancement of plant growth (Kloepper et al. 2004). Species of *Bacillus* are commonly inhabiting inner tissues of various species of plants, including cotton, grape, peas, spruce, and sweet corn, where they play an important role as biocontrol and growth promotor (Berg et al. 2005).

Recently, Choudhary and Johri (2008) reported that mechanisms and role of *Bacillus* species as inducers of systemic resistance in relation to plant-microbe interactions and its pathways involved in their regulation. Kloepper et al. (2004) also reported that specific strains from the species of *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycooides*, and *B. sphaericus* shown significant reductions in the incidence or severity of various diseases on various hosts including greenhouse studies or field trials on plants such as tomato, bell pepper, muskmelon, watermelon, sugarbeet, tobacco, Arabidopsis species, cucumber, loblolly pine, and tropical crops.

Our 16S rRNA identifications showed that the similarity of several isolates, i.e., *B. cereus*, *B. pseudomycooides* *B. anthracis* and *B. toyonensis*. *B. cereus* had been reported as growth promotor and systemic resistance inducers in *Arabidopsis thaliana* (Niu et al. 2011), growth promotor in red pepper seedlings and gibberellin producer (Joo et al. 2004). In addition, *B. toyonensis* had been reported as bacteria, which could be used to promote growth of *Arabidopsis* and *Datura* (Rahmoune et al. 2017) and biocontrol of *Fusarium oxysporum* on tomato (Rocha et al. 2017). On the other hands, *B. pseudomycooides* and *B. anthracis* used as PGPR or biocontrol agents of plant pathogens did not widely report, yet. This means that those could be considered as newfound novel species of PGPR from *Bacillus* genera. Although, our findings did not much prove the effect of PGPR as biocontrol agents to plant pathogens.

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