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Research Article

Bacillus subtilis from Potato Rhizosphere as Biological Control Agent and Chili Growth Promoter

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ABSTRACT

Bacillus subtilis is an antagonist bacteria that inhibits the growth of fungal and bacterial plant pathogens. The *B. subtilis* has roles as biocontrol agents and plant growth promoting rhizobacteria (PGPR). This research aimed to evaluate the potency of *B. subtilis* isolates (B209, B211, and B298) as a biocontrol agent to anthracnose (caused by *Colletotrichum* spp.) and as PGPR to increase the growth of chili plants. The experiments were divided into two batches. The first batch was conducted in the laboratory to evaluate the characteristics of *B. subtilis* (as biocontrol) and PGPR (phosphate solubility, producing IAA and nitrogen). The second batch was conducted in the field in Rempoah Village, Baturraden Regency, Banyumas District, with 5 treatments and 6 replications. Parameters observed were inhibition percentage to *Colletotrichum* spp., disease intensity, the component of PGPR, plant growth of chili, and phatosystem. The results showed that B209, B211, and B298 isolates inhibited the growth of *Colletotrichum* spp, with the highest inhibition percentage on B298. B209, B211, and B298 have characteristics as PGPR, i.e. the ability to soluble phosphate, to produce IAA and nitrogen. The ability of B298 to promote plant growth was shown by the increase of plant height, leaf number, plant dry mass, and dry root mass (38.0%, 54.7%, 61.7%, 61.8%, respectively). B298 and B211 could increase the fresh crop mass (41.2% and 37.1%) and fresh root mass (36.4% and 34.4%). B298 and B209 were similar in increasing the root length (25.2%). Root volume could be increased by 33.3% by applying B211 isolate. B209 was the best isolate to reduce anthracnose up to 80.36%.

Keywords: *Bacillus subtilis*, biocontrol agent, chili growth, PGPR

INTRODUCTION

Bacteria associated with plants may have a role as growth promoters and improve plant health. PGPR (plant growth–promoting rhizobacteria) is a root colonizing rhizosphere bacteria that is able to increase plant growth. The ability of bacteria as PGPR directly is to stimulate plant growth, and indirectly to control pathogens. Biological control is to reduce the use of synthetic pesticides that have a negative impact on the environment, non–target microorganisms and may cause pathogenic resistance. Compant *et al.* (2010) stated that the bacterial-plant association in the natural ecosystem plays a role in improving plant health and growth. Some rhizosphere bacteria (rhizobacteria) can pass the roots and live as endophytic populations by producing IAA (Indole-3-Acetic Acid) to stimulate plant growth (Dawwam *et al.*, 2013; Dwimartina *et al*., 2017) and the others bacteria can pass through the endodermis barrier by the root cortex to the vascular system, and then become endophytic in the stems, leaves, tubers, and other plant organs (Compant *et al.,* 2005). The roles of rhizobacteria according to Kesaulya *et al.* (2015) are as bioprotectant (suppress plant diseases), biofertilizer (improve the absorption of nutrients for plants), and biostimulant (produce phytohormone).Rhizobacteria can suppress disease by antagonistic mechanisms against soil infectious pathogens or induce plant systemic resistance to root and leaf pathogens. This resistance is generally not specific to certain pathogens, yet under natural conditions of several pathogens simultaneously. According to Ahemad & Kibret (2014), siderophore pseudomonine can induce plant resistance associated with salicylic acid.

The number of rhizobacteria that able to spur plant growth is only 2–5% of all the population (Chaiharn *et al.*, 2008). The mechanism of rhizobacteria directly to improve the plant growth are by obtaining phosphate

(dissolve phosphate), fixating N_2, and producing phytohormones such as IAA (Adesemoye *et al.*, 2009). The rhizobacteria may have one or more mechanisms, either sequentially or simultaneously actively affecting different phases of plant growth (Saharan & Nehra, 2011). The mechanism of rhizobacteria indirectly is as a biological control agent of plant pathogens by producing antibiotics, lytic enzymes, hydrogen cyanide, and siderophore, or through nutritional competition and space that significantly improve plant health and stimulate plant growth by increasing germination, vigor and crop yields (Chaiharn *et al.*, 2008; Mishra & Kumar, 2012). Therefore, this study was aimed to evaluate the potential of *B. subtilis* isolates B209, B211, and B298 as biological agents to control anthracnose and as PGPR to increase the growth of chili plants.

MATERIALS AND METHODS

This research was conducted in the Laboratory of Plant Protection, Faculty of Agriculture, Universitas Jenderal Soedirman, in Purwokerto and the experimental field in Rempoah Village, Baturraden District, Banyumas Regency over 7 months. The inhibitory test of anthracnose pathogenic fungi, characteristics of three *B. subtilis* isolates as PGPR were conducted in the laboratory while testing the ability of *B. subtilis* to suppress anthracnose and increase the growth of chili plants were conducted in the field.

B. subtilis *as a Biological Control Agent for Anthracnose Pathogenic Fungi Test*

B. subtilis was tested for its ability as a biological control of plant pathogenic fungi in vitro using the dual culture method by Wang *et al.* (2013) by growing both in a 9 cm diameter petri dish. Inoculation of *B. subtilis* antagonist bacteria was carried out after the pieces of the mycelium *Colletotrichum gloeosporioides* (chili anthracnose pathogen) were cultured. This plate which contained two microbes was incubated at room temperature (28 ± 2)°C for 7 days to observe its inhibition. The percentage of inhibition was calculated by measuring the growth of fungal colonies (Wang *et al.*, 2013):

$$I = \frac{C - T}{C} \times 100\ \%$$

I: inhibition; C: the growth of control fungus mycelium (in the opposite direction to antagonistic bacteria); Q: fungal mycelium growth that leads to antagonists.

Phosphate Solvent Activity Test

The ability of *Bacillus* isolates to dissolve phosphate was conducted using potato-dextrose yeast extract agar (PDYA), containing 50 ml 10% (w/vol) K_2HPO_4 and 100 ml 10% (w/vol) $CaCl_2$. A liter of sterile PDYA was added to produce $CaHPO_4$ precipitation. Each bacterial culture was inoculated in the line middle of the PDYA-CaP at 3 points and incubated at room temperature for 10 days. Phosphate solvents were assessed by measuring the clear zone. Zone calculation is the total clear zone reduced by the diameter of the bacterial colony (de Freitas *et al*., 1997).

IAA Production Test

IAA production test was employed according to Shrivastava & Kumar (2011). The three isolates of *B. subtilis* were grown on YPGA medium with and without L-Tryptophan (0.5%) at room temperature for 48 hours, dropped with 1 ml of Salkowski reagent A [1:50 from 0.5M $FeCl_3$ and 35% perchloric acid (HClO)], then observed until a reddish-pink was formed which indicates diffusion of the agar medium. The variation in color-changing becomes reddish-pink showed the difference in the amount of IAA produced. This test was repeated 3 times to obtain consistency of IAA produced by the isolate.

Nitrogen Producing Activity Test

This test was carried out by growing five isolates of *B. subtilis* on a nitrogen-free broth medium (Xie *et al*., 2003) by shaking for 24 hours at room temperature. The total nitrogen in culture was calculated using the micro Kjeldahl method, which is based on the principle of sample digestion, distillation, and titration. Digestion is the decomposition of organic samples using a solution of sulfuric acid to produce a solution of ammonium sulfate, distillation by adding a base thus changing NH_4^+ to NH_3, followed by boiling and condensing NH_3 gas in the solution. Furthermore, titration that produces ammonium with changes in color and total N concentrations can be measured using autoanalysers (Amin & Flowers, 2004).

Antibiotic Resistance Test

Antibiotic resistance test was conducted to determine whether *B. subtilis* is susceptible or resistant to antibiotics. One plate containing the suitable medium was incubated by spreading with different density bacteria, a paper–disc containing different concentrations of antibiotics was placed on the plate, then incubated at room temperature for 3 days. The presence of inhibition

zones around the paper-disc at different antibiotic concentrations was recorded. Each treatment was repeated 3 times. The antibiotics used were Chloramphenicol 10 and 30 µg/ml, Streptomycin 10 µg/ml, Kanamycin 5 and 30 µg/ml, Penicillin 10 µg/ml, and Tetracyclin 30 µg/ml, and Rifampicin 10 and 30 µg/ml.

Test of Plant Growth Enhancement and Anthracnose Disease Control in the Field

Treatment of *Bacillus* and fungicide (5 treatments) with 6 replications:
K: control (without *B. subtilis* and fungicide application)
A: *B. subtilis* B209 application
B: *B. subtilis* B211 application
C: *B. subtilis* B298 application
D: only fungicide application.
Variables: plant height, number of leaves, fresh and dry plant mass, root length, root volume, fresh and dry root mass. Observation of the pathosystem includes the incubation period and the intensity of anthracnose disease using the formula:

$$DI = \frac{\sum(n \times v)}{N \times Z} \times 100\ \%$$

DI: disease intensity, n: number of plants infected by anthracnose pathogens according to attack category, v: attack category, Z: highest category and N: number of plants observed.

Symptom catagories i.e. 0: plants are asymptomatic; 1: anthracnose symptoms 1–10%; 2: anthracnose symptoms 11–20%, 3: anthracnose symptoms 21–40%, 4: anthracnose symptoms 41–60%, 5: anthracnose symptoms > 60%. The data were analyzed using ANOVA. If there is significantly different, further analyzed by LSD ($\alpha = 5\%$).

RESULTS AND DISCUSSION

The role of *B. subtilis* B298 as a biological control agent of *C. capsici* and *C. gloeosporioides* was shown in Figure 1, with inhibition percentages of 57.6 and 64.6%, respectively against *C. capsici* and *C. gloeosporioides*. In vitro test for controlling of *Colletotrichum sp.* chemically with 800 ppm mancozeb using poisoned food technique showed inhibition of colony growth by 98%, while with cymoxanil 2000 ppm only suppressed 37.2% (Paramita *et al*., 2014). These results showed that *B. subtilis* B298 could be used as a biological control agent of plant pathogenic fungi through the mechanism of antibiosis by producing the enzyme chitinase to inhibit the growth of pathogenic fungi whose cell walls consist of chitin. *B. subtilis* B298 is able to produce the chitinase enzyme as study conducted by Lestari *et al.* (2017), with an activity of 6.937 U/ml at 15 hours incubation, 5.764 U/ml at 40°C, and 6.813 U/ml at pH 5. *B. amyloliquefaciens* SAHA 12.07 showed chitinase activity at pH incubation 5 with an activity of 1.158 U/ml (Azizah *et al*., 2015).

The characteristics of *B. subtilis* as PGPR are its ability to be able to produce secondary metabolites (consisting of enzymes and antibiotics), IAA, and nitrogen; and as a phosphate solvent bacterium. The observation of the ability of *B. subtilis* as PGPR (Table 1) revealed that the three *B. subtilis* isolates capable as PGPR, which are proven to be able to dissolve phosphate and produce IAA, nitrogen and antibiotic resistance. *B. subtilis* B298 showed the strongest isolate as phosphate solvent (the widest clear zone), the highest producer of IAA (dark red color) than other isolates. The total N activity produced by *B. subtilis* B298 isolate was 10.34 µg/ml which was produced based on micro Kjeldahl nitrogen analysis.

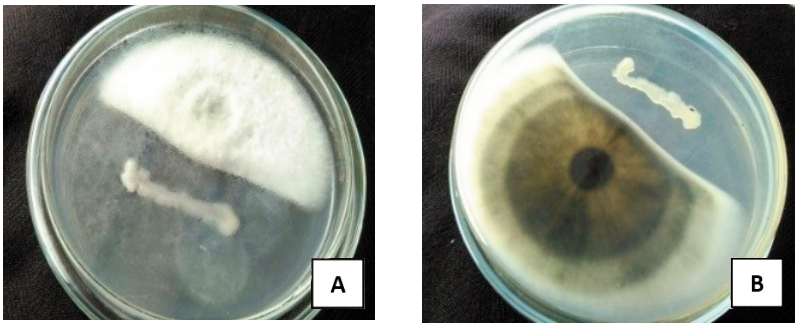

Figure 1. The growth inhibition of *Colletotrichum* by *Bacillus subtilis* B298; (A) *Colletotrichum capsici* and (B) *Colletotrichum gloeosporioides*

The three isolates of *B. subtilis* (B209, B211, and B298) which were explored from the rhizosphere of healthy potatoes showed their ability as phosphate solvents by forming the celar zones around the *B. subtilis* colonies on Pikovskaya with zone sizes varying from 1.2 to 4 mm (Figure 2). P is an important nutrient for plants, abundantly available in the soil, for example in Ultisol or red-yellow podsolic soil or as a result of the use of phosphate fertilizer. However, P is in the form of minerals that are slowly available to plants. According to Gupta *et al*. (2014), the phosphate would bind to Al and Fe in acid soil and Calcium phosphate in alkaline soil. The presence of rhizosphere bacteria can dissolve phosphate from the insoluble P to be dissolved which is characterized by its capacity to decrease pH by secretion of organic acids such as gluconate, citrate, lactate, succinate, and as protons during NH_4^+ assimilation. These rhizosphere bacteria are *Bacillus, Burkholderia, Enterobacter, Klebsiella, Kluyvera, Streptomyces, Pantoea,* and genus *Pseudomonas.*

The ability of *B. subtilis* as IAA-producing was shown in Figure 3, with a red discoloration after adding Salkowski reagent to pure culture of *B. subtilis*, and *B. subtilis* B298 isolate showed the strongest reaction. Antibiotic resistance was tested to justify the resistance of *B. subtilis* to certain antibiotics hence the antibiotic can be used as a marker to test the presence of *B. subtilis* after application. Three *B. subtilis* isolates are resistant to Rifampicin compared to 4 other antibiotics, i.e. Kanamycin, Streptomycin, and Chloramphenicol. Resistance to antibiotics is indicated by the absence of zones around the paper-disc have been added 10 µl antibiotic in an agar plate contained *B. subtilis* with was grown by the pour plate method (Figure 4).

The test of *B. subtilis* as PGPR in chilli field showed that isolate B298 is the best isolate in increasing the

Table 1. PGPR characteristics of the three isolates of *Bacillus subtilis* (B209, B211, and B298)

B. subtilis isolate	Phosphate Solvent	IAA Producing	Nitrogen Producing	Antibiotic Resistance	N total (µg/ml)	Siderophore producing
B209	++	++	++	Rif+	9.343	++
B211	++	++	++	Rif+	9.538	++
B298	+++	+++	++	Rif+	10.34	++

Remarks: ++ strong, +++: very strong

Figure 2. *Bacillus subtilis* as a phosphate solvent shown by a clear zone formed around the colony of *Bacillus subtilis* isolates

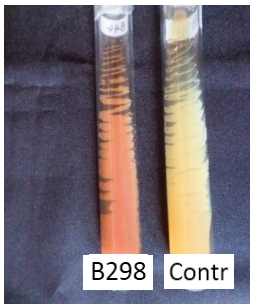

Figure 3. *Bacillus subtilis* produces IAA which is showed in red color, in the pure culture of *Bacillus subtilis*

Figure 4. *Bacillus subtilis* resistance to antibiotics (Rif+) and not resistant to Kanamycin, Streptomycin, and Chloramphenicol

Table 2. Role of *Bacillus subtilis* B209, B211 and B298 as growth promoters of chili plants

Treatment	PH (cm)	Leaf number	FPM (g)	DCM (g)	Root length (cm)	Root volume (ml)	FRM (g)	DRM (g)
Control	27.4c	27.8c	22.5d	3.1d	9.2c	2b	2.3c	0.26d
B. subtilis B209	36.5b	45.7b	29.5b	5.2c	12.3a	2b	2.8b	0.42bc
B. subtilis B211	37.3b	50.3b	35.8a	6.7b	10.3b	3a	3.2a	0.52b
B. subtilis B298	44.2a	61.4a	38.3a	8.2a	12.3a	1c	3.2a	0.68b
Fungicide	27.6c	30.5c	26.5c	3.9d	10.4b	1c	2.4c	0.27cd

Remarks: Values followed by the same letters in the same column were not significantly different according to LSD (α = 5%). PH = plant height, FCM = fresh plant mass, DCM = dry plant mass, FRM = fresh root mass, DRM = dry root mass.

Table 3. The disease intensity of anthracnose in the field after application of *Bacillus subtilis* and fungicide

Treatment	Incubation time (dap)	Disease intensity (%)	Effectivity (%)
Control	36	26.08 a	-
B. subtilis B209	46	5.12 bc	80.36
B. subtilis B211	48	8.08 b	69.01
B. subtilis B298	54	6.91 b	73.50
Fungicide	54	8.97 b	65.61

Remarks: Values followed by the same letters in the same column in the same column were not significantly different according to LSD (α = 5%); DAP = day after planting.

growth of chili compared with control (without *B. subtilis* and without fungicide) and compared with a fungicide treatment (Table 2). Capability of *B. subtilis* B298 in increasing plant growth was shown in plant height, the number of leaves, dry plant mass and dry root mass increased by 38.0%, 54.7%, 61.7%, 61.8%, respectively. The isolates of *B. subtilis* B298 and B211 could increase fresh plant mass (41.2 and 37.1%) and fresh root mass (36.4 and 34.4%), and *B. subtilis* B298 and B209 isolates able to increase root length (25.2%). Furthermore, *B. subtilis* B211 could increase root volume (33.3%). Therefore, the three isolates of *B. subtilis* are able to control anthracnose disease (Table 3). The highest effectiveness of anthracnose disease control was isolate *B. subtilis* B209 by 80.36%.

CONCLUSION

B. subtilis B298 could be used as biological control to control *C. capsici* and *C. gloeosporioides* with inhibition of 57.6 and 64.6%. The three isolates of *B subtilis* (B209, B211 and B298) were able to dissolve phosphate, produce IAA, produce nitrogen, and be resistant to the antibiotic Rifampicin. Isolate *B. subtilis* B298 is the best isolate in increasing plant growth showed by the increase in plant height, number of leaves, dry plant mass and dry root mass (38.04%, 54.75%, 61.7%, and 61.8%, respectively). Isolate *B. subtilis* of B298 and B211 could increase fresh plant mass (41.3% and 37.2%), while B298 and B209 increased the root length as by 25.2%. The three isolates of *B. subtilis* were able to control

anthracnose disease with the highest effectiveness of 80.36% by the treatment of *B. subtilis* B209.

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