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Biological control of maize downy mildew with the antagonistic bacterial consortium

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Abstract. *Mugiastuti E, Manan A, Soesanto L. 2023. Biological control of maize downy mildew with the antagonistic bacterial consortium. Biodiversitas 24: 4644-4650.* Downy mildew is one of the main diseases of maize, which is a limiting factor for maize production in Indonesia. With a consortium of maize-indigenous antagonist bacteria, biological control is expected to reduce downy mildew. The aim of this research was to determine the ability of three antagonistic bacteria *Bacillus amyloliquefaciens* BB.R3, *Bacillus subtilis* BB.B4, *Pseudomonas putida* BB.R1 in suppressing spore germination of *Peronoslerospora* spp., and to evaluate the ability of their consortium in controlling downy mildew and promote the growth of maize. Based on the research results, antagonistic bacteria *B. amyloliquefaciens* BB.R3, *B. subtilis* BB.B4, and *P. putida* BB.R1 were able to suppress spore germination by 76.68-100%. The bacterial consortium of *Bacillus subtilis* BB. B4 + *Pseudomonas putida* BB.R1 was the best consortium of antagonistic bacteria and had the most potential to developed as a downy mildew control and promote the growth of maize. This bacterial consortium delayed the incubation period, lowered the intensity of the disease (85.77%) and AUDPC (83.02%), increased the content of phenols (tannins, glycosides, and saponins), and promoted plant growth (plant height 138.10%, the number of leaves 102.29%, root length 219.89%, fresh plant weight 1091.81%, and dry plant weight 1077.04%) compared to the control. Treatment with antagonistic bacteria showed better results compared to the fungicide metalaxyl. Based on the results, applying antagonistic bacteria consortium is a potential strategy to control maize downy mildew.

Keywords: *Bacillus*, biological control, downy mildew, maize, *Pseudomonas putida*, *Peronoslerospora*

INTRODUCTION

Downy mildew, caused by *Peronosclerospora* spp., is an important disease and has spread widely in various maize-producing countries (Suharjo et al. 2020). *Peronosclerospora* spp. mainly spreads through the wind in the morning and can infect maize from the time the seeds are planted up to 40 days of age, chlorotic symptoms extending parallel to the leaf bones, stunted growth, and does not reproduce seeds (Adhi et al. 2022). This disease can reduce yields by 50-100% (Rustiani et al. 2015; Crandall et al. 2018). In Indonesia, downy mildew is found in all provinces. Three species are reported to cause maize downy mildew in Indonesia, namely *P. maydis*, *P. philippinensis*, and *P. sorghi*. *Peronosclerospora maydis* was reported in maize plantations in Lampung, Central Java, and West Java. *P. philippinensis* was found mostly in maize plantations in South Sulawesi, North Sulawesi, and Gorontalo, while *P. sorghi* most commonly infected maize in North Sumatra (Rustiani et al. 2015; Widiantini et al. 2015).

Downy mildew spreads must be managed considering the huge loss caused; however, downy mildew is relatively difficult to control. Downy mildew spreads rapidly through the air, is spread by seeds, and its oospores can stay in the soil for a long time. *Peronosclerospora* spp. also has high variability and adaptability; hence resistant maize varieties and controlling by fungicides cannot last long (Barbosa et al. 2006; Suharjo et al. 2020). Unwise control of synthetic pesticides can harm the environment, affect non-target organisms, and add residues to food products (Riyaz et al. 2021). Pesticides also trigger the formation of new strains of pathogens and become more resistant to chemicals. For example, the resistance of downy mildew pathogens to metalaxyl fungicide has been reported by Talacca et al. (2021). Therefore, finding effective, safe, and sustainable control alternatives is necessary.

Biological control has the potential to protect plants throughout their life cycle. Biocontrol agents can live and multiply, so their ability in the field can be long-lasting and sustainable (Sharma et al. 2013; Ahanger et al. 2014). Rhizospheric and endophytic bacteria such as fluorescent *Pseudomonad* and *Bacillus* spp. are widely used as biological control agents for soil-borne and airborne diseases. Fluorescent *Pseudomonad* and *Bacillus* spp. have several control mechanisms, including competition, hyperparasitism, producing microbial inhibitor compounds (antibiotics, lytic enzymes, and other physical or chemical disorders), inducing plant resistance, and plant growth promoters (Ahemad and Kibret 2014; Comeau et al. 2021; Saeed et al. 2021)

Generally, biological control only uses one biological agent to control one pathogen; nevertheless, it often gives inconsistent results. The ability of bacteria to produce various biological control compounds and to promote plant growth varies greatly. According to Suryadi (2013) and

Zhou et al. (2015), using a combination of several antagonistic bacteria as a consortium can increase the ability of bacteria as biological controllers and plant growth promoters. The high enhanced disease control activity occurs due to the bacterial metabolic activity of the consortium complementing each other.

Previous studies have isolated maize rhizospheric and endophytic bacteria, including *Bacillus amyloliquefaciens* BB.R3, *Bacillus subtilis* BB.B4, and *Pseudomonas. putida* BB.R1. These antagonistic bacteria can produce microbial inhibitory compounds, such as hydrolytic enzymes (chitinase, proteases, and lipase), siderophores, and HCN. Bacteria can also produce various plant growth-promoting compounds, such as hormones (IAA and gibberellin), phosphate solvent compounds, and some plant-resistance-inducing compounds (phenol, butanediol, and benzothiazole) (Mugiastuti et al. 2020; Mugiastuti 2022). However, the ability of these bacteria to produce various metabolite compounds that inhibit pathogens, promote growth, and induce resistance varies in numbers and types. Therefore, for these reasons, it needs to be used together to increase the effectiveness of its control. Thus, this study aimed to determine the ability of an antagonistic bacterial consortium to control downy mildew and promote plant growth in maize.

MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Protection and Green House of the Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Indonesia, from August to October 2022.

Preparation of *Peronosclerospora* spp. spore suspension and propagation of bacterial antagonist

The suspension of *Peronosclerospora* spp. was prepared by harvesting spores (at 03.00-04.00 AM) from diseased leaves using a fine brush and mixed in sterile water. The antagonistic bacteria were cultured on NB (Nutrient broth) medium and subsequently shaken with Daiki Orbital Shaker at 150 rpm for two days (Muis et al. 2015).

Testing the ability of antagonistic bacteria to suppress spore germination

The tests were performed in a randomized complete block design with four treatments and six repetitions. The treatments included no treatment (control), *B. amyloliquefaciens* BB.R3, *B. subtilis* BB.B4, and *P. putida* BB.R1. The antagonistic bacteria *B. amyloliquefaciens* BB.R3 and *P. putida* BB.R1 obtained from maize rhizosphere, while *B. subtilis* BB.B4 from maize stalks endophyte. The test was performed by mixing 25 µL (10^4 spores mL^{-1}) of *Peronosclerospora* spp. suspension with 25 µL of antagonistic bacterial suspension on an glass object and covered with glass cover. Furthermore, it was incubated in a sealed container lined with moist tissue for 48 hours in dark (Estoppey et al. 2022). Spore germination was carried out at 24 and 48 hours after incubation, and the percentage of spore germination was calculated using the formula of Gordon et al. (2019):

$$\text{Spore germination} = \frac{\text{Number of germinated spores}}{\text{Number of total spores}} \times 100\ \%$$

Testing the ability of antagonistic bacterial consortium to control downy mildew

In this study bonanza variety of sweet maize, susceptible to *Peronosclerospora* spp was used. The research design was conducted in a randomized complete block design with nine treatments, i.e., no treatment (negative control), metalaxyl fungicide (positive control), *B. amyloliquefaciens* BB.R3, *B. subtilis* BB.B4, *P. putida* BB.R1, *B. amyloliquefaciens* BB.R3 + *B. subtilis* BB.B4, *B. amyloliquefaciens* BB.R3 + *P. putida* BB.R1, *B. subtilis* BB.B4 + *P. putida* BB.R1, as well as *B. amyloliquefaciens* BB.R3 + *B. subtilis* BB.B4 +*P. putida* BB.R1. Each treatment unit consisted of 4 plants and was repeated five times.

The antagonistic bacteria were applied by soaking the seeds for 12 hours and spraying when the plants were 6 and 13 days after planting (DAP). The population density of bacteria used was 10^9 cfu mL^{-1} (Yasmin et al. 2017). As a comparison treatment, fungicides were applied through seed coating at a dosage of 2 g/kg of seed. Inoculation of *Peronosclerospora* spp. was carried out when the plant was seven days old by spraying a spore suspension (10^6 spores mL^{-1}) at 03:00 AM on all parts of the plant and growing points.

The variables observed were the incubation period, disease intensity, AUDPC (Area Under Disease Progress Curve), plant height, number of leaves, root length, fresh plant weight, dry plant weight, and leave phenol content (saponin, tannin, and glycoside). The incubation period was observed from inoculation of the pathogen until the appearance of initial symptoms. Disease intensity (DI) was calculated according to the formula from (Ginting et al. 2020):

$$DI = \frac{\sum ni \times vi}{N \times Z} \times 100\ \%$$

Whereas, ni = the total of infected leaves with a specific score, vi = the score category of the symptom, N = total leaves observed, and Z = the highest score used. The scoring categories of symptoms were 0 = no symptom; 1= = symptoms <10%, 2 = symptoms 11-25%, 3 = symptoms 26-50%, and 4 = symptoms >51% (Ravat et al. 2019).

The Area Under Disease Progress Curve was calculated by the formula of Simko and Piepho (2012):

$$AUDPC = \sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i)$$

Whereas, yi = disease intensity at the i^{th} observation, ti = time (days) at the i^{th} observation, n = total number of

observations. Plant height, number of leaves, root length, fresh plant weight, and dry plant weight were measured at the time of the end of the vegetative stage of maize 45 days after planting (DAP). Testing for leave phenol content (saponins, tannins, and glycosides) was carried out when the plants were 40 DAP, according to Rahmania et al. (2018) and Simamora et al. (2021).

Data analysis

Data were analyzed by analysis of variance. If treatment had a significant difference, further tests were carried out using Duncan Multiple Range Test (DMRT) at α 5%. Data analysis using DSAASTAT ver. 1.101.

RESULTS AND DISCUSSION

Testing the ability of antagonistic bacteria to inhibit spore germination

All antagonistic bacteria tested were able to inhibit the germination of *Peronosclerospora* spp. with germination inhibition of 76.68-100% (Table 1). The spore germination of *Peronosclerospora* spp. is shown in Figure 1.

The abilities of *B. amyloliquefaciens* BB.R3, *B. subtilis* BB.B4, and *P. putida* BB.R1 in suppressing the germination of *Peronosclerospora* spp. spores were related to the ability of bacteria to produce compounds that inhibit fungal growth through antibiosis and lysis mechanisms. The results of previous research showed that *B. amyloliquefaciens* BB.R3, *B. subtilis* BB.B4, and *P. putida* BB.R1 can produce protease enzymes, lipase enzymes, chitinase enzymes, siderophores, hydrogen cyanide (HCN), and several volatile toxic compounds (phenol, acetamide, acetic acid, propanoic acid, benzoic acid, pentanoic acid, piperazine, pyrrolidine, and tetrazole) (Mugiastuti 2022). According to Olanrewaju et al. (2017), protease enzymes can degrade fungal cell wall proteins, whereas lipase enzymes can degrade some lipids related to cell walls. The combination of these two enzymes helps antagonistic bacteria to lyse fungal cells. In addition, lipids on the plasma membrane are a vital regulator of fungal pathogenicity, and various glycolipids have been shown to provide virulent properties and endurance in some fungal species (Rella et al. 2016). HCN inhibits microbial growth by inhibiting cytochrome C oxidase, part of the mitochondrial respiratory chain, and other important metalloenzymes (Flury et al. 2017).

The chitinolytic enzyme is also considered necessary in the mechanism of biocontrol agents for pathogenic fungi due to its ability to degrade the cell walls of fungi (Jadhav et al. 2017; Poria et al. 2021). According to Veliz et al. (2017), chitin is an essential component of the cell walls of insects and fungi, nematode eggs, and some protists. The chitinase enzyme will weaken and degrade the cell walls of many pests and pathogens.

B. amyloliquefaciens BB.R3, *B. subtilis* BB.B4, and *P. putida* BB.R1 are also reported to produce volatile toxic metabolite compounds such as phenol, acetamide, acetic acid, propanoic acid, benzoic acid, pentanoic acid, piperazine, pyrrolidine, and tetrazole (Mugiastuti 2022). These compounds can be anti-fungal or anti-microbial to inhibit the growth of fungi (Islam et al. 2012; Ananta et al. 2016; Surya et al. 2020; Roca-Causo et al. 2021).

Testing the ability of antagonistic bacteria to control downy mildew

The result of ability of antagonistic bacteria test to control downy mildew in greenhouse is shown in Table 2. The incubation period of downy mildew was observed from planting to the appearance of initial symptoms of the disease. These initial symptoms were characterized by chlorosis that extended parallel to the leaf bones; a white powdery coating was found under the leaf surface in the morning. According to Adhi et al. (2022), fungi can infect maize from seed and chlorotic symptoms extended parallel to the leaf bones and a white powdery coating is found beneath the leaf surface in the morning. Plant growth stunted and cannot produce seeds.

Table 1. Spore germination of *Peronosclerospora* spp.

Antagonistic Bacteria	Spore Germination (%)	
Pseudomonas putida BB.R1	9.40	b
B. amyloliquefaciens BB.R3	8.19	b
B. subtilis BK. R5	0.00	a
No treatment (control)	42.12	c

Note: Numbers followed by different letters in the same column show a marked difference in DMRT α 5%

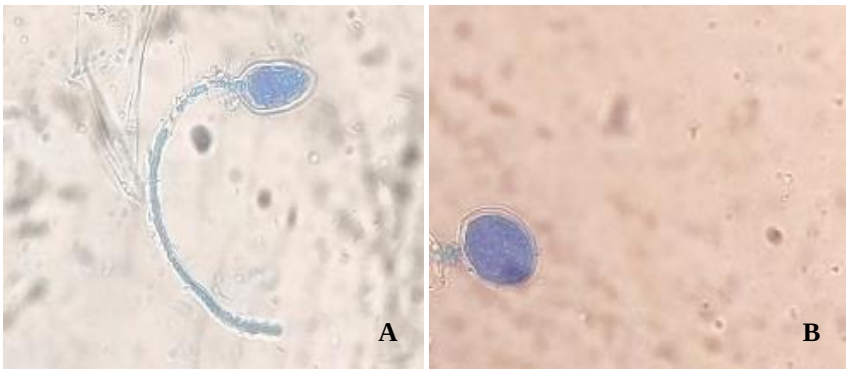

Figure 1. Spores of *Peronosclerospora* spp. A. Spore germinate; B. Spore without germination

Table 2. The incubation period, disease intensity, and AUDPC of downy mildew

Treatments	Incubation period (days)		Disease Intensity (DI) (%)		DI reduction (%)	AUDPC (%.days)		AUDPC reduction (%)
No treatment (control)	5.92	a	100.00	d	-	1212.84	d	-
Metalaksil 35%	6.42	ab	100.00	d	0	1187.98	d	2.05
B.a.R3	8.92	abc	74.36	bcd	25.64	842.54	bcd	30.53
B.s.B4	10.17	cd	69.98	bcd	30.02	834.99	bcd	31.15
P.p.R1	9.17	bcd	81.53	cd	18.47	984.28	cd	18.85
B.a.R3 + B.s.B4	11.58	cd	16.67	a	83.33	259.30	a	78.62
B.a.R3 + P.p.R1	9.25	bcd	53.69	abc	46.31	670.48	abc	44.72
B.s.B4 + P.p.R1	10.33	cd	14.23	a	85.77	205.90	a	83.02
B.a.R3 + B.s.B4 + P.p.R1	12.42	d	35.39	ab	64.61	425.99	ab	64.88

Note: Numbers followed by different letters in the same column show a marked difference in DMRT α 5%. B.a.R3 = *B. amyloliquefaciens* BB.R3, B.s.B4 = *B. subtilis* BB.B4, P.p.R1= *P. putida* BB.R1

The shortest incubation period was found in the control (5.92 days) (Table 2). The treatment of antagonistic bacteria had a more extended incubation period than the control (8.92-12.42 days). This is due to competition and inhibition from antagonistic bacteria, so pathogens experience problems infecting maize. These results align with the previous testing on the ability to inhibit spore germination, showing that antagonistic bacteria can suppress the germination spores of *Peronosclerospora* spp. by 76.68-100% (Table 1).

The ability of antagonistic bacteria to control downy mildew was also shown in variables of disease intensity and AUDPC. Single or consortium application of antagonistic bacteria can reduce the intensity of disease (18.47-85.77%) and AUDPC (18.85-83.02%). The decrease in the disease was related to various antagonism mechanisms of antagonistic bacteria. Antagonistic bacteria could inhibit the germination spore *Peronosclerospora* spp. (Table 1), so *Peronosclerospora* spp. can not infect and colonize maize plants. In addition to the various mechanisms previously described, *Bacillus* and fluorescent *Pseudomonads* have been reported to be able to produce various types of antibiotics. Wang et al. (2015) reported that various strains of *B. subtilis* could produce 68 antibiotics. At the same time, *B. amyloliquefaciens* can produce antibiotics: basilycin, surfactin, iturin A, fengycin A, and fengycin B (Lv et al. 2020; Dhumal et al. 2021). In addition, fluorescent *Pseudomonads* are also reported to produce phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn) and or pyoluteorin (Plt) (Bosire and Rosenbaum 2017; Jaaffar et al. 2017).

The antagonistic bacteria were also reported to produce siderophores (Mugiastuti 2022), the compounds that act as iron chelators, especially in iron-limited conditions. These compounds and their derivatives have wide applications in agriculture to improve soil fertility and biological control of pathogenic fungi. The availability of iron ions is essential for microbial virulence to infect plants (Burbank et al. 2015). Furthermore, HCN inhibits microbial growth by inhibiting cytochrome C oxidase, part of the mitochondrial respiratory chain, and other important metalloenzymes (Flury et al. 2017).

Table 2 shows that application of antagonistic bacterial consortiums (2 or 3 bacteria) had a more extended incubation period, smaller disease intensity, and smaller AUDPC (percentage days) than a single bacterial application. These results showed that the mixture of antagonistic bacteria can improve their performance as biological controllers. According to Zhou et al. (2015), activity of metabolites produced by microbes of the consortium may complement each other and produce synergistic effects. Nevertheless, the lowest disease intensity and AUDPC were observed in consortium (*B. amyloliquefaciens* BB.R3+ *B. subtilis* BB.B4) and consortium (*B. subtilis* BB.B4 and *P. putida* BB.R1). These two bacterial consortiums reduced the highest disease intensity and AUDPC compared to the control (Table 2).

The ability of antagonistic bacteria consortium to suppress the development of downy mildew was revealed to be much better than metalaxyl fungicides. Metalaxyl is one of the recommended fungicides to control corn downy mildew. However, in this test, metalaxyl could not control maize disease (Table 2). This result shows that *Peronosclerospora* spp. from Purwokerto, Central Java, Indonesia, may already be resistant to metalaxyl. Talacca et al. (2011) have also reported the ineffectiveness of metalaxyl in downy mildew control in West Kalimantan and East Java (Kediri). In addition, Rashid et al. (2013) also reported that resistance has evolved in pathogens against metalaxyl fungicides.

The effect of antagonistic bacteria on plant phenol content (tannins, glycosides, and tannins) can be seen in Table 3. Phenol content indicates systemic induction of plant resistance; these tests showed that applying antagonistic bacteria could increase plants' phenol contents. According to Bhattacharyya and Jha (2012) and Rabari et al. (2022), rhizobacteria can induce resistance systemically through increased activity of secondary metabolites such as phenol content.

Generally, treating bacterial consortiums (2 or 3 bacteria) had a higher phenol content than a single bacterial treatment. The amount of phenol in maize can inhibit the development of plant pathogens. The highest phenol contents were recorded in consortium (*B. amyloliquefaciens* BB.R3+ *B. subtilis* BB. B4) and consortium (*B. subtilis* BB.B4 and *P. putida* BB.R1), which was one of the causes of the low disease intensity of these treatments.

The effect of antagonistic bacteria on plant growth components, including the number of leaves, plant height, plant weight, fresh root weight, and root length, are presented in Table 4. Statistical analysis showed that antagonistic bacterial application significantly influences all plant growth variables. All antagonistic bacterial applications, single and consortium, significantly increased plant height (6.94-138.10%), the number of leaves (2.29-102.29%), root length (14.25-219.89%), fresh weight (6.11-1091.81%), and dry weight (5.93-1077.04%) as compared to the control Antagonistic bacteria also showed a better effect then metalaxyl fungicides.

The ability of antagonistic bacteria to promote plant growth may be related to the ability of these bacteria to produce compounds or metabolites that support plant growth. In a previous study, *B. amyloliquefaciens* BB.R3, *B. subtilis* BB.B4 and *P. putida* BB.RI produces phosphate solubilizing compounds and IAA hormones, promoting growth and improving the roots of maize seedlings (Mugiastuti 2022). According to Hassan (2017), phosphate solubilizing compounds convert insoluble organic and inorganic phosphates into forms that can be absorbed. The IAA hormone can increase root surface area and absorption of plant nutrients (Gupta et al. 2015; Olanrewaju et al. 2017).

The ability of antagonistic bacteria to promote plant growth was also related to the ability of bacteria to colonize roots. Root colonization capabilities include rhizosphere, rhizoplane, and root colonization (Santoyo et al. 2021). *Bacillus* spp. and fluorescent *Pseudomonad* are good root colonists because they reproduce by utilizing seeds and root exudate, compete with other microbes, and adapt to the environment (Gadhave et al. 2018; Santoyo et al. 2021; Suresh et al. 2021).

The bacterial consortium (*B. subtilis* BB.B4 + *P. putida* BB.R1) showed the best results on all plant growth variables. The bacterial consortium was able to increase plant height (138.10%), the number of leaves (102.29%), root length (219.89%), fresh plant weight (1091.81%), and dry plant weight by (1077.04%) compared to control. In addition to the role of antagonistic bacteria as a plant growth booster, there was a relationship between plant growth and *Peronosclerospora* spp infection; the magnitude of the pathogen infection rate will affect plant growth. The bacterial consortium (*B. subtilis* BB.B4 + *P. putida* BB.R1) had the least disease intensity, so the plant can still carry out its metabolism and physiology well.

The bacterial consortium (*B. subtilis* BB. B4 + *P. putida* BB.R1) was the best consortium of antagonistic bacteria and had the most potential to developed as a downy mildew control. This bacterial consortium delayed the incubation period, lowered the intensity of the disease (85.77%) and AUDPC (83.02%), increased the content of phenols (tannins, glycosides, and saponins), and promoted plant growth (plant height, number of leaves, root length, fresh weight and dry weight of the plant).

Table 3. Contents of tannins, glycosides, and saponins in maize plants

Treatments	Tannins	Glycosides	Saponins
No treatment (control)	+	+	-
Metalaxyl 35%	-	+	-
B.a.R3	++	+++	++
B.s.B4	+	++	+
P. p.R1	+	++	++
B.a.R3 + B.s.B4	+++	+++	+++
B.a.R3 + P.p.R1	++	++	+++
B s.B4 + P.p.R1	+++	+++	+++
B.a.R3 + B.s.B4+ P.p.R1	+++	+++	++

Note: - = none, + = litte, ++ = medium, +++ = much. B.a.R3 = *B. amyloliquefaciens* BB.R3, B.s.B4 = *B. subtilis* BB.B4, P.p.R1= *P. putida* BB.R1

Table 4. Growth parameters of maize plant

Treatments	Plant Height (cm)		Number of Leaves (sheet)		Root Length (cm		Fresh Plant Weight (g)		Dry Plant Weight (g)	
No treatment (control)	46.83	a	3.50	a	22.17	a	17.83	a	2.70	a
Metalaxyl 35%	50.08	a	3.58	a	25.33	a	18.92	a	2.86	a
B.a.R3	70.17	b	4.75	ab	31.58	b	39.17	b	5.82	b
B.s.B4	73.17	b	5.25	ab	37.17	c	42.00	b	7.45	c
P.p.R1	78.33	b	5.67	ab	54.92	de	67.17	c	13.38	d
B.a.R3 + B.s.B4	104.75	de	6.67	ab	58.25	e	138.75	f	20.83	g
B.a.R3 + P.p.R1	95.33	c	6.25	ab	51.50	d	110.08	d	16.78	e
B.s.B4 + P.p.R1	111.50	e	7.08	b	70.92	f	212.50	g	31.78	h
B.a.B4 + B.s.B4 + P.p.R1	97.83	cd	6.75	ab	56.42	de	126.08	e	19.07	f

Note: Numbers followed by different letters in the same column show a marked difference in DMRT α 5%. B.a.R3 = *B. amyloliquefaciens* BB.R3, B.s.B4 = *B. subtilis* BB.B4, P.p.R1 = *P. putida* BB.R1

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