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Effect of Plant Growth Promoting *Bacillus* spp. on Germination and Seedling Growth of Soybean

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ABSTRACT

Background: *Bacillus* spp., known to promote growth and reduce disease of various field and vegetable crops, are frequently found in soils. The objective of the study was to select effective *Bacillus* spp. isolates with multiple plant growth properties and antifungal activities and to examine their effect on germination of soybean.

Methods: Bacterial isolates were screened for production of indole-3-acetic acid (IAA) and siderophores and solubilization of phosphate. The ability of bacterial isolates to inhibit the growth of seven phytopathogenic fungi affecting soybean was determined using a dual plate assay. *Bacillus* spp. were further selected and examined in a seed germination test.

Result: All *Bacillus* spp. isolates were positive for IAA production, while siderophore production and P-solubilization were observed in 80% and 20% bacterial isolates, respectively. *Bacillus* spp. exhibited the highest antifungal activity against *Diaporthe caulivora*, followed by *Diaporthe sojae, Diaporthe eres, Diaporthe longicolla* and *Macrophomina phaseolina* and the least antagonistic effect toward *Fusarium graminearum* and *Fusarium subglutinans*. Selected isolates of *B. subtilis* significantly affected final germination, shoot length, root length, shoot dry weight and root dry weight of two soybean cultivars. The most effective *Bacillus* spp. isolates could be used as potential inoculants for improving soybean productivity.

Key words: Antifungal activity, *Bacillus*, Germination, PGPR, Soybean.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the most important legumes in the world, providing vegetable protein, oil and nutrients for millions of people and for various purposes. As a legume, soybean has the ability to fix atmospheric nitrogen, thus reducing nitrogen fertilization and proving fit for crop rotation. Global soybean production has increased more than tenfold in the last fifty years and is constantly on the rise. Intensification of agricultural production implies the use of synthetic fertilizers and pesticides which have adverse effects on living organisms and environments (Tripathi *et al.*, 2020). The use of plant growth promoting rhizobacteria (PGPR) represents a promising and environmentally friendly method to minimize the negative effects (Backer *et al.*, 2018; Kaur and Kaur, 2018).

PGPR are a group of bacteria that actively colonize rhizosphere and plant roots and exert beneficial effects on plant growth, increase yield, reduce pathogen infection and mitigate biotic or abiotic stresses (Compant *et al.*, 2010). *Bacillus* spp. are among the most widely reported PGPR for crop production improvement. Numerous studies of a wide range of crops showed a constant increase in the number of *Bacillus* spp. identified as potential plant growth promoters and biocontrol agents (Miljaković *et al.*, 2020). *Bacillus* spp. can be found and successfully isolated from the soil and rhizosphere of plants. However, only a few *Bacillus* spp. of about 200 within the genus exhibit multiple plant growth promoting (PGP) traits as well as biocontrol activity and might be applicable in developing inoculants. Nowadays, application of inoculants in soybean production is a common and widespread agronomic practice. These inoculants mostly contain symbiotic nitrogen fixing bacteria, while the use of PGPR, especially *Bacillus* spp., as biofertilizers and/or biopesticides in soybean production is less studied. Therefore, the aim of the study was to select effective *Bacillus* spp. isolates with multiple plant growth properties and antifungal activities and to determine their effect on seed germination and seedling growth of two soybean cultivars.

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MATERIALS AND METHODS

The experiment was carried out in the Institute of Field and Vegetable Crops Novi Sad (IFVCNS) during 2020. *Bacillus* spp. used in this study were isolated from soil and identified by 16S rDNA analysis and then screening of bacterial isolates for PGP traits and antifungal activity, as well as germination assays were conducted. All assays were performed in the Laboratory for Microbiological Research and Laboratory for Seed Testing (IFVCNS).

Using the serial dilution technique, *Bacillus* spp. isolates were isolated from soil samples collected at different sites of the Vojvodina Province (northern Serbia) (Bjelić *et al*., 2018). In short, soil suspensions (10 g of soil in 90 ml of dH_2O) were serially diluted (10^{-3} - 10^{-6}) and 0.1 ml aliquots were spread on plates containing nutrient agar (NA). After 48 h of incubation at 28°C, isolates were characterized according to morphological and biochemical properties and subjected to molecular identification based on PCR analysis of 16S rDNA sequences (Bjelić *et al*., 2018). Each isolate was deposited in the NCBI GenBank database under a unique accession number (Table 1). Bacterial cultures were collected and stocked on NA slants at 4°C. *Bacillus* spp. isolates for assays were cultured for 24h in nutrient broth (NB), at optimal temperature of 28°C. Culture suspension contained 10^9 of colony forming units per ml (CFU ml^{-1}).

Screening for PGP properties in *Bacillus* spp. isolates included determination of their capability to produce indole-3-acetic acid (IAA) and siderophores and to dissolve sparingly soluble inorganic phosphate. For quantitative analysis of indole-3-acetic acid (IAA) production, a 100 µl 24 h-old bacterial suspension was inoculated in the nutrient broth (NB), supplemented with 0 and 250 µg ml^{-1} of L-tryptophan (HiMedia, India). Salkowski reagent ($FeCl_3$-$HClO_4$: 2% 0.5 M ferric chloride in 35% perchloric acid) was mixed with the supernatant (2:1 v/v) and intensity of the developed color was measured at 530 nm (Glickman and Dessaux, 1995). Bacterial ability to produce siderophores was assayed on Chromeazurol S (CAS) (Himedia, India) medium by observing changes of the color zones (green-blue to orange) (Milagres *et al.,* 1999). The capability of *Bacillus* spp. to dissolve sparingly soluble inorganic phosphate was determined on Pikovskaya medium (PVK) (Pikovskaya, 1948) and National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999) with 0.5% tricalcium phosphate [$Ca_3(PO_4)_2$].

The antifungal activity of *Bacillus* spp. isolates against phytopathogenic fungi originally isolated from soybean seeds was examined using a dual plate assay (Zhao *et al*., 2010). Investigated fungi were: *Macrophomina phaseolina*, *Diaporthe longicolla*, *Diaporthe caulivora*, *Diaporthe sojae*, *Diaporthe eres*, *Fusarium graminearum*, *Fusarium subglutinans*. Bacterial suspension was steaked on potato dextrose agar (PDA), while tested fungus (7-day-old mycelial plugs on PDA, R = 6 mm) was aseptically transferred on the same plate. After incubation, the per cent of growth inhibition (PGI) was calculated according to the formula:

$$PGI\,(\%) = \frac{C - R1}{C} \times 100$$

Where

C and R1 represent growth of mycelium in control and dual culture. Assays for each bacterium and fungus were performed in three replicates.

The effect of *Bacillus* spp. on seed germination and initial seedling development was examined using a standard germination test (ISTA, 2020). Seeds of soybean cultivars Teona and Atlas, developed at the IFVCNS, were used for test. Seeds were surface disinfected in 5% sodium hypochlorite (NaOCl) for 5 min, rinsed with sterile distilled water four times and then dried back on sterile filter paper under aseptic conditions. Inoculation of seeds was performed with 10 ml of *Bacillus* suspension (10^9 ml^{-1}). Non-inoculated seeds were control. Four replicates of 100 treated seeds were germinated on a moistened filter paper in plastic boxes 240 × 150 mm and placed in a germination chamber at 25°C for 8 days. Treatments were arranged in a completely randomized block design. Energy of germination was determined 5 days after sowing, while seed germination, shoot and root length were determined 8 days after sowing. To determine dry weight, samples were oven-dried at 80°C for 24 hours.

Data were analyzed by analysis of variance (ANOVA) using the statistical software package STATISTICA 12.0 (StatSoft Inc., Oklahoma, USA). The differences among various treatment means were compared using the Tukey's test at 5% (P<0.05) probability level.

RESULTS AND DISCUSSION

In this study, ten *Bacillus* spp. isolates were used for PGP and antifungal activity assays (Table 1).

Screening for characteristics commonly associated with plant growth promotion revealed that the tested bacterial isolates were able to produce IAA in a range of 5.41 to 45.09 µg ml^{-1} (Table 2). All *Bacillus* spp. isolates produced IAA in the medium without L-tryptophan and the amount of produced IAA increased with concentration of precursor in the medium. Isolates of *B. subtilis* B5 and B32 were the best IAA producers, in both media. The ability of bacteria to produce IAA indicates their potential use as plant hormones or plant regulators (Spaepen and Vanderleyden, 2011).

PGPR have evolved specialized mechanisms for the assimilation of iron, including the production of siderophores. Additionaly, siderophores act as biocontrol and bioremedation agents (Miljaković *et al*., 2020). The ability of *Bacillus* spp. to produce siderophores was detected for all isolates except *B. pumilus* B22 and B23. The largest

Table 1: Isolates of *Bacillus* spp. from soil samples collected at different locations at northern Serbia.

Isolate code	Species	NCBI accession number
B2	*Bacillus safensis*	KU953932
B3	*Bacillus pumilus*	KU953923
B5	*Bacillus subtilis*	KU953925
B7	*Bacillus subtilis*	KU953927
B11	*Bacillus pumilus*	KU953931
B13	*Bacillus subtilis*	KX444639
B21	*Bacillus pumilus*	KX444647
B22	*Bacillus pumilus*	KX444648
B23	*Bacillus pumilus*	KX444649
B32	*Bacillus subtilis*	KX766373

production zone was measured for *B. subtilis* B5 (>15 mm), followed by *B. safensis* B2, *B. pumilus* B3 and B11, *B. subtilis* B7, B32 (5-15 mm), *B. subtilis* B13 and *B. pumilus* B21 (1-5 mm) (Table 2).

PGPR solubilize insoluble inorganic P and mineralize insoluble organic P, thus enhancing plant growth and P uptake by plants. *Bacillus* spp. are among the most prospective P-solubilization microorganisms (Sharma *et al.,* 2013). In this study, solubility of $Ca_3(PO_4)_2$ on PVK and NBRIP has been determined for *B. subtilis* B5 and B7, with solubilization zone 4-7 mm and 1-4 mm, respectively (Table 2).

The tested isolates of *Bacillus* spp. exhibited strong antifungal activity against seven soybean pathogens (Table 3). The highest antagonistic effect was obtained by confrontation of *Bacillus* spp. with *Diaporthe caulivora*, followed by *Diaporthe sojae, Diaporthe eres, Diaporthe longicolla* and *Macrophomina phaseolina*, while antifungal activity was the lowest against *Fusarium graminearum* and *Fusarium subglutinans* (average PGI ranged from 14.7% to 55.2%). The antifungal activity of particular *Bacillus* spp. depended on the investigated fungi. On average, the highest biocontrol potential toward soybean pathogens was observed for *B. subtilis* isolates B5, B7 and B32 (47.4%, 49.3% and 47.2% reduction in fungal growth, respectively). Previous studies showed very strong antifungal activity of *B. subtilis* B5, B7 and B32 against *Fusarium tricinctum*, *F. oxysporum*f. sp. *cepae*, *F. proliferatum*, *F. verticillioides* and *F. acuminatum*, isolated from infected garlic cloves (Bjelić *et al.*, 2018). Similarly, Rani *et al*. (2021) observed that *Bacillus* showed

Table 2: Plant growth promoting properties of *Bacillus* spp. isolates from soil samples collected at different locations at northern Serbia.

Isolate	IAA (µg ml^{-1} ± SD) at different L-tryptophan concentrations (µg ml^{-1})		Siderophores	P-solubilization	
	0	250		(PVK)	(NBRIP)
Bacillus safensis B2	5.44^{g}	14.78^{g}	++	nd	nd
Bacillus pumilus B3	5.41^{g}	15.30^{e}	++	nd	nd
Bacillus subtilis B5	11.09^{a}	45.09^{a}	+++	++	++
Bacillus subtilis B7	6.28^{e}	16.80^{c}	++	+	+
Bacillus pumilus B11	5.90^{f}	15.67^{d}	++	nd	nd
Bacillus subtilis B13	6.45^{d}	15.66^{d}	+	nd	nd
Bacillus pumilus B21	7.32^{c}	13.01^{h}	+	nd	nd
Bacillus pumilus B22	6.41^{d}	15.14^{f}	nd	nd	nd
Bacillus pumilus B23	6.49^{d}	14.64^{g}	nd	nd	nd
Bacillus subtilis B32	9.57^{b}	18.20^{b}	++	nd	nd

Means with different lowercase letters in the same column are significantly different (p<0.05, Tukey test); Siderophores: (+) 1-5 mm wide of orange zone, (++) 5-15 mm wide of orange zone, (+++) >15 mm wide of orange zone; P-solubilization: (+) 1-4 mm of halo diameter, (++) 4-7 mm of halo diameter; (nd) not detected.

Table 3: Antifungal activity of *Bacillus* spp. isolates from soil samples collected at different locations at northern Serbia.

Isolate	*Macro-phomina phaseolina*	*Diaporthe longicolla*	*Diaporthe caulivora*	*Diaporthe sojae*	*Diaporthe eres*	*Fusarium grami-nearum*	*Fusarium subglutinans*	Average
	Per cent of growth inhibition (%)							
Bacillus safensis B2	31.8^{d}	45.5^{ab}	60.4^{c}	64.7^{b}	47.8^{cd}	51.4^{a}	-	43.1
Bacillus pumilus B3	51.8^{a}	44.3^{abc}	62.3^{bc}	52.9^{d}	53.3^{a}	-	-	37.8
Bacillus subtilis B5	38.8^{b}	42.3^{bcd}	59.2^{c}	58.8^{c}	48.6^{bc}	48.2^{bc}	35.7^{b}	47.4
Bacillus subtilis B7	49.8^{a}	38.8^{d}	60.4^{c}	64.7^{b}	50.9^{ab}	50.6^{ab}	30.2^{c}	49.3
Bacillus pumilus B11	34.1^{cd}	42.3^{bcd}	53.3^{d}	69.4^{a}	45.1^{de}	-	-	34.9
Bacillus subtilis B13	36.5^{bc}	40.0^{cd}	62.3^{bc}	38.8^{e}	49.8^{bc}	-	35.3^{b}	37.5
Bacillus pumilus B21	35.7^{bc}	44.7^{ab}	66.7^{a}	53.3^{d}	44.3^{e}	-	-	35.0
Bacillus pumilus B22	34.5^{cd}	44.3^{abc}	64.7^{ab}	60.4^{c}	48.6^{bc}	-	-	36.1
Bacillus pumilus B23	-	48.6^{a}	-	-	33.7^{g}	-	-	11.8
Bacillus subtilis B32	35.7^{bc}	44.7^{ab}	62.3^{bc}	56.8^{cd}	39.6^{f}	45.9^{c}	45.5^{a}	47.2
Average	34.9	43.5	55.2	52.0	46.2	19.6	14.7	
Isolate	**	**	**	**	**	**	**	

Means with different lowercase letters in the same column are significantly different (p<0.05, Tukey's test); **- Significance at 0.01 probability level.

Table 4: Analysis of variance for germination parameters of soybean under different cultivar and bacterial treatments.

P-value	Germination viability (%)	Final germination (%)	Shoot length (mm)	Root length (mm)	Shoot dry weight (g)	Root dry weight (g)
Cultivar (C)	0.000	0.000	0.007	0.000	0.012	0.107
Bacteria (B)	0.097	0.002	0.004	0.000	0.004	0.000
C × B	0.032	0.017	0.487	0.000	0.386	0.000

Table 5: Effect of selected *Bacillus* isolates from soil samples collected at different locations at northern Serbia on seed germination and seedling growth of soybean.

Cultivar	Treatment	Germination viability (%)	Final germination (%)	Shoot length (mm)	Root length (mm)	Shoot dry weight (g)	Root dry weight (g)
Teona	Control	80.0^{ab}	88.3^{bc}	116.2^{c}	129.6^{e}	0.73^{b}	0.17^{c}
	Bacillus subtilis B5	82.8^{a}	90.3^{abc}	125.6^{abc}	135.1^{de}	0.90^{ab}	0.17^{bc}
	Bacillus subtilis B7	79.0^{ab}	92.8^{ab}	121.9^{abc}	153.1^{bc}	0.88^{ab}	0.21^{a}
	Bacillus subtilis B32	88.0^{a}	98.0^{a}	120.9^{abc}	183.5^{a}	0.87^{ab}	0.22^{a}
	Average	83.3	93.7	122.8	157.2	0.88	0.20
Atlas	Control	65.3^{c}	78.3^{d}	119.1^{bc}	98.1^{f}	0.83^{ab}	0.12^{d}
	Bacillus subtilis B5	72.3^{bc}	86.8^{bc}	130.3^{ab}	166.5^{b}	0.98^{a}	0.21^{ab}
	Bacillus subtilis B7	71.0^{bc}	85.0^{bcd}	133.0^{a}	152.1^{bc}	1.01^{a}	0.20^{abc}
	Bacillus subtilis B32	67.0^{c}	82.8^{cd}	125.3^{abc}	146.3^{cd}	0.89^{ab}	0.19^{abc}
	Average	70.1	84.9	129.5	155.0	0.96	0.20

Means with different lowercase letters in the same column are significantly different ($p<0.05$, Tukey's test).

maximum zone of inhibition to the tested pathogen among the three antagonistic biocontrol agents.

Three isolates of *B. subtilis* B5, B7 and B32 with the highest PGP and biocontrol potential were selected for further examination of their effect on soybean seed germination and seedling growth. Plant growth promotion is crucial in early developmental stages such as germination and seedling growth (Gholami *et al.*, 2009). Establishment of PGPR in plant rhizosphere and soil could lead to an increase in biomass and grain production in later developmental stages (Compant *et al.* 2010). This study showed that investigated cultivars had significant effect on germination viability, final germination, shoot length, root length and shoot dry weight, while bacterial treatments significantly affected final germination, shoot length, root length, shoot dry weight and root dry weight. Interaction of experimental factors also had significant effect on tested germination parameters, except shoot length and shoot dry weight (Table 4).

Application of selected *B. subtilis* isolates improved seed quality compared to control, in both cultivars (Table 5). On average, bacterial treatments resulted in an increase in germination viability (4% and 7%), final germination (6% and 8%), shoot length (6% and 9%), root length (21% and 58%), shoot dry weight (21% and 16%) and root dry weight (18% and 67%). The highest increase of germination viability and final germination was achieved by *B. subtilis* B32 (10% and 11%) of cv. Teona and B5 (11% and 11%) of cv. Atlas. Isolate *B. subtilis* B5 led to the highest increase of shoot length and shoot dry weight (8% and 23%) of cv. Teona, as well as root length and root dry weight (70% and 75%) of cv. Atlas. Isolate *B. subtilis* B32 had the best effect on root length and root dry weight (42% and 23%) of cv. Teona, while treatment with isolate B7 caused the highest increase in shoot length and shoot dry weight (12% and 22%) of cv. Atlas.

Similarly to these results, Bahadir *et al.* (2018) reported that the best P-solubilizing and IAA-producing isolates of *B. subtilis* had positive effects on the seed germination and vegetative growth parameters of eggplant, pepper and tomato, while the studies of Yu *et al.* (2011) indicated that siderophores produced by *Bacillus* spp. were involved in suppression of plant diseases along with promotion of plant growth. *Bacillus* species perform dual functions by showing antagonism against plant pathogens and displaying plant growth promoting properties, which could be highly advantageous in developing the best possible inoculants for soybean, especially if applied in combination with the symbiotic bacteria. Recently, a beneficial effect of *Bacillus subtilis* and *Bradyrhizobium japonicum*on plant growth was reported by Kiruthika and Arunkumar (2020), probably due to the IAA production, P-solubilization and siderophore production. Similarly, Singh and Singh (2018) observed that co-inoculation with PGPR and rhizobia is a promising strategy for the enhancement of plant absorption of P, as well as plant growth and grain yield.

CONCLUSION

Based on our results, the indigenous *Bacillus* spp. isolates from soil generally had good PGP and biocontrol potential. Application of the selected *B. subtilis* isolates resulted in an increase of germination parameters in the two tested

soybean cultivars. In general, the best effect on the tested germination parameters was observed after seed inoculation with *B. subtilis* B5 and B32 isolates as superior IAA and siderophore producers. These isolates have the potential as PGP and biocontrol agents of soybean. Further selection of the most effective strains through field trials will be necessary to establish their efficiency as individual and combined inoculants in certain environmental conditions.

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