

The effect of bacterial isolates from rhizosphere soils on wheat and barley seed germination

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ABSTRACT

Soil microorganisms, which are usually found in plant rhizosphere, have a wide spectrum of beneficial effects on the promotion of plant growth. The most comprehensively studied bacteria with these effects belong to the *Bacillus* genus. In this study, seven *Bacillus* isolates from *Medicago sativa* rhizosphere soils were isolated. Plant growth promoting characteristics of these isolates, such as production of indole-3-acetic acid, siderophores and hydrogen cyanide were tested. The induction of wheat (*Triticum aestivum*) and barley (*Hordeum jubatum*) seed germination was evaluated *in vitro*. Isolate BMG2 produced the highest indole-3-acetic acid of 24.89 µg/mL. The length of roots of barley increased up to 60%, while the length of shoots of barley increased 2.23 times after applying isolates PAZE-6 and BMG1, respectively. In addition, isolates BMG1, BMG2 and PAZE-6 improved germination of both types of seeds and showed ability to produce useful substances such as siderophores and indole-3-acetic acid. Further, these isolates could be used in the production of liquid crop additives that can improve the total yield of cultivated plants, especially barley.

Keywords: *Bacillus* sp., seed germination, indole-3-acetic acid (IAA), siderophores, *Triticum aestivum*, *Hordeum jubatum*.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that have the capability of synthesizing plant growth-promoting substances and decrease or prevent the deleterious effects of phytopathogenic microorganisms. They can be found in the rhizosphere, root surfaces and in association with roots, such as in symbiotic association in root nodule formation. Soil microorganisms are important for agriculture as they promote the circulation of plant nutrients on the one side and reduce the need for chemical fertilizers on the other side (Çakmakçi et al. 2007; Yadav et al., 2010). A large number of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Rhizobium* (*Bradyrhizobium*, *Sinorhizobium*) and *Serratia* have been reported as PGPR to improve plant growth (Yadav et al., 2010; Buntić et al., 2019, Stajkovic et al., 2011, Kumawat et al., 2019). They can improve the quality of plant growth directly and/or indirectly. Directly by the synthesis of phytohormones, vitamins, enzymes, inhibiting plant ethylene synthesis, enhancing stress resistance, improving nutrient uptake, fixing atmospheric nitrogen, solubilising inorganic phosphate, iron and mineralising organic phosphate (Canbolat et al., 2006; Dobbelaere et al., 2003, Çakmakçi et al. 2007). Plant growth benefits due to the addition of PGPR include increases in germination rate, root growth, yield, leaf area, chlorophyll content, nitrogen content, protein content, tolerance to drought, shoot and root weight, and delayed leaf senescence (Dobbelaere et al., 2003; Çakmakçi et al. 2005, 2007). One of the most commonly reported PGPR is *Bacillus* species. It has range of reported properties, including nitrogen fixation, P solubilisation, antibiotic and cytokinin production (Hu et al., 2019; Singh et al., 2015; Fira et al., 2018; Dimkic et al., 2015). It is also reported that *Bacillus* isolates can increase root and shoot growth of wheat (Knezevic et al., 2019). For example, *Bacillus megaterium* increased grain yield of rice and barley and reduced the required P fertilization of sugarcane by 25% (Sundara et al., 2002; Çakmakçi et al. 2007).

The aim of this study was to evaluate the efficiency of novel bacterial isolates from *Medicago sativa* rhizosphere soils (*Bacillus* isolates) on induction of wheat (*Triticum aestivum*) and barley (*Hordeum jubatum*) germination (*in vitro*). The length of the roots and shoots, and the relative seed germination index (RSGI) of the seed were calculated. In

addition, simple characterization of isolates and quantified indole-3-acetic acid (IAA) production were performed.

MATERIAL AND METHODS

The bacterial strains used in this research were isolated from rhizosphere soils gathered from local grassland. Soil sample was diluted to 10^{-6} , and heated on 80°C during 15 min to isolate *Bacillus* spores. The seven obtained isolates (B3, B4, B5, BMG1, BMG2, PAZE-4 and PAZE-6), preliminary characterized as species of *Bacillus* genus, based on the cell morphology and Gram staining, were grown on Nutrient agar and used for further tests.

The ability of bacterial isolates to produce siderophores was tested by azurol S (CAS) agar plate assay, as described by Milagres (1999). A loopfull of bacterial culture, previously grown on a nutrient agar, was transferred to the borderline between CAS and NA medium. The inoculated Petri dishes were incubated for 7 days at 28°C . The change of CAS agar color from blue to yellow was regarded as a positive reaction. The experiment was performed in 3 independent repetitions.

The production of indole-3-acetic acid was tested in a liquid nutrient broth enriched with 0.5mg/mL and 2mg/mL of tryptophan. Isolates were grown in liquid medium for 24h at 28°C and centrifuged (13.000 rpm, 5min). After centrifugation, 1 mL of supernatant was mixed with 2mL of Salkowski reagent (0.5M FeCl_3 in 35% HClO_4 solution) and placed in a dark place during 30 min. Optical density (OD) was recorded at 530 nm for each sample. The results were expressed as $\mu\text{g/mL}$ of IAA based on the comparison to the standard curve of auxin solution (0, 5, 10, 20, 50, 100 and 120 rpm).

The production of Hydrogen cyanide (HCN) was tested using Cyantesmo paper. Bacterial isolates were streaked on Petri dishes containing a nutrient agar, and pieces of Cyantesmo paper (approximately 2cm) were placed on the inner top of the Petri dish. Plates were incubated for 7 days at 28°C . The color change of Cyantesmo paper from white to gray was considered as a positive result.

The API-ZYM system (bioMérieux, USA) was applied according to the manufacturer's recommendations. A pure overnight bacterial culture from the nutrient medium was centrifuged. The suspension, with a turbidity of 5-6 McFarland in API Suspension Medium (2 mL), was prepared using precipitate. The inoculum (65 μL) was

pipetted into each well of the kit, and then the API test strip was covered and incubated for 4.5 h at 30°C. After incubation, one drop of reagents “ZymA” and “ZymB” were added to stop the reaction, and the resulting color was developed over 5 min. The development of color was scored as positive reaction.

The ability of bacterial isolates to induce the germination of wheat and barley seeds was tested *in vitro* in Petri dishes. A sterile filter paper was moistened with 1 mL of sterile distilled water and placed with 40 wheat or 20 barley seeds in a Petri dish. The control sample consisted of wheat or barley seeds, without any bacterial treatment applied. The test treatment sample was prepared by immersing seeds in overnight bacterial culture. The experiment was performed in triplicate. The Petri dishes were placed in a transparent sealed box at room temperature (22°C) to provide humidity. The results were scored after seven days and expressed by the length of shoots and roots and the relative seed germination (%) index (RSGI) (Buntić et al., 2017). The percentage of RSGI is expressed by Eq.1:

$$\text{RSGI}(\%) = \frac{\text{SG}_s}{\text{SG}_c} \times 100 \quad (1)$$

where SG_s is seeds germination in samples and SG_c is seeds germination in control.

RESULTS AND DISCUSSION

The results of CAS agar assay showed that all tested isolates, except B5 and PAZE-6, have the ability to synthesize siderophores (Table 1).

Table 1. Production of siderophores, IAA and HCN by *Bacillus* isolates

Isolate	Siderophores	IAA (µg/mL)*	HCN
B3	+	4.22	–
B4	++	6.00	–
B5	–	0.37	–
BMG1	+	3.85	–
BMG2	+	24.89	–
PAZE-4	+	3.19	–
PAZE-6	–	8.67	–

*- in the presence of tryptophan (2 mg/mL)

Table 2. Isolates characterization (API-ZYM test)

Enzyme	<i>Bacillus</i> isolate						
	B3	B4	B5	BMG1	BMG2	PAZE-4	PAZE-6
<i>Phosphatase:</i>							
Alkaline phosphatase	+	–	+	–	–	–	+
Acid phosphatase	+	+	+		+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+
<i>Esterase:</i>							
C4 esterase	–	+	+	+	+	+	+
C8 esterase lipase	–	–	+	–	+	+	+
C14 lipase	–	–	–	–	–	–	–
<i>Amino peptidase:</i>							
Leucine arylamidase	+	+	+	+	+	+	+
Valine arylamidase	–	–	–	–	–	+	+
Cystine arylamidase	–	–	–	–	–	–	–
<i>Protease:</i>							
Trypsin	+	–	+	–	–	–	+
α -chymotrypsin	+	–	–	+	+	+	–
<i>Glycosyl hydrolase:</i>							
α -galactosidase	–	+	–	–	–	–	–
β -galactosidase	+	+	–	+	+	+	–
β -glucuronidase	–	–	–	–	–	–	–
α -glucosidase	+	+	–	–	–	+	–
β -glucosidase	–	–	+	–	–	–	+
N-acetyl- β -glucosaminidase	+	–	+	–	–	–	–
α -mannosidase	+	+	–	–	–	–	–
α -fucosidase	–	–	–	–	–	–	–

Isolate B4 had the largest halo zone and showed the best potential in the production of siderophores. However, none of the tested isolates could produce HCN. A study conducted by Singh et al. (2015) showed that *Bacillus* strains isolated from rhizosphere soil could not produce HCN and IAA, but they are potent producers of siderophores.

In addition, all tested isolates produced IAA in the presence of tryptophan concentration of 2 mg/mL (Table 1). However, the production of IAA was low in the presence of 0.5 mg/mL of tryptophan. Isolate BMG2 showed the highest IAA production of 24.89 μ g/mL, and was therefore characterized as the most potent producer. Isolates PAZE-6

and B4 also produced significant amounts of IAA, 8.67 $\mu\text{g/mL}$ and 6.00 $\mu\text{g/mL}$, respectively. As all other tested *Bacillus* isolates showed lower IAA producing ability they could be characterized as weak producers. Research performed by Mohite (2013) also indicated that bacteria isolated from rhizosphere soils could be very efficient in the production of plant growth stimulating substances, such as IAA. Furthermore, a research conducted by Djordjevic et al. (2017) indicated that the inoculation of maize seeds with indolacetic acid from *Bacillus* isolates has direct positive effects on root and shoot growth. *Bacillus* isolate used in their study had the ability to produce 8,86 $\mu\text{g/mL}$ of IAA, which was similar as in the case of isolate PAZE-6 used in this research (Djordjevic et al., 2017).

According to API-ZYM test, all seven tested *Bacillus* isolates, showed the ability to produce various enzymes form phosphatase, esterase, amino peptidase, protease and glycosyl hydrolase groups (Table 2). Isolates B3, B5 and PAZE-6 had the richest enzymatic profile with the ability to produce 10 (B3) and 9 (B5 and PAZE-6) different enzymes. The production of these enzymes could be useful for the indirect promotion of plant growth, especially proteases which could improve antagonist effects of PGP bacteria by interacting with the cell walls of plant pathogenic fungi.

The effect of seven *Bacillus* isolates on *T. aestivum* and *H. jubatum* seeds germination and the length of shoot and root are shown on Table 3 and Fig. 1, respectively.

Table 3. Germination of wheat (*T. aestivum*) and barley (*H. jubatum*) seeds treated with *Bacillus* isolates

Seeds	RSGI (%)							
	<i>Bacillus</i> isolate							
	B3	B4	B5	BMG1	BMG2	PAZE-4	PAZE-6	Control
Barley	90.00	85.00	95.00	100.00	100.00	95.00	100.00	95.00
Wheat	85.00	95.00	95.00	97.75	97.50	95.00	97.50	95.00

According to the RSGI, all tested bacterial isolates showed good effect on *T. aestivum* and *H. jubatum* seeds germination. However, the lowest seed germination was obtained after applying isolates B3 and B4 on wheat and barley seeds, respectively. On the contrary, isolates BMG1, BMG2 and PAZE-6 improved the seed germination process (Table 3) in comparison to control. In the literature, there are studies which also confirmed that *Bacillus* species were

capable to improve seed germination including wheat and barley seeds (Hu et al., 2019; Abbasdokht and Gholami, 2010; Yadav et al., 2010; Canbolat et al. 2006).

In addition, all applied *Bacillus* isolates significantly improved the growth of shoots and roots of barley, recorded over seven days (Figs.1 and 2). The increase in the length of shoot of barley was between 1.39 (PAZE-6) to 1.99 (B3) times and 2.23 (BMG1) times in relation to control (Figs 1 and 2). While, the length of root of barley was increased by 31%, 39%, 54%, 55% and 60% with the applied PAZE-4, B5, BMG2, BMG1 and PAZE-6 respectively. The isolate BMG1 greatly increased the length of shoot and on the same time it contributed to the significant improvement of root length (Fig.1).

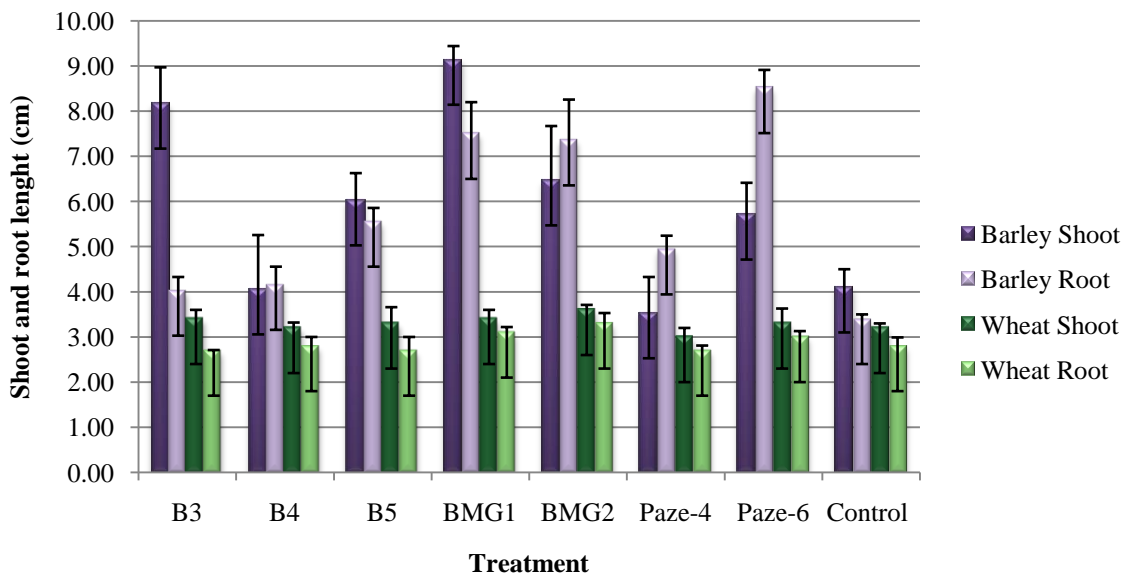


Figure 1. The effect of *Bacillus* isolates on the length of shoot and root of *T. aestivum* and *H. jubatum* seeds

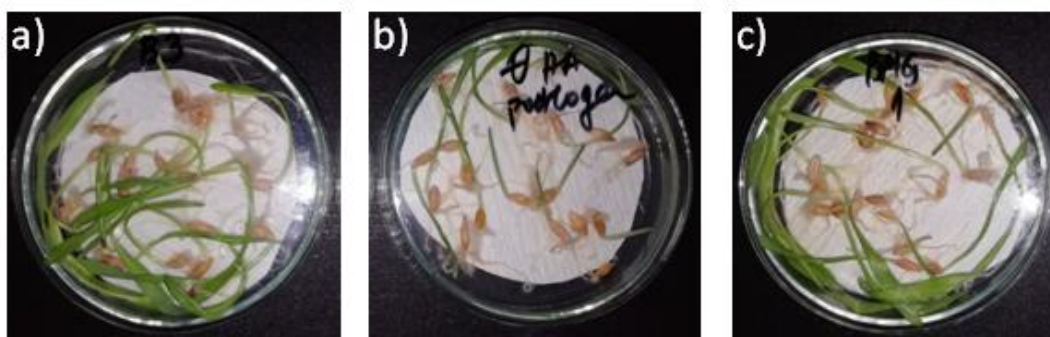


Figure 2. The growth of shoots of *H. jubatum* seeds treated with B3 (a) and BMG1 isolate (c) and control (b)

On the other hand, isolate B3 improved the growth of shoot and less growth of roots of barley seeds. Canbolat et al. (2006) also reported that *Bacillus* strains (RC01, RC02, RC03 and M-13) improved the growth both roots and shoots of barley seeds. The strain *Bacillus* RC01 showed the highest increase in root length of 8.88% (Canbolat et al. 2006). In addition, Çakmakçi et al. (2007) studied the effect of plant growth promoting rhizobacteria on barley seed using *Rhodobacter capsulatus* RC04, *Paenibacillus polymyxa* RC05, *Pseudomonas putida* RC06, *Bacillus* OSU-142, *Bacillus* M-13, *Bacillus megaterium* RC01 and *Bacillus licheniformis* RC02. 10.1%. The highest improvement in the length of shoot and root in relation to control sample was obtained by strain *Bacillus* OSU-142 with values of 5.4% and 7%, respectively (Çakmakçi et al. 2007).

However, applied *Bacillus* isolates showed low *T. aestivum* seeds germination. The highest increase in the length of shoot and root was given by BMG2 and it was 11.2% and 15.2 % in comparison to control, respectively (Fig.1). Taking into account the types of seeds, the isolate BMG2, with very good RSGI and good results of increasing the length of shoot and root for both seeds, can be considered as a good plant growth promoting bacteria for wheat and barley. In addition, this isolate showed ability for the production of IAA (24.89 µg/mL) and siderophores. Growth promotion may be due to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities. These abilities depend on the availability of precursors for their production by microorganisms and their uptake by plant (Yadav et al., 2010).

CONCLUSION

Bacillus isolates BMG1, BMG2 and PAZE-6 showed the strongest potential in the induction of barley and wheat seed germination. All tested isolates improved the growth of shoots and roots of barley. None of the isolates has the ability to produce HCN. Isolates BMG2 and PAZE-6 produced the highest amount of IAA, while B4 showed the best potential in the production of siderophores. Based on the obtained results it can be considered that isolates BMG1, BMG2 and PAZE-6 could be used as PGP agents.

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Uticaj bakterijskih izolata iz zemljišta rizosfere na indukciju klijavosti semena pšenice i ječma

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Zemljišne bakterije iz rizosfere korena biljaka najčešće imaju širok spektar pozitivnog dejstva na biljke i mogu poboljšati njihov rast. Vrste roda *Bacillus* jedne su od najistraživanijih bakterija koje mogu doprinosti poboljšanju rasta biljaka. U ovom radu je izolovano sedam *Bacillus* izolata iz rizosfere lucerke (*Medicago sativa*). Ispitivana je sposobnost produkcije jedinjenja kao što su vodonik-cijanid, indol-3-sirćetna kiselina i siderofore. Procenjena je i sposobnost indukcije klijavosti na semena pšenice (*Triticum aestivum*) i ječma (*Hordeum jubatum*). Izolat BMG2 je proizveo najviše indol-3-sirćetne kiseline (28.89 µg/mL). Dužina korenčića je povećana za 60%, dok je dužina izdanka uvećana 2.23 puta primenom izolata PAZE-6 i BMG1. Pored toga, izolati BMG1, BMG2 i PAZE-6, su poboljšali klijavost obe vrste semena i pokazali sposobnost da proizvedu korisne supstance kao što su siderofore i indol-3-sirćetna kiselina. Na dalje bi ovi izolati mogli da se koriste u proizvodnji tečnih aditiva koji bi povećali prinos kultivisanih biljaka, a posebno ječma.

Ključne reči: *Bacillus* sp., klijavost semena, indol-3-sirćetna kiselina (IAA), siderofore, *Triticum aestivum*, *Hordeum jubatum*.