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CHARACTERIZATION OF ENDOPHYTIC BACTERIA OF THE GENUS *BACILLUS* AND THEIR INFLUENCE ON THE GROWTH OF MAIZE (*ZEA MAYS*) *IN VIVO*

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ABSTRACT

Bacteria with positive properties on plant vitality are also called PGPB (Plant Growth Promoting Bacteria). Their presence can be observed not only in the root area but also in the above-ground parts of plants like endophytic bacteria. The aim of our study was to characterize promoting features of bacteria from *Bacillus* genus and compare them with *Pseudomonas simiae* WCS417 (plant growth promoting strain). The work was carried out in locality Kolíňany near Nitra (40°26´46´´N, 79°58´56´´W) and root samples were taken from 6 randomly selected plants of maize (*Zea mays* L.) in vegetative plant growth stage BBCH 14-15. Bacteria isolated from plant roots were identified and tested to biochemical parameters. From the biochemical features, we observed the detection of siderophores, determination of indole-3-acetic acid (IAA), monitoring the ability to dissolve phosphates and antifungal activity. Bacterial suspensions were applied to maize seeds and tested *in vivo* controlled conditions. Tested isolates were identified as *Bacillus flexus*, *Bacillus megaterium* and *Bacillus subtilis*. All 3 strains achieved the middle – class of phosphate solubilization index ($2.00 \leq SI < 4.00$), produced phytohormone IAA and showed positive production of siderophores and inhibited growth of *Fusarium culmorum* to more than 50%. All differences between tested strains and control strain *P. simiae* WCS417 were also statistically confirmed. All strains showed positive results in monitoring plant growth promoting properties. The effect of three *Bacillus* strains on maize seeds *in vivo* conditions showed significant differences in root length ($P<0.0001$) and weight of the young plant ($P<0.001$) compared to control.

Keywords: bacteria, plant vitality, identification, maize

INTRODUCTION

Bacterial strains that have beneficial effects on plant growth and vitality are characterized as beneficial plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes), and the inside of plant tissues (endophytes) **(Kobayashi and Palumbo, 2000)**. Depending on their effect on the host plant, bacteria can be categorized into three groups: plant-growth promoting (PGPB), plant-growth inhibiting, and plant-growth neutral **(Sturz *et al.*, 2000)**. Numerous microbes are naturally beneficial to plants and help to sustain plant growth during abiotic and biotic stresses **(Sharma *et al.*, 2014)**.
The beneficial effects of PGPBs are generally observed by the occurrence of an increase in germination rates, root growth, yield, leaf area, chlorophyll content, magnesium content, nitrogen content, hydraulic activity tolerance to drought, shoot and root dry weights. PGPB can promote plant growth directly or indirectly **(Glick, 1995)**. Recent studies showed that the direct mechanisms involve improved uptake of nutrients such as nitrogen and phosphorus, production of plant hormones such as indole-3-acetic acid (IAA), gibberellins and cytokinins. On the other hand, indirect mechanisms mainly consist of production of iron chelating agents, cyanides and siderophores. Besides, production of various antimicrobial metabolites is also grouped into the indirect mechanisms **(Tonelli *et al.*, 2017; Radhakrishnan and Lee, 2016)**. Plants are constantly involved in interactions with a wide range of bacteria, which belong to the *Acetobacter, Azospirillum, Azotobacter, Bacillus or Pseudomonas* genera **(Jones *et al.*, 2007)**. Among several species of PGPB, the *Pseudomonas* and *Bacillus* spp. have been identified as the predominant genus **(Kang *et al.*, 2015)**. *Bacillus* spp. are gram positive, ubiquitous in nature and recovered from all niches in the environment. These species have also been used to prepare industrial and agricultural products **(Lyngwi and Joshi, 2014)**. Endophytic bacteria are isolated from various parts of the plant above and below ground but the highest number of endophytic bacteria were observed in the roots **(Afzal *et al.*, 2019)**. The *Bacillus* spp. associated with plant roots and rhizosphere promoted plant growth **(Beauregard *et al.*, 2013)**.
The aim of this study was to test endophytic bacterial strains isolated from maize roots for plant growth promoting traits and to find differences between these strains and plant growth promoting strain *Pseudomonas simiae* WCS 417. We further monitored whether the results of analyses with the assumption of plant growth support cooperate with the application of bacteria *in vivo* to the model plant. In this study we perform the following steps: (1) identification of species isolated from maize roots, (2) testing all *Bacillus* strains to biochemical properties that promote plant growth and (3) monitoring of changes in plant growth after application of *Bacillus* strains to the maize seeds under *in vivo* conditions compared to *Pseudomonas simiae* WCS 417 (Netherlands).

MATERIALS AND METHODS

Collection of root samples

Plants of maize (*Zea mays* L.) for the isolation of endophytic bacteria were collected from SUA University Farm Kolíňany (40°26'46"N, 79°58'56"W), Slovakia (Fig. 1), in plant growth stage BBCH 14-15 (growth of 2-4 true leaves). The sowing of maize was established in April 2017 and maize was once treated with the herbicides Laudisode and Lontrel.. Six randomly selected plants of maize with roots were carefully taken by sterilized spade from soil and transferred into laboratory Department of Microbiology, Faculty of Biotechnology and Food Sciences, SUA, Nitra. In the laboratory were plants separated into stems and roots. Roots were cleaned thoroughly with tap water, rinsed with deionized water and drained on absorbent towels for futher sterilization and isolation steps.

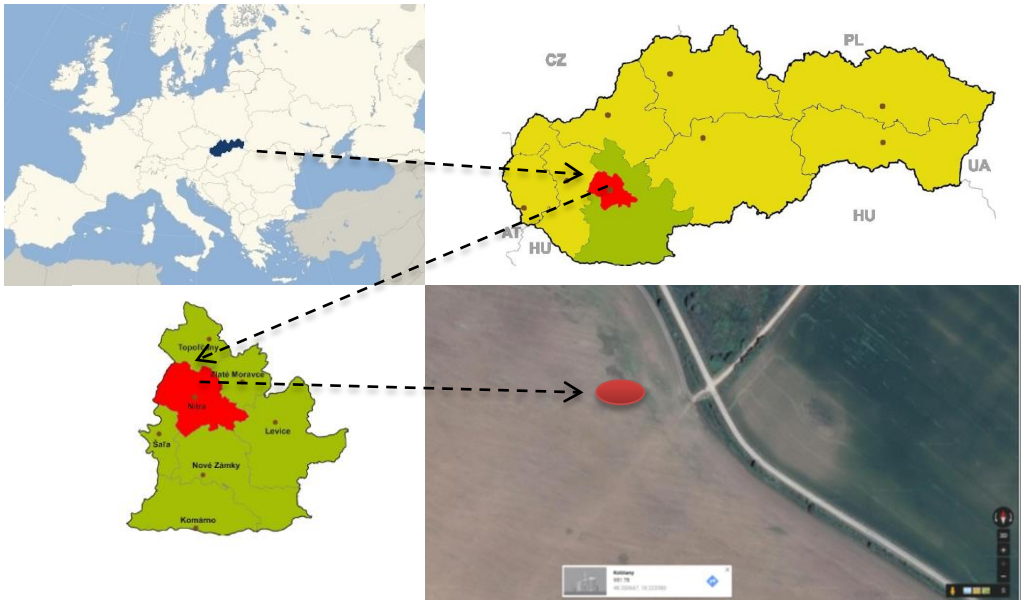

Figure 1. Location of sampling point

Isolation of bacterial strains

Root surface sterilization included the followings steps **(Sun *et al.*, 2008):** to wash out the mechanical impurities roots were washed under distilled water and then surface sterilized in 99% ethanol for 1 minute and 3.125% sodium hypochloride solution for 6 minutes and followed by final wash in sterile distilled water for 3 times. Sterilized root samples were aseptically cat to small fragments which were placed on Luria-Bertani (LB) agar plates in 3 repetitions and incubated for 24 h at 30 °C. After incubation, distinct bacterial colonies were streaked on LB agar plates to get single colonies. Thus prepared bacterial isolates were used for further testing.

Molecular characterization

DNA was extracted from 1-day-old bacterial isolates prepared by the TSA agar cultivate method. Approximately 50 mg of each bacterial culture was placed in 200 µl of PrepMan solution (Life technologies) and homogenized with glass beads on BeadBug homogenizer (Benchmark scientific). DNA from all samples was use as a DNA template in PCR reaction. The bacterial 16S rRNA genes were amplified through PCR using the universal bacterial primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (**Nardi *et al.*, 2004).** Cycling conditions for PCR amplification (performed in thermocycler MJ Mini (Biorad, USA) were as follows: 95°C for 3 min. followed by 40 cycles of 95°C for 30 s, annealing at temperature 56°C for 30 s, 72°C for 90 s and a final elongation at 72°C for 10 min. Amplification products were sequenced and performed by Macrogen (South Korea). Acquired sequences were assembled and processed in MEGA 7 software **(Kumar *et al.*, 2018)**. Alignment was made in MUSCLE (**Edgar, 2004**). We used the reference sequences from Genbank database for phylogenetic analysis. A phylogenetic tree was constructed using method of Maximum likehood with substitute model **Tamura-Nei (Tamura *et al.*, 2007)**.

Morphological, physiological and biochemical characterization

We monitored cell morphology, gram property using Gram´s staining, endospore formation, oxidase and catalase activity (Collins et al., 2004).

***In vitro* tests for direct growth promotion traits**

Bacterial isolates were tested for these direct growth promotion traits: production of phytohormone indole-3-acetic acid (IAA) and phosphate solubilisation. *Pseudomonas simiae* WCS417 (Netherlands) was used as a positive control.
Production of phytohormone Indole-3-acetic acid (IAA) was evaluated according to **Gordon and Weber (1951)**. Bacterial cultures were grown for 24 hours at 30 °C on Trypton Soya Agar (TSA) plates and than the concentration of each bacterial strain was standardized to 0.5 McFarland (10^8 CFU.ml^{-1}). Bacterial suspension was incubated in Pikovskaya's broth with 0.2% L-tryptophan for 7 days at 30 °C. The supernatant was mixed with Salkowski reagent. The mixture was incubated in the dark for 30 minutes and then examined for the development of pink colour as an indication of IAA production. The colorimetric measurement of IAA was done spectrophotometrically at 530 nm.
Activity of bacteria to dissolve phosphates was evaluated on Pikovskaya's agar with bromophenol blue **(Gupta *et al.*, 1994).** Plates were inoculated with tested bacteria and incubated for 7 days at 30 °C. The yellow/clear zone around bacterial colonies showed positive solubilisation of phosphates and Solubilisation index (SI) was calculated according to **Kumar and Narula (1999).**

***In vitro* test for indirect growth promotion traits**

Bacterial isolates were evaluated for these 2 traits associated with indirect growth promotion: antagonism against phytopatogenic fungi and siderophore production. In tested methods was used *Pseudomonas simiae* WCS417 as a positive control.
Phytopathogenic fungi were inoculated on plates with Potato Dextrose agar (PDA) and incubated for 10 days at 25 °C. Tested bacteria were inoculated on TSA plates and incubated 24 hours at 30 °C. Bacterial suspensions were diluted to 0.5 McFarland (10^8 CFU.ml^{-1}) and spreaded onto the surfaces of TSA plates using sterile bacterial cell spreaders. The centre of each plate was inoculated with 9 mm diameter fungal plug cut with a sterilized cork-borer from a *Fusarium culmorum* culture. Petri dishes were incubated for 10 days at 25 °C. *Pseudomonas simiae* WCS417 (Netherlands) was used as a positive control, sterile TSA plates as a negative control. The pathogenic fungus *Fusarium culmorum 819.* was obtained from Department of Plant Protection, Faculty of Agrobiology and Food Resources, SUA, Nitra.
Production of siderophores was tested according to **Schwyn and Neilands (1987)** using the Chrome Azurol S (CAS) medium. Each bacterial isolate was inoculated on agar plates and incubated for 7 days at 30 °C. The orange halo zone around a bacterial colony indicated siderophore production by bacteria.

Growth promotion effects *in vivo*

The potential PGPB strains isolated from maize roots were analysed for their ability to exhibit plant growth promotion. Firstly, the maize seeds were surface sterilized with 3.125% NaOCl for two min. and followed by three washes in sterile distilled water. The organically poor soil was sterilized in autoclave in followed conditions: pressure 0.1 MPa and temperature 121 °C for 20 minutes. Inoculants of the selected strains were prepared on TSA agar plates and incubated at 30 °C for 24 h. Concentration of bacteria was standardized to 0.5 McFarland (10^8 CFU.ml^{-1}) for each strain. Sterilized seeds were immersed in the bacterial suspensions for 1 hour. After soaking, the seeds were sown in the soil, 1 cm below the surface. A growth chamber experiment lasted 4 weeks and was carried out under controlled conditions: changes in temperature depending on the light period - 16 hours, 28 °C and dark period – 8 hours, 22 °C and constant humidity in both phases was 80%. As a control, seeds were treated in distilled water. As a positive control we used the strain *Pseudomonas simiae* WCS417 (Netherlands). 30 days after sowing plants were removed from the soil and their roots were carefully washed. The root length (cm) and weight of the young plant (g) were evaluated.

STATISTICAL ANALYSIS

All results are the means of three independent replicates. Analysis was carried out using the GraphPad Prism where standard error was evaluated. We used One-way analysis of variance ANOVA to compare values from the analysis of plant growth promoting characteristics and analysis of the effect of these bacteria on maize growth performance (root length and weight of the young plant). A comparisons were done using Dunnett's test and the significant level was set at **** $P < 0.0001$ and *** $P < 0.001$.

RESULTS AND DISCUSSION

During last few years many studies focused on the isolation and characterization of bacterial strains isolated from soil or plant parts with plant growth promotion potential (**Ramakrishna *et al.*, 2019; Majeed *et al.*, 2018; Numan *et al.*,2018; Olanrewaju *et al.*, 2017; Olivares *et al.*, 2017**). These types of bacteria were classified as PGPB - Plant Growth Promoting Bacteria and we can divide them to three main groups like rhizospheric bacteria, phyllospheric bacteria and endophytic bacteria **(Kuan *et al.*, 2016).** According to this fact we tested endophytic bacteria isolated from plant roots to their plant growth promoting traits. The maize root samples were taken from SUA University Farm Kolíňany. *Bacillus* strains are mostly dominant in the soil around the roots (rhizospere) and inside the roots **(Hallmann and Berg, 2006).** Studies on the diversity of root-associated bacteria in maize revealed extensive colonization by *Bacillus* strains during the active vegetative plant growth stage of the plants **(Lalande *et al.*, 1989).**

Our root samples were collected during active growth stage of maize BBCH 14-15. From all samples we isolated 10 bacterial strains and 3 of them were identified as *Bacillus megaterium* (231), *Bacillus flexus* (239), and *Bacillus subtilis* (246). Similar result are observed for different plant species, including sugar cane (**Ratón *et al.*, 2012**), maize (**Pereira *et al.*, 2011**) and rice (**Nautiyal *et al.*, 2013**). Detailed data about taxonomic affiliation of tested strains and GenBank accession numbers are situated in Table 1. The phylogenetic tree of bacterial strains constructing by using their 16S rRNA sequences is shown on Figure 1.

Table 1 Taxonomic affiliation of active strains and GenBank accession numbers

Sample Code	Accession Number	Closet relative species	% Identity
231	NR_116873.1	*Bacillus megaterium* strain ATCC 14581	99.65%
239	NR_113800.1	*Bacillus flexus* strain NBRC 15715	100%
246	NR_112116.2	*Bacillus subtilis* strain IAM 12118	99.89%

Legend: 231 – *Bacillus megaterium*, 239 – *Bacillus flexus*, 246 – *Bacillus subtilis*

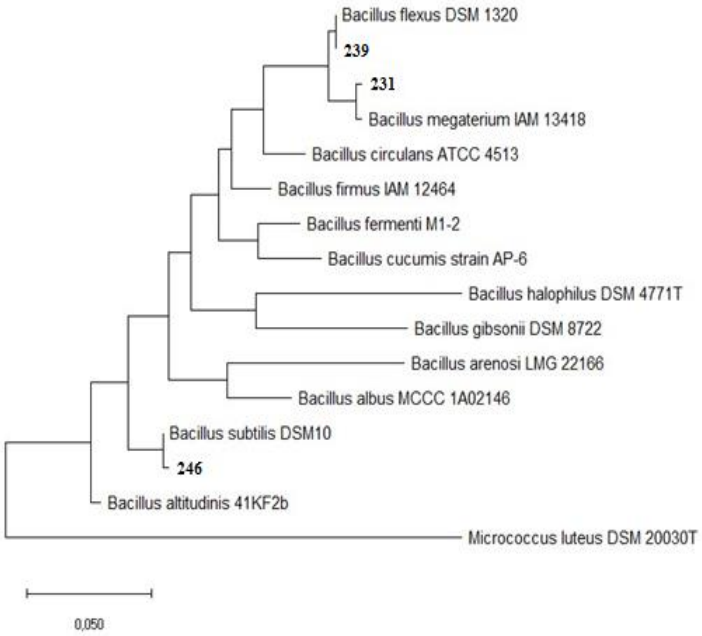

Figure 2 Phylogenetic tree of 16S rDNA gene sequences showing the relationships among the isolates from maize roots

Bacillus strains were examined for their morphological, physiological and biochemical properties. Relative results including cell morphology, Gram property and endospore formation, oxidase and catalase activity. All results are summarized in Table 2. In all properties we tested reference strain *Pseudomonas simiae* WCS417 (Netherlands).

Table 2 Morphological, physiological and biochemical characterization of tested isolates

Sample code	Cell morphology	Gram property	Endospore formation	Oxidase activity	Catalase activity
231	Rod	+	+	+	+
239	Rod	+	+	+	+
246	Rod	+	+	+	+
525	Rod	-	-	+	+

Legend: 231 – *Bacillus megaterium*, 239 – *Bacillus flexus*, 246 – *Bacillus subtilis*, 525 – *Pseudomonas simiae WCS417*

The aim of our work was to determine the effectiveness of bacteria by testing their ability to promote plant growth and apply them to seeds under *in vivo* conditions. For growth promotion, we tested the following: the ability to solubilize phosphates into a plant-accessible form, the production of phytohormone indole-3-acetic acid (IAA), the production of siderophores and antifungal activity. As a positive control we tested *Pseudomonas simiae WCS 417* (Netherlands) strain. In the last years many publications described biological mechanisms involved in the ability of the PGPB strain *P. simiae* WCS417 to promote plant growth and health (**Desrut *et al.*, 2020; Pieterse *et al.*, 2020; Stringlis *et al.*, 2019; Yu *et al.*, 2019; Williams *et al.*, 2018**). The results of plant growth promoting direct and indirect potentials of tested *Bacillus* strains and reference strain *Pseudomonas simiae* WCS417 are shown in Table 3.

Seed germination and plant growth are influenced by the nutrients available in the soil. Plants absorb phosphorus (P) from the soil through root transporters. Available forms of P are also limited **(Bidondo *et al.*, 2012).** *Bacillus* spp. convert the complex form of essential nutrients, such as P to a simple available form that is used during uptake by plant roots. All tested strains solubilize phosphate. They are included in the group with middle phosphate solubilizaton index (2.00<SI>4.00) according to **Kumar *et al.* (1999).** A statistically significant difference (P<0.01) was observed between strain 239 (*B. flexus*) and *P. simiae* WCS417.

The presence of tryptophan and other bacterial food source compounds induces the synthesis of indole-3-acetic acid (IAA) and other hormones in bacterial populations **(Glick, 2014).** Plant-growth-promoting substance IAA is synthesized by *Bacillus* spp. and increase root and shoot cell division and elongation **(Radhakrishnan and Lee, 2016).** According to the IAA production assay results all 3 *Bacillus* isolates and *Pseudomonas simiae* WCS417 produce auxin from 2.07 to 5.60 $\mu g.ml^{-1}$. A statistically significant difference was observed between all *Bacillus* strains to the *P. simiae* WCS417. The P value was as follows 525-231 and 525-239 (P<0.001) and 525-246 (P=0,034). The iron-chelating properties of *Bacillus* spp. via siderophore production help to solubilize iron from minerals and organic compounds in rhizosphere **(Nadeem *et al.*, 2012).** In the case of *Pseudomonas simiae* WCS417, its biological activity is mainly associated with the production of siderophores (**Berendsen *et al.*, 2015).** In our study was production of siderophores positive in all tested isolates.

Maize is one of the crops that is attacked by a number of pests at all growth stages. Among the most common are microscopic fungi, especially representatives of the genus *Fusarium* **(Javoreková *et al.*, 2020).** Genus *Bacillus* is well known for their antagonistic behavior by producing siderophores, HCN, hydrolytic enzymes and antibiotics. In the Srivastava's study nine of the soybean bacterial endophytes that belong to *Bacillus* sp. were reported to have antifungal activities against major soil-borne plant pathogens like *Rhizoctonia*, *Fusarium* and *Sclerotinium* **(Srivastava *et al.*, 2016).** *Bacillus* spp. attaches to the mycelial cell walls and damage fungal mycelium **(Akram *et al.*, 2016).** In our study was protection against pathogens more than 50% in all tested isolates. Statistically significant differences were observed between all tested strains to the control strain *P. simiae* WCS417.

Table 3 Plant growth promoting characteristics of the bacterial isolates (means±standard error)

Sample Code	Phosphate Solubilization Index	IAA Production [µg.ml^{-1}]	Siderophore Production	Protection against pathogens [%]
231	2.14±0.08	5.60±0.13	+	55.93±0.03
239	2.52±0.19	2.81±0.18	+	59.70±0.12
246	2.01±0.04	2.07±0.06	+	57.04±0.06
525	2.03±0.09	2.15±0.02	+	52.73 ±0.08

Legend: 231 – *Bacillus megaterium*, 239 – *Bacillus flexus*, 246 – *Bacillus subtilis*, 525 – *Pseudomonas simiae* WCS417 (Netherlands), IAA - indole-3-acetic acid

The application of *Bacillus* strains can enhance the plant-available forms of nutrients, promote plant growth (roots elongation) and control disease-causing pathogenic microbial growth **(Garcia-Fraile *et al.*, 2015).** We applied *Bacillus* strains on maize seeds. The plants were placed in the cultivation chamber and growth under controlled conditions. All 3 *Bacillus* strains tested in this study exhibit the potential to increase plant growth in *in vivo* experiments (root length, weight of the young plant) which was also confirmed statistically. The length of the root system increased by more than 18.52% (*B. megaterium*), 29.63% (*B. flexus*) and 36.6% (*B. subtilis*) compared to the control. Weight of the young plant increased by more than 400% after application of *B. subtilis* and almost 600% after application of *B. megaterium* and *B. flexus* strain. According to **Zamioudis *et al.* (2013)** study, colonization of *Arabidopsis* roots by *Pseudomonas simiae* WCS417 promotes plant growth by driving auxin-dependent developmental changes in root architecture, resulting in the stimulation of lateral root emergence and root hair formation. In our study, all strains produced IAA, although in different amounts. *Pseudomonas simiae* WCS417, which was used as positive control, promoted plant growth less than other tested bacteria. The length of the root system was extended by 12% and the amount of weight of young plant increased almost threefold compared to the control. *Bacillus* strains that produced lower levels (246 - *Bacillus subtilis*, 239 - *Bacillus flexus*), had bigger influence on root elongation and biomass, characteristics of great interest that provide greater surface area for the absorption of nutrients. According to **Arshad and Frankenberger (1991)** the effects of the auxin depend on its concentration, i.e. when it is low it can stimulate growth and when it is high can be inhibitory, same for seed germination. Microbial synthesis and secretion of IAA significantly increase the root length in all cases and play important role in the germination event of various plant species. Weight of the young plant increases in all tested samples, too.

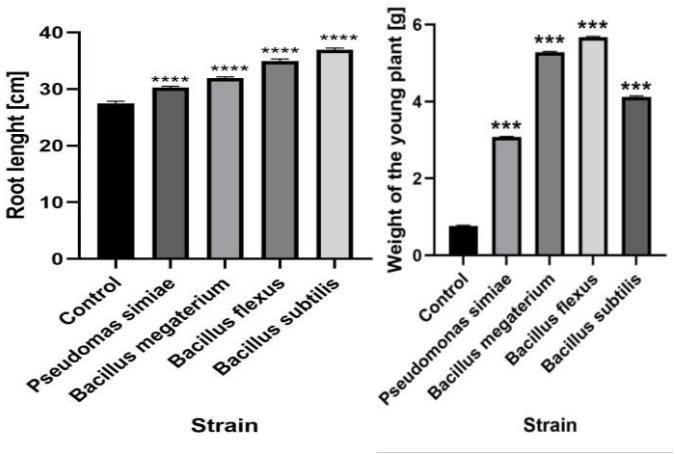

Figure 2 Effects of *Bacillus* tested strains and reference strain *Pseudomonas simiae* WCS417 on maize growth (root length and weigth of the young plant) according to the control

According to the all tested direct and indirect plant growth promoting traits and potential of bacteria to promote root length and weight of the young plant we evaluated as the most productive *Bacillus flexus* strain (239). Results of all tested strains are shown in Table 4.

Table 4 Summarizing the results of the tested bacteria strains – plant growth promoting trates and promotion of the root length and weight of the young plant

Sample Code	IAA	PSI	SP	PAP	RL	YP	Total
525	3.	3.	+	4.	4.	4.	4.
231	1.	2.	+	3.	3.	2.	2.
239	2.	1.	+	1.	2.	1.	**1.**
246	4.	3.	+	2.	1.	3.	3.

Legend: IAA- Indole-3-acetic acid, PSI-Phosphate Solubilization Index, SP-Siderophore Production, PAP-Protection against Pathogens, RL-Root Length, YP - Weight of the young plant, 525 – *Pseudomonas simiae* WCS 417, 231 – *Bacillus megaterium*, 239 – *Bacillus flexus*, 246 – *Bacillus subtilis*

CONCLUSION

Several strains of *Bacillus* genera are well-established model organisms for research on molecular plant-microbe interactions. Many species are well-known plant-growth promoters. In our study we tested *Bacilus flexus*, *Bacillus subtilis* and *Bacillus megaterium* strains isolated from maize roots. All tested strains showed positive results in laboratory experiment and *in vivo* experiment. According to this fact application of *Bacillus* as biofertilizer for agriculture improvement can reduce the use of agrochemicals and support eco-friendly crop production. *Bacillus* sp. having antagonistic and resistance-inducing trait might be useful in formulation of novel bioinoculants leading to almost effective for biocontrol strategies for improving crop growth. Continued research with *Bacillus* genera has potential for developing biofertilizer and biocontrol agents.

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