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Biocontrol of *Botrytis cinerea* and promotion of tomato growth by local soil-borne *Bacillus* isolates

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Abstract

Grey mould, which is caused by *Botrytis cinerea* Pers., is among the most damaging diseases of cultivated plants worldwide. The use of fungicides dominates in the protection of tomatoes against grey mould. Due to the emergence of resistance of pathogens to the active ingredients of fungicides, the possibility of using bacteria of the genus *Bacillus* was studied. A total of 666 isolates were isolated from different agricultural soils, and a preliminary screening dual culture test with *Alternaria dauci* was done. A total of 77 isolates were dual culture tested against *Fusarium tricinctum* and *F. proliferatum*. Based on preliminary dual culture testing, eight *Bacillus* isolates were selected for testing their biological potential against two isolates of *B. cinerea.* The effect of *Bacillus* spp. isolates was studied for seed germination and plant growth promotion (PGP) on the seedlings of tomato (*Solanum lycopersicum* L.). Using molecular identification (16S rDNA), five bacterial isolates were identified as *Bacillus subtilis*, two isolates as *B. amyloliquefaciens,* and one isolate as *B. pumilus*. Using the Internal transcribed spacer (ITS) region, two fungal isolates from an infected leaf and a tomato fruit were identified as *B. cinerea*. The results of the dual culture test showed that eight bacterial isolates could significantly inhibit the growth of *B. cinerea* mycelia from 50% to 80%. Two isolates *B. amyloliquefaciens* 28.3 and *B. amyloliquefaciens* 21/IV increased seed germination by 85.66% and 86.16%, respectively, compared to the control (82.66%). All bacterial isolates positively affected the morphological parameters of the seedlings. In the greenhouse experiment, three isolates *B. subtillis* 20.10, *B. amyloliquefaciens* 28.3, and *B. amyloliquefaciens* 21/IV and their mixture increased all measured PGP parameters. Isolates Bac 20.10 and Bac 28.3 reduced the severity of grey mould disease by 27.47% and 30.36%, respectively. These results show that *Bacillus* isolates have the potential for biological control of grey mould; also, that they have a positive effect on the growth of tomato plants.

Keywords: antifungal activity, *Bacillus* spp. *Botrytis cinerea*, biocontrol, plant growth promotion.

Introduction

Tomato (*Solanum lycopersicum* L.) is the most cultivated vegetable crop, grown on more than 5.03 million ha worldwide with an average yield of 35.93 t ha^{-1} (FAO, http://www.fao.org/home/en/; https://www.fao.org/faostat/en/#data/QCL). In 2019, in Serbia, the tomato was grown at nearly 8.000 ha with an average yield of 14.15 t ha^{-1} (RZS; https://data.stat.gov.rs/). The huge yield difference could be partially explained by outdated cultivation technology, inadequate hybrid selection, and plant pathogenic microorganisms. During the growing season, several pathogens can affect tomato plants reducing yield and quality (Williamson et al., 2007). One of the most devastating diseases of tomatoes is grey mould caused by *Botrytis cinerea* Pers. This fungus can cause diseases of over 230 host plants infecting different plant organs: flowers, fruits, leaves, and shoots (Fernández-Ortuño et al., 2014). The pathogen has a necrotrophic life cycle and can survive as a saprophyte or produce survival structures and sclerotia. In addition, the fungus forms conidiophores and conidia, apothecia, and ascospores that allow the spread of the pathogen and can survive as a mycelium in infected plant tissue or on plant seeds (Holz et al., 2007).

Due to diverse survival mechanisms, broad host range, and various modes of attack, *B. cinerea* is challenging to control. Preventive control measures in protected areas include ventilation, air circulation, keeping the relative humidity below 85%, and sanition practices (Gibson et al., 2014). However, some environmental manipulations may not be feasible due to inadequate or obsolete production facilities (Elad, Steward, 2007). Despite various preventive methods, the application of fungicides remains the most important and effective measure of controlling *B. cinerea* on tomatoes (Wang et al., 2018). Continuous application of chemicals has raised consumers' concerns over fungicide residues on tomato fruits and their effect on human health (Lamichhane et al., 2016). For example, Elgueta et al. (2020) showed that in reported samples of tomatoes and lettuces, maximum residue limits (MRLs) were between 7.5% and 10.4%. Furthermore, repeated use of fungicides leads to resistant *B. cinerea* populations. Resistance to fungicides intended for *B. cinerea* control on tomato has been reported in China for pyrimethanil (Liu et al., 2016) and cyprodinil (Fan et al., 2017), in Germany for iprodione and fludioxonil (Rupp et al., 2017), and in Greece for multiple resistance to carbendazim and cyprodinil (Konstantinou et al., 2015).

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Public concern for chemical residues in vegetables and the emergence of resistant pathogen populations has increased interest in finding alternative control measures for *B. cinerea* (Pal, McSpadden Gardener, 2006). One of the alternatives includes using biological control agents such as antagonistic bacteria. This method of disease management uses beneficial organisms that are introduced or already established in an environment (Pal, McSpadden Gardener, 2006). The promising biocontrol bacteria include *Bacillus* species, primarily because of diverse antagonistic mechanisms (Kilani-Feki et al., 2016) and adaptation to different external conditions (Köhl et al., 2019). Different members of this genus were previously used for biological control of tomato grey mould (Lee et al., 2006; Elad, Steward, 2007; Wang et al., 2018). However, pathogen diversity and genomic heterogeneity drive a perpetual need to study the relationship of the locally present, natural, and soil-associated microbiome to discover new, specific, and more effective biological control agents. Also, *Bacillus* spp. are known to stimulate plant growth by producing numerous metabolites that can increase the availability of nutrients as well as by producing phytohormones (Blake et al., 2020).

Thus, this study aimed to isolate local soil-borne *Bacillus* spp. from various locations in Serbia and assess their antifungal activity against local *B. cinerea* isolates both *in vitro* and in the greenhouse. In addition, the aim was to examine their ability to promote tomato seed germination, growth of seedlings, and plants in the greenhouse.

Materials and methods

Isolation and identification of Botrytis cinerea. Samples of tomato (*Solanum lycopersicum* L.) plants with symptoms of V-shaped blotch on the leaves and plants with soft rot of fruits were collected at two distant localities in Serbia: Paraćin (43°52′ N, 21°25′ E) (Pomoravlje district) and Gložan (45°17′ N, 19°34′ E) (South Bačka district) and used for isolation of *B. cinerea* Pers. using standard mycological methods (Dhingra, Sinclair, 1995). A sample taken from the leaf was marked as 436G-19, and the sample taken from the fruit was marked as 103-20. Symptomatic plant parts were washed under tap water, their surface disinfected with 2% NaOCl (sodium hypochlorite) solution for 40 s, rinsed in sterile distilled water (SDW), and air-dried on sterile filter paper. A small piece (3 × 3 mm) from the healthy and infected tissue border was placed on potato dextrose agar (PDA) medium and incubated at 25°C temperature in the dark. After seven days, a fungal colony around plant fragments obtained pure cultures for monosporic isolates. Koch's postulates were conducted by wound inoculating five surface-sterilized tomato leaves with a conidial suspension (4×10^5 conidia ml^{-1}) on the abaxial surface of the leaf. As a negative control, tomato leaves inoculated with SDW were used. Inoculated plants were incubated in air-tight containers at room temperature of 23 ± 2°C with a 16/8 h light/dark photoperiod and examined for the presence of the symptoms. From all symptomatic leaves, the pathogen was re-isolated using the same methods as for isolation. Based on macroscopic and microscopic characteristics, two isolates 463-19 and 103-20 were then identified as *B. cinerea*. Of the macroscopic characteristics, the morphology and appearance of colonies grown on a PDA medium at 24°C temperature for seven days were studied. Microscopic characteristics were studied on temporary microscopic slides. Molecular identification was carried out by polymerase chain reaction (PCR) amplification and sequencing using internal transcribed spacer (ITS) primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes, Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990). Amplified fragments of two isolates were directly sequenced in both directions using an automated sequencer ABI 3730XL (Automatic Sequencer Macrogen Inc., Korea) and the same primers as for amplification. Consensus sequences were computed using a programme *CLUSTAL W* (Thompson et al., 1994) integrated with software MEGA6 (Tamura et al., 2013) and deposited in the GenBank (http://www.ncbi.nlm.nih.gov). Generated sequences were compared with each other by calculating nucleotide (nt) similarities and previously deposited isolates 436G-19 and 103-20 available in the GenBank using the similarity search Basic Local Alignment Search Tool (BLAST).

Soil sampling and isolation of bacteria Bacillus spp. During 2018, 93 samples of soil from the field, vegetable, and fruit plant species were collected from 63 locations across Serbia (Figure 1).

Figure 1. Localities of collected soil samples with eight selected *Bacillus* spp. isolates in Serbia

Samples were placed in polyethene bags and stored in the fridge for 24 h at a temperature of 4°C. Isolation of *Bacillus* spp. was performed by suspending 10 g of soil in 90 ml of sterile 0.85% NaCl (sodium chloride) solution on a rotary shaker for 30 min at 150 rpm. Suspensions were serially diluted, and 1 ml of 10^{-4} to 10^{-6} dilutions were spread on nutrient agar (NA) plates. To obtain pure cultures, after 24–48 h of incubation at 30°C temperature, *Bacillus*-like colonies were recultivated on NA (Collins, Lyne, 1984). Morphological characteristics were observed under a microscope, and identification of isolates was performed following Berge's manual for determinative bacteriology (Vos et al., 2009).

Preliminary in vitro dual culture. Antifungal activity of 666 *Bacillus* isolates was preliminarily tested against *Alternaria dauci*. Based on the results of the dual culture test, 77 isolates were selected to be dual culture tested against *Fusarium tricinctum* and *Fusarium proliferatum* from garlic. For seed testing collection, phytopathogenic fungi were obtained from the Laboratory for Microbiological Researchs of the Institute of Field and Vegetable Crops, Novi Sad, Serbia. Based on preliminary screening, for further research, eight isolates: Bac 18/IV, Bac 57.2, Bac 18.8, Bac 65.5, Bac 5.2, Bac 21/IV, Bac 28.3, and Bac 20.10, were selected. Three isolates Bac 20.10, Bac 28.3, and Bac 21/IV and their mixture were selected for their biological efficacy and effect on the growth of tomato plants based on dual culture testing with two isolates of *B. cinerea* for molecular identification, total seed germination, and morphological parameters of seedlings.

In vitro dual culture. Antifungal activity of the eight *Bacillus* isolates against two *B. cinerea* isolates were performed by dual culture test (Suparman et al., 2002). First, bacterial isolates were streaked on PDA along the edge of the Petri dish (R = 85 mm). Discs of week-old fungal cultures, 6 mm in diameter, were placed on the opposite side. Antagonism testing was performed in three replicates. After seven days of incubation at 25°C temperature, the percentage of growth inhibition (PGI) was calculated according to the formula of Korsten and De Jager (1995). The inhibition zone was measured (in mm) as the distance between the *Botrytis* isolates and the antagonist growth area.

Molecular identification of antagonistic Bacillus spp. Bacterial DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's

instructions. To identify the species of the genus *Bacillus*, 16S rDNA sequence was amplified in Mastercycler PCR (Eppendorf, Germany) using the universal primers fD1 (27F) (AGAGTTTGATCM(C)TGGCTCAG) and rP3 (1492R) (TACGGYTACCTTGTTACGACTT) (Weisburg et al., 1991). PCR products were stained with ethidium bromide, separated by horizontal electrophoresis in 1.5% agarose gel, and visualised on a UV transilluminator. The appearance of fragments of 1460 bp was considered a positive reaction. Amplified DNAs from the respective regions were sent for sequencing to Macrogen Ltd., The Netherlands. Using FINCHTV, version 1.4.0, and BLAST analyses, multiple comparisons of the obtained sequences with the GenBank database were performed. The sequences were deposited in the National Center for Biotechnology Information (NCBI) database with corresponding NCBI accession numbers. Phylogenetic analysis was performed using the software MEGA X (Pennsylvania State University, USA). Bootstrap analysis was used to estimate the topology of the data tree that was merged with 3.000 random data sets, and the relationships between the sequences were analysed by the neighbour-joining method.

In vitro total germination of tomato seeds. To investigate the effect on seed germination and seedling growth, tomato seeds were coated by immersion in the suspension of each of the eight isolates. The experiment was performed following the standard germination test (ISTA, 2016). One hundred tomato seeds of the cultivar 'Novosadski jabučar' (Institute of Field and Vegetable Crops, Novi Sad) were immersed in bacterial suspension (10^8 CFU ml^{-1}) for 2 min, air-dried and placed on sterile filter paper that was soaked with sterile water in a Petri dish (R = 140 mm). Control seeds were immersed in 10 ml of 0.2% KNO_3 (potassium nitrate) solution. Inoculated seeds were incubated in a germination chamber with 95% relative humidity and a 16/8 h day/night regime at 25°C temperature. Each treatment was repeated four times. Total germination (%) of seeds was recorded 14 days after the treatment (ISTA, 2016). Following the total germination assessment, ten seedlings were randomly selected from each treatment: the stem and root length (cm) and the fresh (FM) and dry (DM) mass (g) of the stems and roots were measured.

Plant growth promotion (PGP) on tomato plants. Based on antifungal activity and the effect of seed total germination, *Bacillus* spp. isolates Bac 20.10, Bac 28.3, and Bac 21/IV and their mixture were tested for PGP on tomato plants in a greenhouse using a modified method of Xu et al. (2016). Used tomato plants were grown in greenhouse conditions at 25°C temperature and 16/8 h photoperiod. Four-week-old tomato seedlings (BBCH 14) were transplanted in 20 cm diameter pots filled with the substrate "Florabella" (Klasmann, Germany) with NPK value 12:11:18, pH value 5.5–6.5, and soil electrical conductivity of 45 mS m^{-1}. Following transplantation, tomato plants were drenched three times in the following ten days with 55 ml of bacterial inocul (10^9 CFU ml^{-1}, OD_{620nm} = 0.3) from individual isolates Bac 20.10, Bac 28.3, and Bac 21/IV and their mixture. To test the effect of the bacterial consortium, the plants were drenched in the same manner with a mixture of the three isolates blended in equivalent proportions. The inocula were prepared by mixing 48-hour-old bacterial cultures grown in nutrient broth (NB) and SDW. Control plants were drenched with 50 ml SDW and 5 ml NB. After the treatment, tomato plants were watered regularly until the pre-flowering stage (BBCH 61), when the following morphological characteristics were measured: shoot and root length, the FM and DM of the shoot, the FM and DM of the root, and the number of leaves and internodes. The experiment was set up according to a randomized complete block design (RCBD) with five replications.

Biological control on tomato grey mould in the greenhouse. For biological control of *B. cinerea* under greenhouse conditions, individual isolates Bac 20.10, Bac 28.3, and Bac 21/IV and their mixture were tested using a modified method of Xu et al. (2016). Two-month-old tomato plants (BBCH 59) of the cultivar 'Novosadski jabučar' were treated with two different doses of each isolate and their mixture of the same concentration (10^9 CFU ml^{-1}) until the water drained once a week for three consecutive weeks. The first dose was prepared with 5 ml of bacterial cultures and 25 ml of SDW, i.e., 10 ml of suspension per pot. The second dose was prepared with 5 ml of bacterial cultures and 50 ml of SDW, approximately 18.33 ml of suspension per pot. One day after the last bacterial treatment, all plants, both treated and nontreated, were foliar-inoculated with *B. cinerea* isolate 463-19 (10^5 conidia ml^{-1}) obtained from a 14-day-old culture grown on PDA. Following the application of the pathogen, systemic fungicide Switch 62.5 WG with the active ingredients cyprodinil and fludioxonil (Syngenta, Switzerland) was used with concentration 0.23% as a positive control and SDW as a negative control on nontreated plants. The plants were monitored daily for symptoms, and the disease severity (%) was evaluated on days 7, 10, and 14 after inoculation on a scale of 0 – no disease symptoms, 1 – 0.1–5%, 2 – 5.1–20%, 3 – 20.1–40%, and 4 – 40.1–100% (Lee et al., 2006). The experiment was set up according to a RCBD with three replications with six plants per individual isolates and their mixture as well as control per replication.

Statistical analysis. Experimental data were processed using a one-way analysis of variance (ANOVA). Values of *p*-ANOVA were shown for a dual culture test and seed total germination. Using the Tukey's method for a *p*-value of multiple comparisons results for morphological parameters of seedlings were shown. All analyses were performed with software Statistica, version 10 (TIBCO Software Inc.) for 95% confidence levels (StatSoft Inc., USA). The phylogenetic tree was constructed with the eight selected isolates and eight strains from the NCBI database by the software MEGA X.

Results

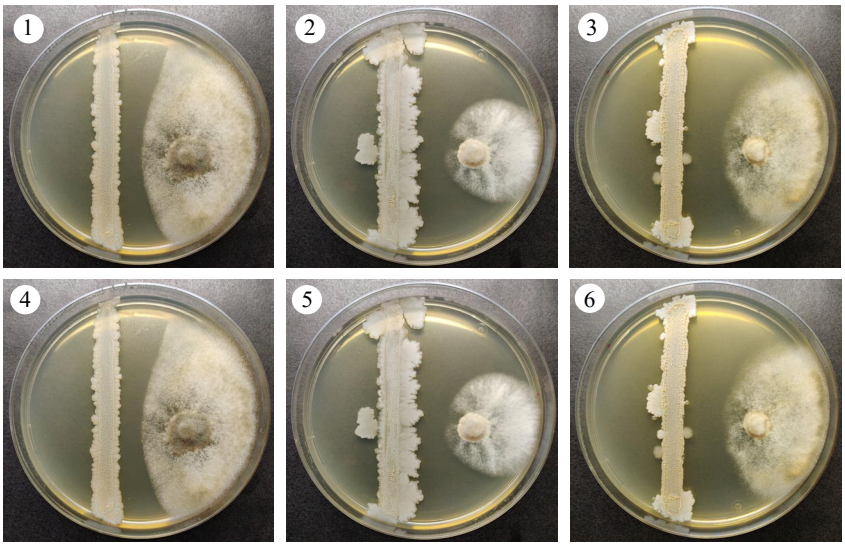
Isolation and identification of the fungus. After seven days on PDA, isolates 463-19 and 103-20 formed white to fluffy grey colonies with an average daily growth rate of 11.6 mm, pale brown to grey, smooth, ellipsoidal to globose, single conidia (9.7–9.4 µm) in mass on tree-like conidiophores. Four weeks post-inoculation, both isolates formed black, round sclerotia (0.5–3.0 mm) scattered unevenly throughout the colonies and immersed in the PDA. All the listed characteristics confirm the identification of two isolates. Five days post-inoculation, both isolates caused symptoms on inoculated leaves that resembled natural infection, from which original isolates were regularly recovered. Negative control leaves remained symptomless. The isolates 463-19 and 103-20 (accession Nos. MZ597847 and MZ597851) shared nucleotide (nt) sequence similarity of 100%, while BLAST analysis revealed the nt sequence similarity of 99.46–100% with over 100 sequences of *B. cinerea* from different parts of the world and different host plants. Isolate 463-19 had 99.82% sequence similarity with sequence of *B. cinerea* isolate KU992694 from a tomato from Malaysia, while isolate 103-20 had 100% sequence similarity with sequence of *B. cinerea* isolate MN158622 from *Fragraea ceilanica* from China.

Isolation and preliminary screening of antagonists. A total of 666 Gram-positive bacterial isolates were selectively isolated based on macroscopic morphological characteristics from the soil of various agricultural plants. All isolates had white, round, or oval colonies with irregular edges. Under the microscope at 500× magnification, the isolates had rod-shaped cells often arranged in pairs or chains and contained endospores. Based on cell and colony morphological characteristics, the isolates were identified as *Bacillus* spp. Based on the preliminary dual culture screening test, all 666 isolates were tested against *A. dauci*, and 77 isolates with the highest PGI value were selected (results not shown). These 77 isolates were further dual culture tested against *F. tricinctum* (accession No. MH496030.1) and *F. proliferatum* (accession No. MH496028.1) (results not shown), from which eight perspective isolates were chosen for further research.

Dual culture test. In the *in vitro* screening, the eight bacterial isolates were able to inhibit the mycelial growth of both *B. cinerea* isolates by resulting in an inhibition zone of the fungal growth. The isolates showed a negative effect on the development of *B. cinerea* isolates in the dual culture test with relatively lower values, and the extent of inhibition was different among the isolates, as shown in the Figure 2.

Among them, the highest antagonistic activity was observed by isolate Bac 20.10 with PGI of 80% and inhibition zone of 18.66 ± 2.30 mm against *B. cinerea* isolate 463-19, and with PGI of 77.77% and inhibition zone of 17.33 ± 0.57 mm against *B. cinerea* isolate 103-20 (Table 1).

The inhibition rate for isolate Bac 28.3 was 77.22%, and against *B. cinerea* isolates 463-19 and 103-20 was 73.33%. Finally, isolate Bac 21/IV achieved PGI of 77.22% and 69.44% against *B. cinerea* isolates 463-19 and 103-20, respectively.



1 – *B. amyloliquefaciens* Bac 21/IV, 2 – *B. subtilis* Bac 20.10, 3 – *B. amyloliquefaciens* Bac 28.3 against *Botrytis cinerea* isolate 463-19; 4 – *B. amyloliquefaciens* Bac 21/IV, 5 – *B. subtilis* Bac 20.10, 6 – *B. amyloliquefaciens* Bac 28.3 against *B. cinerea* isolate 103-20

Figure 2. Antifungal activity of the isolates of *Bacillus* spp.

Table 1. Bacterial isolates used and antifungal activity isolates against two *Botrytis cinerea* isolates

Bacterial isolate	*Bacillus* species	Location, soil source	*B. cinerea* 463-19		*B. cinerea* 103-20		NCBI accession number
			PGI % ± SD	IZ mm ± SD	PGI % ± SD	IZ mm ± SD	
Bac 18/IV	*B. subtilis*	Vrbas, walnut orchard	50.00 d ± 1.15	1.20 b ± 2.30	53.33 d ± 5.77	2.33 c ± 4.04	MT559805
Bac 57.2	*B. subtilis*	V. Šiljegovac, unknown	51.11 d ± 4.81	1.33 b ± 3.46	55.55 d ± 5.09	4.00 bc ± 1.15	MT559804
Bac 18.8	*B. pumilus*	S. Karlovci, hazelnut orchard	52.77 d ± 1.92	2.00 b ± 2.30	61.66 cd ± 6.00	8.00 bcd ± 3.60	MT559555
Bac 65.5	*B. subtilis*	Veliki izvor, walnut orchard	65.55 a ± 0.96	9.66 a ± 0.57	62.22 bcd ± 3.46	8.00 bcd ± 2.64	MT559809
Bac 5.2	*B. subtilis*	Lipar, soybean field	68.33 ac ± 6.00	12.33 ac ± 4.16	67.22 abc ± 3.46	11.66 abd ± 2.51	MT559524
Bac 21/IV	*B. amyloliquefaciens*	Družetić, raspberry field	77.22 bc ± 4.81	17.00 c ± 2.30	69.44 abc ± 1.92	12.00 ad ± 1.73	MT559810
Bac 28.3	*B. amyloliquefaciens*	B. Palanka, hazelnut orchard	77.22 bc ± 2.54	15.33 ac ± 1.00	73.33 ab ± 0.00	15.00 ad ± 0.00	MT559807
Bac 20.10	*B. subtilis*	S. Karlovci, vineyard	80.00 b ± 4.40	18.66 c ± 2.30	77.77 a ± 1.92	17.33 a ± 0.57	MT559808
		Average	66.66 ± 3.63	9.54 ± 2.56	65.06 ± 4.02	9.79 ± 2.03	

Note. PGI – percentage of growth inhibition, IZ – inhibition zone; mean values of fungal growth inhibition and zones of inhibition with standard deviation (SD) are shown; the values followed by the same letter in the columns did not differ significantly ($p < 0.00$) according to ANOVA.

Molecular identification of bacteria. To further identify, eight antagonistic isolates 16S rDNA was sequenced and analysed using the online BLAST search in the GenBank database (Table 2).

The phylogenetic tree showed that all eight isolates belong to the genus *Bacillus*. Isolate Bac 18.8 is the closest to the reference strain (X60637.1); isolates Bac 65.5, Bac 5.2, Bac 18/IV, and Bac 57.2 are clustered together. One reference strain (JQ900623.1) and three isolates (Bac 21/IV, and Bac 20.10, and Bac 28.3) do not form any clusters and show distance in a phylogenetic tree (Figure 3).

Tomato seed treatment. The percentage of total germination of the tomato seeds treated with *Bacillus* isolates ranged from 68.66% to 86.16% (Table 3). Two isolates of *B. amyloliquefaciens*, Bac 21/IV and Bac 28.3, showed an increased percentage of total germination by 86.16 ± 4.35% and 85.66 ± 1.71%, respectively, compared to the control (82.66 ± 9.29%).

Tested isolates showed a different effect on morphological parameters of tomato seedlings (Table 4).

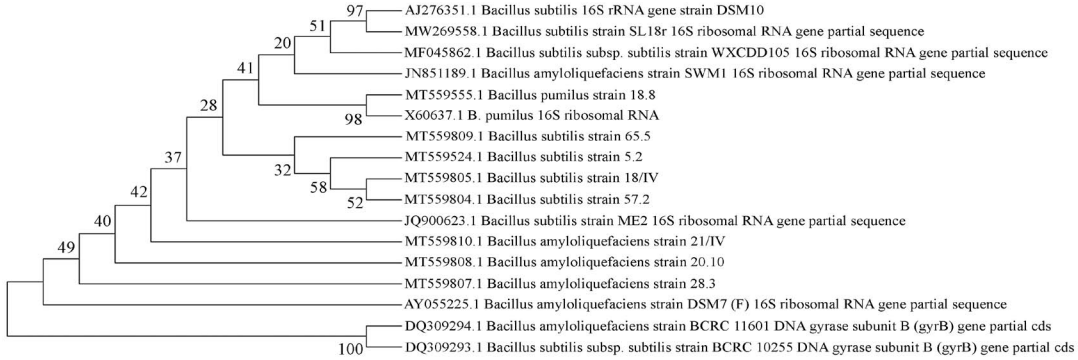
The most substantial increase in the shoot length was measured after seed treatment with isolate Bac 65.5 (7.48 *vs* 5.96 cm for the control), which corresponded to an increase of 25.50%. The largest increase in the root length was obtained by isolate Bac 57.2, i.e., 3.27 cm for the treatment *vs* 2.78 cm for the control, corresponding to 17.62%. Five isolates Bac 28.3, Bac 18.8, Bac 5.2, Bac 65.5, and Bac 57.2 showed a significant increase in the shoot FM from 0.093 to 0.099 g corresponding to an increase of 12.90% to 22.22%, respectively, compared to the control. Four isolates Bac 18.8, Bac 57.2, Bac 20.10, and Bac 65.5 increased the root FM from 0.080 to 0.082 g correlating to an increase of 2.56% to 5.12%, respectively, compared to the control. No significant changes were observed in promoting the shoot DM and root DM, compared to the control.

Effect of bacteria on the growth of tomato plants. In the greenhouse experiment, three selected isolates and their mixture positively affected all measured PGP parameters (Figure 4). However, the best results were obtained in a treatment containing a mixture of three *Bacillus* isolates. This consortium increased the shoot length (71.80), the shoot FM (32.68), and the shoot DM (4.78), which corresponds to an increase of 18.87%, 39.18%, and 125.47%, respectively, compared to the control. Isolate Bac 28.3 increased the root length (23.60), the root FM (2.37), and the root DM (0.19), and the number of internodes (8.40) corresponding to an increase of 50%, 70.50%, 90%, and 31.25%, respectively, compared to the control. An increase in the number of leaves was observed, when plants were treated with isolate Bac 20.10 (81.40), compared to the control (61.20), equivalent to an increase of 33%.

Biological efficacy of bacterial isolates. The results of the current experiment concluded that using a dose of 55 ml, individual bacterial isolates showed an effective reduction in

Table 2. Antagonistic isolates of bacteria and sequence analysis using the online BLAST search in the GenBank database

Bacterial isolate		Percentage of similarity	Compared strain in GenBank database
B. subtilis	Bac 18/IV	99.62%	*B. subtilis* BY45 (MN133892.1) isolated from rice rhizosphere in Pakistan
B. subtilis	Bac 57.2	99.44%	*B. subtilis* (KY820920.1) isolated from cotton rhizosphere in Pakistan
B. subtilis	Bac 65.5	99.63%	
B. pumilus	Bac 18.8	99.14%	*B. pumilus* QT-79 (MT065805.1) isolated in China
B. subtilis	Bac 5.2	100%	*B. subtillis* H34 (KU922339) isolated from air in China
B. subtilis	Bac 20.10	99.44%	*B. subtilis* bacs5 (MN252546.1)
B. amyloliquefaciens	Bac 28.3	98.92%	*B. amyloliquefaciens* W36 (MN922613.1) isolated from activated sludge in China
B. amyloliquefaciens	Bac 21/IV	99.4%	



Note. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (3. 000 replicates) are shown next to the branches; the evolutionary distances were computed using the Maximum composite likelihood estimation (MCLE) method and are in the units of the number of base substitutions per site; evolutionary analyses were conducted by the software MEGA X.

Figure 3. Phylogenetic relationship of eight identified *Bacillus* isolates based on 16S rDNA gene sequences compared with the reference sequences from GenBank

Table 3. Effect of *Bacillus* spp. on total germination (%) of tomato seeds

Bacterial isolate	Total germination ± SD	*p*-value
Bac 18.8	68.66 a ± 2.06	0.0254
Bac 20.10	74.66 a,c ± 4.99	0.1798
Bac 5.2	77.66 a,b,c ± 2.06	0.3335
Bac 65.5	74.33 a,c ± 3.70	0.1462
Bac 18/IV	82.00 b,c ± 3.30	0.8966
Bac 57.2	78.00 a,b,c ± 3.27	0.3796
Control	82.66 b,c ± 9.29	–
Bac 28.3	85.66 b ± 1.71	0.5484
Bac 21/IV	86.16 b ± 4.35	0.5187

Note. Mean values of fungal growth inhibition and zones of inhibition with standard deviation (SD) are shown; the values followed by the same letter in the columns did not differ significantly ($p < 0.00$) according to ANOVA.

the severity of the grey mould in tomatoes, compared to the negative control (Figure 5). Two isolates Bac 28.3 and Bac 20.10 reduced the severity of the disease by 30.63% and 27.42%.

Also, it was observed that the same isolates reduced the severity of the grey mould in tomatoes by 14% and 10%, respectively, compared to the fungicide Switch 62.5 WG. Thus, both isolates Bac 28.3 and Bac 20.10 have a good biocontrol effect on *B. cinerea*. Individual bacterial isolates and their mixture in treatments with a dose of 30 ml effectively reduced the severity of grey mould, compared to the negative control, but also showed lower efficiency in suppressing grey mould, compared to treatments with a dose of 55 ml. In treatments with a dose of 30 ml, individual bacterial isolates Bac 21/IV, and Bac 20.10 and their mixture showed lower efficacy, compared to the fungicide Switch 62.5 WG.

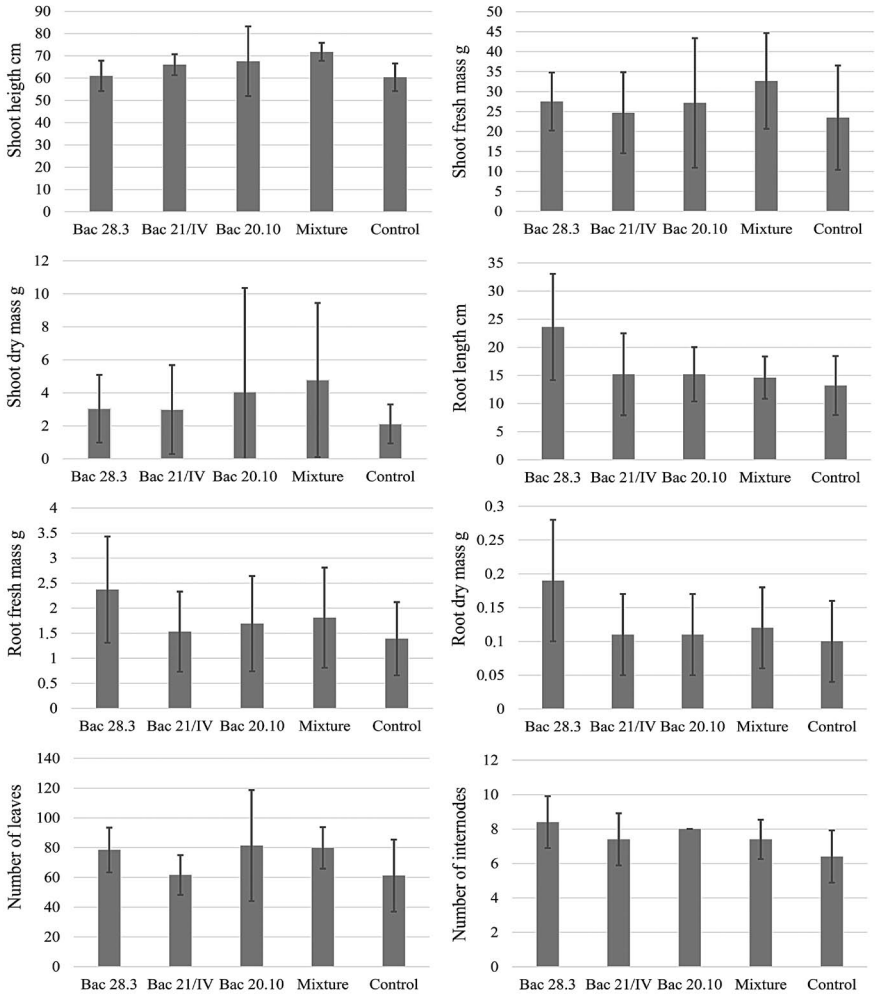
Table 4. Effect of *Bacillus* spp. isolates on the morphological parameters of tomato seedlings

Bacterial isolate	Shoot length cm ± SD	Root length cm ± SD	Fresh mass of shoot g ± SD	Dry mass of shoot g ± SD	Fresh mass of root g ± SD	Dry mass of root g ± SD
Bac 18/IV	5.19 b ± 0.50	2.07 ab ± 0.62	0.019 a ± 0.001	0.003 a ± 0.001	0.009 a ± 0.003	0.002 b ± 0.001
Bac 57.2	6.18 ab ± 1.95	3.27 a ± 1.39	0.099 c ± 0.003	0.071 bc ± 0.004	0.080 b ± 0.004	0.071 b ± 0.004
Bac 18.8	4.85 b ± 0.84	1.90 ab ± 0.08	0.094 c ± 0.001	0.073 bc ± 0.004	0.080 b ± 0.003	0.070 b ± 0.004
Bac 65.5	7.48 a ± 0.13	2.04 ab ± 0.64	0.098 c ± 0.001	0.072 bc ± 0.001	0.082 b ± 0.001	0.072 b ± 0.001
Bac 5.2	5.87 ab ± 0.52	1.91 ab ± 0.45	0.095 c ± 0.003	0.072 bc ± 0.003	0.079 b ± 0.003	0.070 b ± 0.003
Bac 21/IV	4.70 b ± 0.54	2.62 ab ± 0.67	0.091 bc ± 0.005	0.072 c ± 0.033	0.054 b ± 0.036	0.047 b ± 0.033
Bac 28.3	5.69 ab ± 0.28	1.59 b ± 0.27	0.093 c ± 0.002	0.072 bc ± 0.005	0.077 b ± 0.005	0.069 b ± 0.005
Bac 20.10	4.84 b ± 0.63	2.57 ab ± 0.56	0.090 bc ± 0.003	0.067 c ± 0.004	0.081 b ± 0.004	0.072 b ± 0.004
Control	5.96 ab ± 0.62	2.78 ab ± 0.51	0.081 b ± 0.002	0.106 b ± 0.001	0.078 b ± 0.001	0.388 a ± 0.001
Average	5.64 ± 0.66	2.30 ± 0.57	0.27 ± 0.00	0.06 ± 0.00	0.38 ± 0.04	0.28 ± 0.00
p value	0.0014	0.0332	0.00	0.00	0.00	0.0083

Note. The average morphological parameters of tomato seedlings with standard deviation (SD) and the *p*-value according to ANOVA are shown.

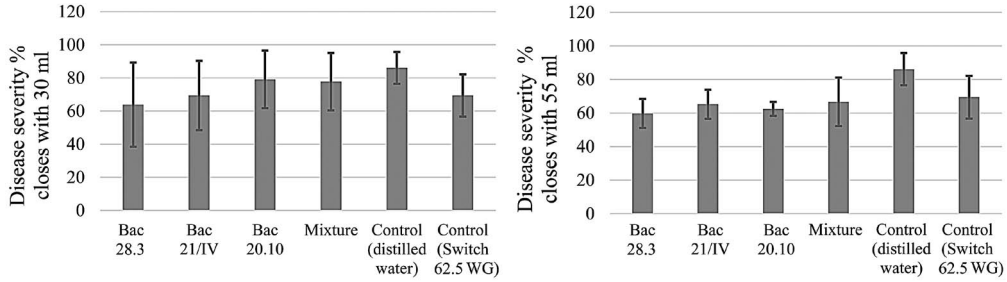
Discussion

Many *Bacillus* species have been identified as PGP (Saxena et al., 2019) and biocontrol agents (Bolivar-Anillo et al., 2021). In the last few decades, interest in the genus *Bacillus* is a consequence of the use of increasing amounts of pesticides, mineral fertilizers, and the emergence of resistance of pathogens to some active ingerdients of pesticides. The isolates were isolated from the soil, where the genus *Bacillus* is most prevalent. For further studies, preliminary dual culture screening tests selected eight promising isolates out of 666 isolated isolates. All eight isolates showed antifungal activity against two *B. cinerea* isolates with PGI values ranging from 50% to 80%. These results confirm that eight effective isolates by preliminary screening tests were selected. With much higher PGI values, this finding proved promising, compared to similar studies, in which inhibition of pathogen growth was measured from 27% to 53% (Kefi et al., 2015). However, the Ge et al. (2016) indicates that *Bacillus methylotrophicus* strain NKG-1 showed an inhibition ratio of 81.67% versus *B. cinerea*, compared to the control. Also, *B. subtillis* strain WXCDD105 had an inhibition rate of 71.57% according to *B. cinerea* in a dual culture screening test (Wang et al., 2018).



Note. The average values shown in columns with standard deviation (SD) as error bars on morphological parameters on tomato plants in the greenhouse at the pre-flowering stage.

Figure 4. Effect of three *Bacillus* spp. isolates on the morphological parameters of tomato plants



Note. The average values shown in columns with standard deviation (SD) as error bars on disease severity with 30 and 55 ml doses on tomato plants in the greenhouse at the pre-flowering stage.

Figure 5. Effect of three *Bacillus* spp. isolates and their mixture on tomato grey mould disease severity (%)

A comparison of our PGI readings with the previous three studies (Kefi et al., 2015; Ge et al., 2016; Wang et al., 2018) shows the strong antifungal activity of three isolated strains and indicates their potential in the biological control of *B. cinerea* in *in vivo*. The zone of inhibition can be a good indicator of the production of antifungal compounds by antagonists such as antibiotics, volatile ingredients, or enzymes (Köhl et al., 2019). The isolates showed a zone of inhibition towards both isolates *B. cinere*a from 1.20 to 18.66 mm. Studies are underway to discover the mode of action behind the antagonistic action of isolated bacteria transmitted by the soil against *B. cinerea*. In addition, it is important to discover the principle of biocontrol to exclude biological control agents that produces antimicrobial metabolites with the potential to interfere with antibiotics in human and veterinary medicine.

The highest percentage of total germination of tomato seeds was achieved by two isolates Bac 28.3 and Bac 21/IV ranging from 85.66% to 86.16%, respectively, compared to the control. The results obtained from the application of two isolates are important, because germination and the initial growth of plants at the first stages of tomato development are essential. Early uniformity of plants ensures a good set and ensures higher yields both in the open field and in the greenhouse. Also, the result of stimulating the germination of tomato seeds can be stimulated by the role of the plant hormone gibberellin, which has a mechanism of stimulating the synthesis of hydrolytic enzymes that helped the initial germination. Similar studies described improved tomato seed germination with inoculation of *Pseudomonas fluorescens*, different *Bacillus* strains, and *Brevibacillus brevis* from 81% to 93% (Konappa et al., 2020).

After the end of total germination of tomato seeds, it was concluded that isolate *B. subtillis* Bac 65.5 increased the shoot length by 25.50% and isolate *B. subtillis* Bac 57.2 increased the root length by 17.62%, compared to the control.

Five isolates Bac 28.3, Bac 18.8, Bac 5.2, Bac 65.5, and Bac 57.2 showed a significant increase in the shoot FM as much as 22%, compared to the control. Four isolates Bac 18.8, Bac 57.2, Bac 20.10, and Bac 65.5 increased the root FM correlating to an increase of 2.56% to 5.12%, respectively, compared to the control. The antagonistic *B. subtilis* strain WXCDD105 stimulated both the seed germination and seedling growth of tomatoes. Using the fermentation liquid of the mentioned strain (10^8 CFU ml^{-1}) for seed treatment increased the germination rate and root length (Wang et al., 2018).

Results of the greenhouse experiment indicate that by applying a mixture of three isolates, the largest height of the shoot, the shoot FM, and the shoot DM were obtained. Isolate Bac 20.10 increased the number of tomato leaves. Using isolate Bac 28.3 significantly increased the root length, the root FM, the root DM, and the number of internodes. This indicates that in current experiment, three bacteria isolated from the soil could promote plant growth and yield through the mechanism of biostimulation and the production of various plant hormones. The isolate Bac 28.3 stimulated the root growth in tomato plants, and this ability may be related to the production of the phytohormone indole-acetic acid (IAA). This can enable the plant's tolerance of abiotic factors, especially moisture stress, because increasing the length of the roots promotes plant growth during stress. Also, the IAA plays an important role in plant adaptation to the impact of heavy metals and salinity stress (Wani et al., 2016). Earlier reports by Xu et al. (2016) show that *Bacillus* strains SG08-09 and SG09-12 isolated from soil stimulated the growth of tomato plants by 10% to 42% and reduced the intensity of the disease caused by grey mould by 66%.

Using strain NKG-1 in the greenhouse experiment, the FM of tomato plants (27.4%), the length of the shoot (12.5%), and the length of the roots (57.7%) significantly increased, compared to the control. The use of *Bacillus* strains as potent agents for biological control has been reported in many studies (Castaldi et al., 2021; Salehin et al., 2021).

The results of the current experiment suggest that isolates Bac 28.3 and Bac 20.10 affect the reduction of grey mould in the greenhouse when applying a bigger (55 ml) dose, which is extremely important for indoor cultivation of tomatoes. Also, in our experiment, the effect of two isolates on the reduction of tomato grey mould may be associated with an increase in plant immunity referred to as induced systemic resistance (ISR). It is one of the important mechanisms for protecting plants from a wide range of pathogens (Shafi et al., 2017), which should be studied in further experiments. Wang et al. (2018) report that strain WXCDD105 isolated from the rhizosphere of tomatoes has shown control efficacy (74.70%) in the control of *B. cinerea*. *B. subtilis* strain QST713 produces lipopeptides that exhibit a fungicidal effect and is used to treat cotton seeds, legumes, and other species in the control of *Fusarium* sp., *Alternaria* sp., and *Aspergillus* sp. Foliar is also applied in the control of *B. cinerea* on blue eggplant and tomato (Tomlin, 2006). By using strain NKG-1 in a preventive spray to control *B. cinerea* on tomato leaves, biocontrol efficacy was 60%. Strain NKG-1 also promoted the growth of tomato seedlings in greenhouse and field conditions (Ge et al., 2016).

To our knowledge, in Serbia, the effects of antagonistic bacteria of the genus *Bacillus* on the occurrence of grey mould in tomatoes have not been studied so far. However, Zdravković et al. (2015) studied the influence of *Pseudomonas* spp. and *Bacillus* sp. strain Q10 on phytopathogenic fungi *F. acuminatum*, *B. cinerea*, and *A. niger* isolated from cucumber plants.

This research shows that local isolates of *Bacillus* from the soil have a strong biocontrol potential and could be used to control grey mould and stimulate tomato plant growth. Further research should focus on field testing to determine their efficacy as biofungicides and biostimulants. The results confirm the need for continuous isolation of new isolates from the soil to find effective isolates, as even in our experiment, only eight selected isolates out of 666 showed antifungal activity against this significant tomato pathogen.

Conclusions

1. The isolates of *Bacillus subtilis* Bac 20.10, *B. amyloliquefaciens* Bac 28.3, and *B. amyloliquefaciens* Bac 21/IV showed very strong antifungal activity against both isolates of grey mould (*Botrytis cinerea* Pers.).

2. Two isolates Bac 28.3 and Bac 21/IV affected total germination of tomato seeds by 85.66% and 86.16%, respectively.

3. Isolate *B. subtillis* Bac 65.5 increased the tomato shoot length, and isolate *B. subtillis* Bac 57.2 increased the root length. Five isolates Bac 28.3, *B. pumilus* Bac 18.8, *B. subtillis* Bac 5.2, Bac 65.5, and Bac 57.2 showed a significant increase in the fresh mass of the shoot, and four isolates Bac 18.8, Bac 57.2, 20.10, and Bac 65.5 increased the fresh mass of the root.

4. Two isolates Bac 28.3 and 20.10 reduced the severity of *B. cinerea* in the greenhouse by 30.36% and 27.47%, respectively, and have a high biocontrol potential.

5. The results of our experiment showed that *Bacillus* spp. isolates isolated from the soil have a very strong potential to control *B. cinerea* of tomatoes and improve plant growth.

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Botrytis cinerea biokontrolė ir pomidorų augimo skatinimas iš dirvožemio išskirtais *Bacillus* izoliatais

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Santrauka

Kekerinis puvinys, kurį sukelia *Botrytis cinerea* Pers., yra viena žalingiausių augalų ligų visame pasaulyje. Apsaugai nuo pomidorų kekerinio puvinio dažniausiai naudojami cheminiai fungicidai. Dėl didėjančio atsparumo fungicidų veikliosioms medžiagoms tirta *Bacillus* genties bakterijų panaudojimo galimybė. Iš įvairių žemės ūkio paskirties dirvožemių buvo išskirti 666 izoliatai ir atliktas preliminarus dvigubos kultūros tyrimas su *Alternaria dauci*. Iš jų atrinkti 77 izoliatai, kurie tirti nuo *Fusarium tricinctum* ir *Fusarium proliferatum*. Remiantis preliminariais dvigubų kultūrų eksperimentų duomenimis, buvo atrinkti 8 izoliatai, turintys biologinį potencialą prieš du *B. cinerea* izoliatus. Įvertintas *Bacillus* spp. izoliatų poveikis valgomojo pomidoro (*Solanum lycopersicum* L.) sėklų daigumui ir daigų augimą skatinančiam poveikiui. Taikant molekulinį identifikavimą (16S rDNA) nustatyta, kad 5 bakterijų izoliatai buvo *Bacillus subtilis*, 2 izoliatai – *B. amyloliquefaciens* ir vienas izoliatas – *B. pumilus*. Panaudojus vidinio transkribuojamo tarpiklio (angl. *ITS region*) sritį, du grybų izoliatai iš pažeisto lapo ir pomidoro vaisiaus buvo identifikuoti kaip *B. cinerea*. Dvigubos kultūros eksperimento rezultatai parodė, kad 8 bakterijų izoliatai *B. cinerea* micelio augimą galėjo reikšmingai slopinti nuo 50 iki 80 %. Du izoliatai *B. amyloliquefaciens* 28,3 ir *B. amyloliquefaciens* 21/IV sėklų daigumą paskatino atitinkamai 85,66 ir 86,16 %, lyginant su kontroliniu variantu (82,66 %). Visi bakterijų izoliatai turėjo teigiamą įtaką daigų morfologiniams rodikliams. Šiltnamio eksperimente trys izoliatai – *B. subtillis* 20.10, *B. amyloliquefaciens* 28.3 bei *B. amyloliquefaciens* 21/IV – ir jų mišinys padidino visus pomidorų augimo skatinimo rodiklius. Izoliatai Bac 20.10 ir Bac 28.3 kekerinio puvinio paplitimą sumažino atitinkamai 27,47 ir 30,36 %.
Eksperimento rezultatai rodo, kad *Bacillus* spp. turi biokontrolės potencialą ir daro teigiamą įtaką pomidorų augalų augimui.

Reikšminiai žodžiai: priešgrybinis aktyvumas, *Bacillus* spp. *Botrytis cinerea*, biokontrolė, augalų augimo skatinimas.