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ANTIFUNGAL ACTIVITY OF INDIGENOUS *Bacillus* spp. ISOLATED FROM SOIL

ABSTRACT: Biocontrol using plant growth-promoting rhizobacteria (PGPR) represents an alternative approach to disease management, since PGPR are known to promote growth and reduce diseases in various crops. Among the different PGPR, members of the genus *Bacillus* are prefered for most biotechnological uses due to their capability to form extremely resistant spores and produce a wide variety of metabolites with antimicrobial activity. The objective of this research was to identify antagonistic bacteria for management of the plant diseases. Eleven isolates of *Bacillus* spp. were obtained from the soil samples collected from different localities in the Province of Vojvodina. The antifungal activity of bacterial isolates against five fungal species was examined using a dual plate assay. *Bacillus* isolates exhibited the highest antifungal activity against *Fusarium proliferatum, Fusarium oxysporum* f. sp. *cepae* and *Alternaria padwickii*, while they had the least antagonistic effect on *Fusarium verticillioides* and *Fusarium graminearum*. Molecular identification showed that effective bacterial isolates were identified as *Bacillus safensis* (B2), *Bacillus pumilus* (B3, B11), *Bacillus subtilis* (B5, B7) and *Bacillus megaterium* (B8, B9). The highest antagonistic activity was exhibited by isolates B5 (from 39% to 62% reduction in fungal growth) and B7 (from 40% to 71% reduction in fungal growth). These isolates of *B. subtilis* could be used as potential biocontrol agents of plant diseases.

KEYWORDS: *Bacillus*, biocontrol, *Fusarium*, *Alternaria*, antifungal activity, isolation, soil

INTRODUCTION

Fusarium and *Alternaria* species are among major pathogens that infect plants throughout the year at all growth stages and cause destructive and eco-

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nomically damaging diseases responsible for high yield reductions (James, 1981). Control of plant diseases is largely based on genetic resistance in host plants, cultural practices and synthetic pesticides (Lazarovits *et al*. 2014). Beside environmental impact and potential health risk related to the chemical pesticides application in agriculture, chemical control also creates imbalances in the microbial community, which may be unfavorable to the activity of beneficial organisms and lead to the development of resistant strains of pathogens (Aktar *et al*. 2009).

The need for alternative control strategies, particularly those involving biological control, has greatly increased over the past two decades. Biocontrol using plant growth-promoting rhizobacteria (PGPR) represents an alternative approach to disease management, since PGPR are known to promote growth and reduce disease in crops (Lugtenberg and Kamilova, 2009). The most common approach to biological control consists of selecting antagonistic microorganisms and developing a biological control product (Alabouvette *et al*. 2006).

Several antagonistic microorganisms have been tested for their ability to inhibit phytopathogenic fungi, including *Fusarium* and *Alternaria* species (Jain and Pandey, 2016; Li *et al*. 2017). Although some fungal antagonists showed effective inhibition, bacterial antagonists mainly from the genus *Bacillus* have shown by far the most promising results (Pane and Zaccardelli, 2015; Zalila-Kolsi *et al*. 2016). Due to their capability to form extremely resistant spores and produce a wide variety of metabolites with antimicrobial activity, members of the *Bacillus* genus are generally found in soil. *Bacillus* spp. strains inhibit pathogen growth primarily through the production of antibiotics, cell wall degrading enzymes, competition for nutrients and/or inducing systemic resistance (Lugtenberg *et al*. 2013).

The objective of this study was to isolate *Bacillus* spp. from soil and to examine their *in vitro* antifungal activity toward *Fusarium* and *Alternaria* species.

MATERIAL AND METHODS

Soil sample collection

Different soil samples were randomly collected from various parts of the Province of Vojvodina (northern Serbia). Several diverse locations were selected for the collection of soil samples, which included the rhizosphere of plants, agricultural and non-agricultural soils. Soil samples differed in their cropping and tillage history, physical and chemical properties. Samples were taken up to a depth of 20 cm. After removing approximately 3 cm of the soil surface, as well as large roots and stones, the remainder was passed through an autoclave-sterilized brass sieve with a 2 mm aperture size and then stored at 4 °C until further examination.

Isolation of Bacillus spp.

Soil dilutions were prepared with 1 gram of each soil sample suspended in 9 mL of 0.85% NaCl in sterile test tubes. A 0.1 ml aliquot of each dilution

(10^{-3}–10^{-6}) was spread aseptically on Nutrient Agar (NA) and incubated at 30 °C for 24 hour. After the incubation, colonies showing resemblance with *Bacillus* spp., roughly identified based on their morphology, were transferred and recultivated five times to obtain pure cultures. The bacterial isolates were characterized by their morphological and biochemical characteristics using standard methods (Jarak and Đurić, 2006).

Antifungal activity assay

Antifungal activity of *Bacillus* spp. isolates against five fungal isolates was tested *in vitro* using a dual plate assay (Zhao *et al.* 2010). In addition to three fungal isolates identified from garlic cloves (*Fusarium proliferatum*, *Fusarium verticillioides, Fusarium oxysporum* f. sp. *cepae*) (Ignjatov *et al.* 2016a), another two were isolated from seeds of soybean (*Fusarium graminearum*) (Ignjatov *et al.* 2016b) and rice (*Alternaria padwickii*). Bacterial isolates were grown for 24h in nutrient broth (NB) at 30 °C, while potato dextrose agar (PDA) was used for the cultivation of fungi. The mycelial plugs (6 mm in diameter) of each fungus were sampled from the 7-day-old cultures and aseptically transferred on the PDA, about 25 mm from the edge of each Petri dish. A broth culture of the tested bacteria was then streaked 30 mm away from the mycelial plugs in the same dish. The controls consisted of cultures of the tested fungi without the presence of *Bacillus* spp. isolates. All dual cultures and controls were incubated for 7 days at 25 °C. Antifungal activity assay was done in three repetitions for each treatment. The percent of growth inhibition (PGI) was calculated using the following formula: PGI (%) = [(KR-R1)/KR] x 100, where KR represents the fungal growth (measured in mm) in the control dishes, and R1 is the fungal growth in the treated dishes (Dimkić *et al.* 2015).

Molecular species identification

Bacillus isolates for DNA extraction were grown on NA plates for 24 h. DNA was extracted using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany), according to the manufacturer's recommendations. For the amplification of 16S rDNA gene fragments, primers fD1 (27F) (AGAGTTTGATCMTGGCTCAG) and rP3 (1492R) (TACGGYTACCTTGTTACGACTT) were used (Weisburg *et al.* 1991). The polymerase chain reaction (PCR) was done in 25-µl aliquots using S-thermal cycler (Eppendorf, Germany). The PCR reactions were performed with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50 °C primer annealing for 1 min, and 72 °C extension for 30 s, followed by a final extension step at 72 °C for 7 min. Amplicons were electrophoresed in 1.5% agarose gel (Invitrogen) with ethidium bromide. Purification and sequencing of the PCR-amplified DNA fragments were done in the company MACROGEN, Seoul, South Korea (http://dna.macrogen.com). FinchTV Version 1.4.0. was used for sequence analysis and nucleotide sequences were

filed in the GenBank Database at the National Center for Biotechnology Information (NCBI).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using software STATISTICA 12.6 (Statsoft, Tulsa, Oklahoma, USA). Means were separated using Tukey's HSD (honest significant difference) test at the $P < 0.05$ level.

RESULTS AND DISCUSSION

This study confirmed the presence of *Bacillus* spp. in soil samples collected from diverse locations in Vojvodina. Because of their fast growth and ability to sporulate under unfavorable conditions, *Bacillus* spp. isolates are attractive candidates for application as biocontrol agents. Analysis of antagonistic activity of newly-isolated strains against phytopatogenic fungi showed that *Bacillus* spp. isolates exhibited the highest antifungal activity against *Fusarium proliferatum*, *Fusarium oxysporum* f. sp. *cepae* and *Alternaria padwickii*, while they had the least antagonistic effect on *Fusarium verticillioides* and *Fusarium graminearum* (Table 1). The highest antagonistic activity was exhibited by isolates B5 (39–62%) and B7 (40–71%) which inhibited the growth of all tested fungal isolates except *F. verticillioides* (Figure 1). Antagonistic effect toward *F. proliferatum* and A. *padwickii* was also observed through confrontation with the isolates B2 (35–42%), B3 (31–38%), and B11 (3–37%). Isolate B8 exhibited antifungal activity against *F. proliferatum* (45%), while isolate B9 inhibited the growth of *F. proliferatum* (42%) and *Fusarium oxysporum* f. sp. *cepae* (33%). Antifungal activity of isolates B1, B4 and B10 was not detected. Significant variability within the same fungal species was found in different isolates, except for *F. graminearum*. The results obtained in this study showed different sensitivity of fungal species tested. Different degrees of fungal inhibition by individual *Bacillus* spp. isolates were also observed.

Similar findings about fungal growth inhibition and possible application of *Bacillus* spp. isolates as biocontrol agents have been found in numerous studies. Isolates of *Bacillus* spp. showed strong *in vitro* inhibition, as well as plant disease suppression of *Fusarium*, *Alternaria*, *Rhizoctonia*, *Aspergillus*, *Cryphonectria*, *Phytophthora*, etc. (Mnif and Ghrib, 2015). Dimkić *et al.* (2015) reported that *Fusarium* species were more resistant to *Bacillus* spp. isolates, while *Alternaria* were among the most sensitive fungi tested. *Bacillus* spp. isolates efficient in biocontrol of various plant pathogens have been mostly found positive for production of lytic enzymes and lipopeptide antibiotics (Abdallah *et al.* 2017). Beside their role in biocontrol, *Bacillus* species can enhance plant nutrition and promote plant growth and development via associative nitrogen fixation, phosphate solubilization, production of phytohormones and siderophores, or enzymatic activities (Borriss, 2011). Therefore, the use of *Bacillus* spp.

as biopesticides and biofertilizers is a promising approach which may result in reduced application of chemical pesticides and fertilizers and improved quality of agricultural products.

Table 1. Antifungal activity of *Bacillus* spp. isolates

Isolate	*Fusarium proliferatum*	*Fusarium verticillioides*	*Fusarium oxysporum* f. sp. *cepae*	*Fusarium graminearum*	*Alternaria padwickii*
PGI (%) ± SD					
B1	nd	nd	nd	nd	nd
B2	41.57 ± 2.96 b	nd	nd	nd	35.29 ± 1.18 c
B3	30.98 ± 2.72 c	nd	nd	nd	38.04 ± 0.68 c
B4	nd	nd	nd	nd	nd
B5	61.57 ± 2.96 a	nd	46.27 ± 4.45a	39.20 ± 1.01 a	58.04 ± 2.72 b
B6	nd	nd	nd	nd	nd
B7	64.71± 2.04 a	nd	42.94 ± 2.61 a	39.80 ± 0.68 a	71.37 ± 2.96 a
B8	44.71 ± 3.11 b	nd	nd	nd	nd
B9	41.96 ± 2.45 b	nd	33.33 ± 1.80 b	nd	nd
B10	nd	nd	nd	nd	nd
B11	3.14 ± 0.96 d	nd	nd	nd	36.83 ± 0.68 c

Mean values of fungal growth inhibition (n = 3) with standard deviation (SD) are shown. Values followed by the same letter within columns are not significantly different ($P < 0.05$), according to Tukey's HSD test. PGI: percent of growth inhibition; nd: not detected.

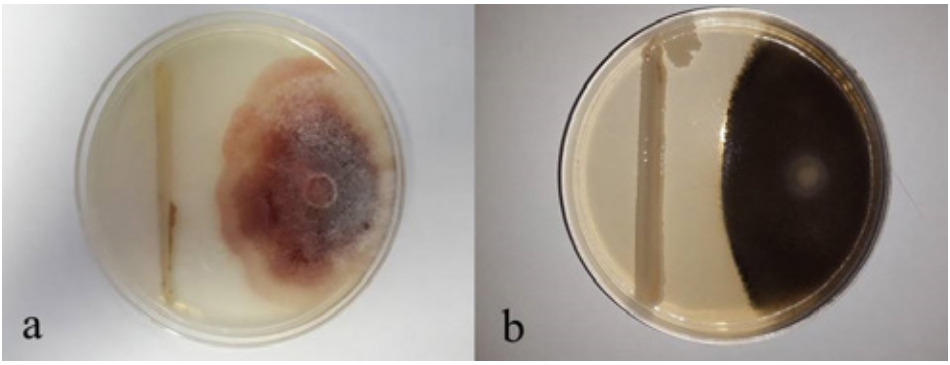

Figure 1. Antifungal activity of *Bacillus subtilis* B7 against *Fusarium proliferatum* (a) and *Alternaria padwickii* (b)

By comparing the sequences with the *Bacillus* ID-database, bacterial isolates effective in fungal growth inhibition were identified as *Bacillus safensis* (B2), *Bacillus pumilus* (B3, B11), *Bacillus subtilis* (B5, B7) and *Bacillus megaterium* (B8, B9). Non-effective isolates were identified as *Lysinibacillus fusiformis* (B1, B4, B10) and *Bacillus cereus* (B6) (Table 2).

Table 2. Isolates of *Bacillus* species from soil in Vojvodina (Bjelić *et al*. 2016)

Isolate	Isolation source	Locality	*Bacillus* species	NCBI
B1	Agricultural soil	Rumenka	*Lysinibacillus fusiformis*	KU953922
B2	Non-agricultural soil	Banatski Dvor	*Bacillus safensis*	KU953932
B3	Rhizosphere (wheat)	Bukovac	*Bacillus pumilus*	KU953923
B4	Non-agricultural soil	Petrovaradin	*Lysinibacillus fusiformis*	KU953924
B5	Rhizosphere (sunflower)	Bački Petrovac	*Bacillus subtilis*	KU953925
B6	Non-agricultural soil	Šangaj	*Bacillus cereus*	KU953926
B7	Rhizosphere (maize)	Rimski Šančevi	*Bacillus subtilis*	KU953927
B8	Rhizosphere (pepper)	Rimski Šančevi	*Bacillus megaterium*	KU953928
B9	Rhizosphere (alfalfa)	Perlez	*Bacillus megaterium*	KU953929
B10	Non-agricultural soil	Pančevo	*Lysinibacillus fusiformis*	KU953930
B11	Forest soil	Vršačka kula	*Bacillus pumilus*	KU953931

Bacillus-based plant disease biocontrol products usually contain one or two strains which belong to species of *B. subtilis*, *B. licheniformis*, *B. pumilus* and *B. amyloliquefaciens* (Berg, 2009). In this study, the strongest and broadest antagonistic activity against all tested fungi was exhibited by isolates of *B. subtilis*. High genetic heterogeneity of different *Bacillus* species, particularly *B. subtilis* allows to suggest that search and identification of new strains from different sources may expand the number of practically important strains and improve our understanding of mechanisms involved in antagonistic interactions (Mardanova *et al.* 2017).

CONCLUSION

This study confirmed that most of the isolates of *Bacillus* spp. from the soil were found positive for antifungal activity by *in vitro* test. Significant variability within the tested fungal species was found in different isolates. The most effective isolates, identified as *Bacillus subtilis* (B5 and B7), could be used as potential biocontrol agents of plant diseases. Further selection of these isolates through greenhouse and field trials will be necessary in order to establish their efficiency as biopesticides in different crops.

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АНТИФУНГАЛНА АКТИВНОСТ ПРИРОДНИХ *Bacillus* spp. ИЗОЛАТА ИЗ ЗЕМЉИШТА

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РЕЗИМЕ: Биоконтрола фитопатогена представља алтернативу примени пестицида, с обзиром да бактерије означене термином PGPR (Plant Growth Promoting Rhizobacteria) стимулишу биљни раст и штите биљке од болести. Захваљујући способности да формирају веома резистентне ендоспоре и продукују широк спектар антимикробних супстанци, врсте рода *Bacillus* су веома заступљене у земљишту и погодне за примену у биотехнологији. Циљ истраживања био је да се утврди антифунгална активност једанаест *Bacillus* spp. изолата из земљишта с различитих локалитета у Војводини. Способност бактеријских изолата да инхибирају раст пет изолата гљива испитана је методом двојне култивације. Изолати *Bacillus* spp. испољили су највећу антифунгалну активност према *Fusarium proliferatum, Fusarium oxysporum* f. sp. *cepae*, и *Alternaria padwickii*, док је најмањи антагонистички ефекат утврђен према *Fusarium verticillioides* и *Fusarium graminearum*.

Ефективни изолати идентификовани су као *Bacillus safensis* (B2), *Bacillus pumilus* (B3, B11), *Bacillus subtilis* (B5, B7) и *Bacillus megaterium* (B8, B9). Највећу антифунгалну активност испољили су изолати *B. subtilis* B5 (39–62%) и B7 (40–71%). Ови изолати могу се користити као потенцијални агенси за биолошку контролу биљних болести.

КЉУЧНЕ РЕЧИ: *Bacillus*, биоконтрола, *Fusarium*, *Alternaria*, антифунгална активност, изолација, земљиште