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ORIGINAL SCIENTIFIC PAPER

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SCREENING OF *Bacillus* spp. AS POTENTIAL BIOCONTROL AGENTS AGAINST SUNFLOWER PATHOGENS

ABSTRACT: *Bacillus* spp. are well known to protect plants from seed or soil-borne pathogens by the synthesis of various metabolites with antimicrobial activity, such as hydrolytic enzymes and antibiotics. This study aimed to select the most effective *Bacillus* spp. from a group of ten antagonistic strains by antifungal activity assay. *Bacillus* strains were primarily isolated from the soil and identified as *B. safensis*, *B. pumilus* and *B. subtilis* by 16S rDNA sequencing. The four analyzed fungi: *Macrophomina phaseolina*, *Alternaria alternata*, *Cladosporium cladosporoides*, and *Sclerotinia sclerotiorum*, were obtained from sunflower seeds and identified using PCR analysis and primers specific for ITS region. The antifungal activity of bacterial strains was examined in a dual plate assay. *Bacillus* spp. demonstrated the highest antagonism against *S. sclerotiorum*, followed by *C. cladosporoides*, *M. phaseolina*, and *A. alternata*, with an average percentage of growth inhibition (PGI) of 77%, 70%, 64% and 59%, respectively. Overall, *Bacillus* spp. included in this study demonstrated a rather strong biocontrol potential, although the effect of particular strain varied depending on the tested fungi. The highest antagonistic effect toward *M. phaseolina* and *A. alternata* was exhibited by *B. safensis* B2 and *B. pumilus* B3. *B. pumilus* B11 and *B. subtilis* B32 were the most efficient against *C. cladosporoides*, whereas *B. pumilus* B3 and *B. subtilis* B7 had the highest antifungal activity against *S. sclerotiorum*. Findings point to the fact that the most effective *Bacillus* spp. could be used as potential biocontrol agents for improving plant health and productivity.

KEYWORDS: *Alternaria*, antifungal activity, *Bacillus*, biocontrol, *Cladosporium*, *Macrophomina*, *Sclerotinia,* sunflower

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INTRODUCTION

Sunflower is one of the most important industrial crops in the world, cultivated in more than 70 countries, with a total production of 50 million tons and average yield of 1.8 tons per hectare (FAOSTAT, 2020). In Serbia, sunflower is grown on 230,000 hectares, with total production of about 700,000 tons and average yield of 3.1 t/ha (Statistical Yearbook of RS, 2021). Plant diseases are the main limiting factors in sunflower production (Tančić Živanov et al., 2021). So far, more than 30 different economically important sunflower pathogens have been identified worldwide (Škorić, 2016). Phytopathogenic fungi are major pathogens that infect sunflower and cause even 80% diseases responsible for high yield reductions, which, depending on environmental conditions, may range from 30% to 50% (Jurković and Ćosić, 2004).

Due to environment and health issues related to the chemical pesticides, efficient management strategy should be based on preventive measures. It is important to identify pathogen, limit its spread and conditions favorable for a disease. Disease control implies cultural, physical, biological and chemical measures such as site selection, sanitation, soil, rotation and water management, using of resistant varieties and healthy seeds, as well as promotion of plant growth and health through proper fertilization and biological control (Poleatewich, 2018). Biological control is defined as the use of beneficial organisms or biological control agents to mitigate the negative effects of plant pathogens (Lazarovits et al., 2014). The most common approach to biological control involves isolation and identification of biocontrol agents, evaluation of biocontrol potential of strains in laboratory, greenhouse and field, bioprocess engineering of selected strains, and development of biopesticides (Poleatewich, 2018).

Bacillus species are among the most investigated biocontrol agents and plant growth-promoting bacteria (PGPB). They are dominant soil, rhizosphere, and endophytic bacteria, able to produce bioactive metabolites and extremely tolerant endospores. *Bacillus*-based biopesticides are developed worldwide and usually contain beneficial strains of *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, *B. velezensis*, and *B. thuringiensis* (Mazzola and Freilich, 2017). Inhibition of pathogen growth by *Bacillus* entails the mechanisms such as competition for nutrients and space, production of antibiotics, hydrolytic enzymes, siderophores, and inducing systemic resistance (ISR). Moreover, *Bacillus* spp. facilitate the uptake of nutrients from the environment via nitrogen fixation and phosphate solubilization, or provide the plant with certain compounds such as plant hormones (Aloo et al., 2019).

The objective of this study was to select most effective antagonistic strains of *Bacillus* spp. against four phytopathogenic fungi affecting sunflower.

MATERIAL AND METHODS

Phytopathogenic fungi

The phytopathogenic fungi were obtained from culture collection of Laboratory for Seed Testing (Institute of Field and Vegetable Crops Novi Sad, IFVCNS). The fungi were originally isolated from sunflower seeds using filter paper method (Mathur and Kongsdal, 2003). Seeds with symptoms were transferred on potato dextrose agar (PDA). After morphological identification, confirmation of pathogenicity was conducted on seedlings using Knop's agar test tube method (Tuite, 1969). Molecular identification was done using PCR analysis of ITS region and ITS1/ITS4 primers (Takamatsu and Kano, 2001). The four analyzed fungi were identified as *Macrophomina phaseolina*, *Alternaria alternata*, *Cladosporium cladosporoides*, and *Sclerotinia sclerotiorum*. Sequences of analyzed fungi are available in the National Center for Biotechnology Information (NCBI) GenBank Database (Table 1).

Table 1. Phytopathogenic fungi used in the study

Isolate code	Isolation source	Species	NCBI
14Sun	Sunflower seeds	*Macrophomina phaseolina*	MH496040
45Sun	Sunflower seeds	*Sclerotinia sclerotiorum*	MH496034
54Sun	Sunflower seeds	*Cladosporium cladosporoides*	MH496035
82Sun	Sunflower seeds	*Alternaria alternata*	MH496037

Antagonistic *Bacillus* spp. strains

Antagonistic strains of *Bacillus* spp. were obtained from culture collection of Laboratory for Microbiology (IFVCNS). In brief, bacterial strains were isolated from soil samples collected in several locations in Serbia, using serial dilution and streak plate methods on nutrient agar (NA) (Bjelić et al., 2018). Morphologically different colonies were recultivated on the same medium to obtain pure cultures. After microscopic observation, strains were selected for molecular identification using 16S rDNA sequencing and universal primers 27F and 1429R (Weisburg et al., 1991). Ten antagonistic strains were identified as *B. safensis* (B2), *B. pumilus* (B3, B11, B21, B22, B23) and *B. subtilis* (B5, B7, B13, B32). Sequences of the examined *Bacillus* spp. were deposited in the NCBI GenBank Database (Table 2).

Table 2. Antagonistic *Bacillus* strains used in the study

Isolate code	Isolation source	Species	NCBI
B2	Non-agricultural soil	*Bacillus safensis*	KU953932
B3	Rhizosphere (wheat)	*Bacillus pumilus*	KU953923
B5	Rhizosphere (sunflower)	*Bacillus subtilis*	KU953925
B7	Rhizosphere (maize)	*Bacillus subtilis*	KU953927
B11	Non-agricultural soil	*Bacillus pumilus*	KU953931
B13	Rhizosphere (maize)	*Bacillus subtilis*	KX444639
B21	Agricultural soil	*Bacillus pumilus*	KX444647
B22	Rhizosphere (maize)	*Bacillus pumilus*	KX444648
B23	Rhizosphere (wheat)	*Bacillus pumilus*	KX444649
B32	Non-agricultural soil	*Bacillus subtilis*	KX766373

Antifungal activity assay

Antifungal activity of ten antagonistic *Bacillus* spp. strains against four phytopathogenic fungi was examined in a dual plate assay, which implies the simultaneous cultivation of bacterial and fungal culture on PDA. Prior to confrontation, *Bacillus* spp. were cultivated overnight in nutrient broth (NB), while the fungi were grown on PDA for 7–10 days, depending on the fungal species. *Bacillus* strains were streaked by bacteriological loop on PDA along the edge of the Petri dish (R = 85 mm), while fungal discs (6 mm in diameter) were placed on the opposite side. After incubation for 7 days at 25 °C, the fungal growth (in mm) in the control (C) and treated dishes (R1) was measured, and the percent of growth inhibition (PGI) was calculated according to the following formula: PGI (%) = [(C-R1)/C] × 100 (Dimkić et al., 2015).

Statistical analysis

Data was 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

Bacillus spp. are able to control fungal growth and prevent fungal disease, while simultaneously enhancing plant growth and yield (Radhakrishnan et al., 2017). This study confirmed that examined bacterial strains possess very strong potential for the biocontrol of phytopathogenic fungi (Table 3). Antifungal activity assay showed that *Bacillus* spp. exhibited the highest antagonistic effect against *S. sclerotiorum*, with an average PGI of 77%. Moreover, PGI obtained via confrontation of *Bacillus* spp. with *C. cladosporoides*, *M. phaseolina*, and

A. alternata, was 70%, 64% and 59%, respectively. The results also showed different effect of particular strain depending on the tested fungal species. The highest inhibition of *M. phaseolina* and *A. alternata* was recorded by *B. safensis* B2 and *B. pumilus* B3. Strains *B. pumilus* B11 and *B. subtilis* B32 had the highest antagonistic effect on the growth of *C. cladosporoides*, whereas *B. pumilus* B3 and *B. subtilis* B7 showed the largest decrease in growth of *S. sclerotiorum*. Similarly, Dimkić et al. (2015) reported different sensitivity of analyzed fungi in antifungal activity assay using *Bacillus* strains.

Table 3. Antifungal activity of *Bacillus* strains

Bacillus strains	Fungal isolates			
	Macrophomina phaseolina	*Alternaria alternata*	*Cladosporium cladosporoides*	*Sclerotinia sclerotiorum*
	PGI (%) – Percent of growth inhibition			
B. safensis B2	70.83^{a}	65.00^{a}	69.17^{cd}	77.50^{a}
B. pumilus B3	70.83^{a}	65.00^{a}	71.67^{bc}	80.83^{a}
B. subtilis B5	65.00^{a}	60.00^{a}	68.33^{cd}	76.67^{a}
B. subtilis B7	65.00^{a}	61.67^{a}	65.83^{de}	80.83^{a}
B. pumilus B11	64.17^{a}	62.50^{a}	76.67^{a}	76.67^{a}
B. subtilis B13	67.50^{a}	63.33^{a}	74.17^{ab}	79.17^{a}
B. pumilus B21	68.33^{a}	60.83^{a}	73.33^{ab}	78.33^{a}
B. pumilus B22	70.00^{a}	55.00^{ab}	66.67^{d}	78.33^{a}
B. pumilus B23	35.83^{b}	33.33^{b}	62.50^{e}	65.83^{b}
B. subtilis B32	65.00^{a}	63.33^{a}	75.00^{ab}	75.83^{a}
Average	64.25	59.00	70.33	77.00

Mean values (n = 3) of fungal growth inhibition are shown. Values followed by the same letter within columns are not significantly different ($P < 0.05$), according to Tukey's HSD test.

In vitro assay is a good method to examine the antagonistic effect of a large number of strains and provides insight into potential candidates for biological control that need further testing. However, most strains that are effective *in vitro* are not able to inhibit pathogens under environmental conditions, most often because they cannot survive under specific conditions and colonize plant. As spore-forming bacteria, *Bacillus* spp. easily survive and adapt in all habitats, including soil and plant rhizosphere or phyllosphere (Wu et al., 2015). Biocontrol action of *Bacillus* spp. is mainly based on their ability to synthesize antifungal peptides such as surfactin, iturin, fengycin, pumilacidin, mixirin, and/or hydrolytic enzymes such as chitinases, glucanases, cellulases, etc. Due to these antifungal metabolites, bacilli cause changes in the fungal cell wall, cell membrane and intracellular structures. *Baciillus* spp. involved in this study produced various lytic enzymes, while four strains (B5, B7, B13, B32) also produced lipopeptide surfactin (Bjelić et al., 2018). In addition, these antagonistic strains demonstrated broad antifungal and anitbacterial activity against species of *Fusarium*, *Alternaria*, *Diaporthe*, *Xanthomonas*, and other plant pathogens (Bjelić et al., 2017; Bjelić et al., 2018; Spremo et al., 2018; Miljaković et al., 2022). Successful biological control depends primarily on the interactions between plants, antagonists and pathogens, as well as the environment. Therefore, it is necessary to evaluate the activity of the most effective antagonists *in planta*, after inoculation of pathogens directly into plant tissue and monitoring the development of infection on plants.

CONCLUSION

Findings point to the fact that the most effective *Bacillus* spp. could be used as potential biocontrol agents for improving plant health and productivity. Furthermore, different sensitivity of the analyzed fungi to the action of individual *Bacillus* spp. indicates the possibility of selecting the strain appropriate for each disease and its causative agent. Further evaluation of effective *Bacillus* strains in greenhouse and field experiments is needed to determine their effectiveness in disease suppression and growth promotion of sunflower and/or other field and vegetable crops.

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ОРИГИНАЛНИ НАУЧНИ РАД

ИСПИТИВАЊЕ *Bacillus* spp. КАО ПОТЕНЦИЈАЛНИХ АГЕНАСА БИОКОНТРОЛЕ ПАТОГЕНА СУНЦОКРЕТА

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РЕЗИМЕ: *Bacillus* spp. сузбијају фитопатогене синтезом различитих метаболита са антимикробним деловањем као што су литички ензими и антибиотици. Циљ ових истраживања била је селекција најефективнијих *Bacillus* spp. из групе антагонистичких сојева испитивањем њихове антифунгалне активности. *Bacillus* сојеви су првобитно изоловани из земљишта и идентификовани као *B. safensis*, *B. pumilus* и *B. subtilis* секвенцирањем 16S rDNA. Анализиране гљиве, *Macrophomina phaseolina*, *Alternaria alternata*, *Cladosporium cladosporoides* и *Sclerotinia sclerotiorum*, изоловане су са семена сунцокрета и идентификоване PCR анализом уз примену прајмера специфичних за ITS регион. Антифунгална активност бактерија према фитопатогеним гљивама испитана је методом двојне култивације. *Bacillus* spp. испољили су највећи антагонизам према *S. sclerotiorum*, затим *C. cladosporoides*, *M. phaseolina* и *A. alternata*, са просечним процентом инхибиције раста 77%, 70%, 64% и 59%. Испитивани сојеви *Bacillus* spp. показали су веома јак биоконтролни потенцијал, иако је ефекат одређеног соја варирао у зависности од испитиване гљиве. Највећи антагонистички ефекат према *M. phaseolina* и *A. alternata* имали су сојеви *B. safensis* B2 и *B. pumilus* B3. Најефективнији сојеви против *C. cladosporoides* били су *B. pumilus* B11 и *B. subtilis* B32, док је највећа антифунгална активност према *S. sclerotiorum* утврђен применом сојева *B. pumilus* B3 и *B. subtilis* B7. Резултати ових истраживања показују да се најефективнији сојеви *Bacillus* spp. могу користити као потенцијални агенси биоконтроле за побољшање здравља и продуктивности биљака.

КЉУЧНЕ РЕЧИ: *Alternaria*, антифунгална активност, *Bacillus,* биоконтрола, *Cladosporium*, *Macrophomina*, *Sclerotinia*, сунцокрет