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Bacillus isolates as potential biocontrol agents of *Fusarium* clove rot of garlic

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Abstract

Clove rot caused by *Fusarium* spp. is a very important disease of common garlic (*Allium sativum* L.) occuring in many areas of the world. However, there is a lack of data about biocontrol of these pathogens. *Bacillus* species are attractive for research due to their potential use in the biological control of fungal diseases. The aim of this study was to select effective biocontrol agents from a series of indigenous *Bacillus* spp. isolated from soil. Bacterial isolates positive for hydrolytic enzymes production were screened for antifungal activity against *Fusarium* spp. isolated from infected garlic cloves. Polymerase chain reaction (PCR) analyses were used for molecular identification of bacterial (16S rDNA gene) and fungal (EF-1α gene) isolates, and detection of biosynthetic genes for antimicrobial lipopeptides (surfactin, iturin, bacillomycin D and fengycin) in *Bacillus* spp.

The obtained results confirmed the presence of *Fusarium tricinctum*, *F. oxysporum* f. sp. *cepae*, *F. proliferatum*, *F. acuminatum* and *F. verticillioides* as the causal agents of garlic clove rot. Four bacterial isolates identified as *Bacillus subtilis* exhibited the highest antagonistic effect during *in vitro* testing of antifungal activity (up to 71% reduction in fungal growth), and caused a significant suppression of garlic clove infection (up to 58% reduction in rot symptoms) *in situ*. Analysis of the antifungal compounds involved in the antagonistic activity of the examined isolates revealed their ability to produce the antibiotic lipopeptide surfactin. The most effective isolates of *B. subtilis* could be used as potential biocontrol agents of garlic clove rot.

Key words: *Allium sativum*, antifungal activity, *Bacillus subtilis*, lipopeptides.

Introduction

Common garlic (*Allium sativum* L.) is one of the major vegetable crops grown on average on an area of 1.5 million ha, with an annual production of 24.9 million tons (FAO, 2014, http://www.fao.org/faostat/en/#data/QC). Beside for culinary purposes, garlic is also widely used as a prophylactic as well as therapeutic medicinal plant. Different compounds in garlic are thought to have antimicrobial, anticancer, antidiabetic, antiinflammatory, antioxidant and cardioprotective activities (Martins et al., 2016). The unique flavour and health-promoting functions are usually attributed to organosulfur compounds in garlic, such as allicin, ajoene and diallyl sulfides (Rahman, 2007). Regardless, garlic is prone to several diseases, which indicates the severity of the causing pathogens.

Rot disease caused by *Fusarium* spp. is one of the most serious threats to garlic production worldwide. Fungi are transmitted by soil and can persevere for a long time in the soil and plant residues (Koleva, 2004). The disease may occur both in the field and in the storage, with intense development when environmental conditions are suitable and plants are susceptible to infection (Palmero et al., 2013). Disease is favoured by higher (20–30°C) temperatures and high humidity. Garlic bulbs are quite perishable because of their high moisture content (Schwartz, Mohan, 2008). Plants may show decreased germination and necrosis of leaves, while infected bulbs become undersized, softened, brown and watery, with white, light purple or red mycelium on the cloves (OSU, 2008). Additionally, *Fusarium* spp. lead to a serious reduction in yield and represent an important safety interest due to their ability to produce numerous mycotoxins such as fumonisin, moniliformin, beauvericin, fusaric acid and fusaroproliferin (Stanković et al., 2007).

Different strategies have been used for the suppression of garlic rot, including fungicide treatments and preventive detection of infection before seeding, but none of them are enough to control the disease (Dugan et al., 2007). The demand for alternative control strategies, particularly the use of biopesticides, has been increasing steadily due to environmental awareness

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and potential health hazards from chemical pesticides (Ansari et al., 2012). *Bacillus* spp. are attractive for research due to their possible utilization in the biological control of fungal diseases (Mardanova et al., 2017). They offer several preferences over other microorganisms because of their ability to form endospores and tolerate adverse environmental conditions (Islam et al., 2012). Furthermore, their long term viability and rapid growth in a liquid medium simplify the production of commercial preparations (Wu et al., 2015).

Antimicrobial activities of *Bacillus* spp. were usually associated to the synthesis of secondary metabolites with antibiotic properties such as cyclic lipopeptides, namely surfactin, iturin and fengycin (Cawoy et al., 2015). These lipopeptides can also influence the root colonization and interaction of *Bacillus* spp. with plants by stimulating host defence mechanisms (Ongena, Jacques, 2008). Other biocontrol mechanisms of *Bacillus* spp. involve the excretion of cell wall hydrolases, competition for nutrients and/or elicitation of induced systemic resistence (Lugtenberg et al., 2013). Moreover, *Bacillus* spp. had the ability to promote plant growth through asymbiotic nitrogen fixation, phosphate solubilization, production of phytohormones, siderophores and enzymes (Borriss, 2011).

It has been found that *Bacillus* spp. exert beneficial effects on plant growth and disease suppression of various crops (Mnif, Ghribi, 2015). However, the lack of data about biocontrol of garlic pathogens is evident in literature. Considering the increasing occurrence of *Fusarium* rot on garlic in Serbia (Stanković et al., 2007; Lević et al., 2009; Ignjatov et al., 2017 a; b), it is necessary to investigate the possibilities of using *Bacillus* spp. as potential biocontrol agents of the disease. The aim of this study was to isolate *Bacillus* spp. from soil and to evaluate their antifungal activities toward *Fusarium* spp. isolated from infected garlic cloves.

Materials and methods

Isolation and molecular identification of *Bacillus* and *Fusarium* species were performed during 2015–2016, while polymerase chain reaction (PCR) detection of antimicrobial lipopeptides, hydrolytic and antifungal activity assays were conducted during 2016–2017. All experiments were performed in the Laboratory for Microbiology and Laboratory for Seed Testing of Institute of Field and Vegetable Crops, Novi Sad, Serbia.

Isolation of Bacillus spp. Bacillus spp. were isolated from soil samples collected in several locations in Serbia as described by Bjelić et al. (2017). Soil samples included the rhizosphere of plants, agricultural and non-agricultural soils, and differed in their physical and chemical properties, cropping and tillage history. In short, soil samples were taken up to a depth of 20 cm, sieved and stored at 4°C until processed. Soil suspensions (1 g of soil in 9 ml of 0.85% NaCl) were serially diluted (10^{-3}–10^{-6}) and 0.1 ml aliquots were spread on plates containing nutrient agar. Followed by 24 h incubation at 30°C and several recultivations of single colonies on nutrient agar, the bacterial isolates were characterized according to morphological and biochemical properties (Cappuccino, Welsh, 2016).

Isolation of Fusarium spp. *Fusarium* spp. were isolated from rot symptomatic garlic bulbs collected from several storages in Vojvodina Province, northern Serbia. Cloves were separated from the bulbs, and surface disinfested in 1% NaOCl for 2 to 3 min, rinsed with sterilized distilled water three times, and then dried on sterile filter paper under aseptic conditions. Pieces of infected clove tissues (3 to 4 mm) were cut and plated onto a potato dextrose agar (PDA) amended with 300 mg l^{-1} streptomycin sulfate (w/v). Plates were incubated at 25°C in the dark. Seven days later, *Fusarium* colonies were recognized morphologically and isolates were subcultured in PDA using the single spore technique.

Pathogenicity. Confirmation of pathogenicity was conducted for twenty *Fusarium* spp. isolates. Cloves of the common garlic (*Allium sativum* L.) cultivar 'Bosut' (developed at the Institute of Field and Vegetable Crops, Novi Sad, Serbia) were surface sterilized in 0.5% NaOCl for 1 min, rinsed in four changes of sterile water and wounded to a depth of 4 mm using a 1-mm diameter probe (Palmero et al., 2012). Each of the 7 day-old fungal isolates was inoculated into five wounded cloves and incubated in a growth chamber at 25°C for 3 weeks. Six randomly selected isolates of *Fusarium* spp. were re-isolated from artificially inoculated garlic cloves fulfilling Koch's postulates, and incubated at 25°C for 7 days before use. Morphology of macroconidia, microconidia and chlamydospore was assessed from cultures grown on PDA and carnation leaf agar (CLA) (Leslie, Summerell, 2006).

In vitro hydrolytic activity assay. Screening for lytic enzyme production in indigenous *Bacillus* spp. isolates included determination of lipase, phospholipase, pectinase and cellulase activity (Djuric et al., 2011). Lipase production was confirmed on a medium with the addition of polysorbate Tween 80 (Sigma-Aldrich, USA), after 7 days of incubation at 28°C, by the formation of dark and opaque zone around the colonies. Production of phospholipase was assayed on a medium supplemented with egg yolk, after 48 h of incubation at 28°C, by the appearance of an opaque zone around the colonies. Pectinase production was determined on a pectin agar after 48 h of incubation at 28°C and pouring with 2 M HCl, by the appearance of a clear zone around the colonies. Production of cellulase was assayed on the carboxyl methyl cellulose (CMC) agar, after 7 days of incubation at 28°C and pouring 0.1% Congo Red solution into 1 M NaCl, by the appearance of a clear zone around the colonies.

Molecular species identification. Bacillus spp. isolates for DNA extraction were grown on nutrient agar plates for 24 h, while DNA from *Fusarium* spp. isolates was extracted from the 7-day-old mycelium (100 mg wet weight) grown on PDA plates. DNA from bacterial and fungal isolates was extracted using a DNeasy Mini Kit (QIAGEN Inc., Germany). Universal primers fD1 (27F) (AGAGTTTGATCMTGGCTCAG) and rP3 (1492R) (TACGGYTACCTTGTTACGACTT) were used for the amplification of 16S rDNA gene fragments of *Bacillus* isolates (Weisburg et al., 1991). The translation elongation factor-1 *alpha* (EF-1α) gene region of *Fusarium* isolates was amplified with the primer pair EF1 (ATGGGTAAGGAGGACAAGAC) and EF2 (GGAAGTACCAGTGATCATGTT) (Geiser et al., 2004). Amplifications were performed in a Mastercycler PCR device (Eppendorf, Germany). Amplicons were electrophoresed in a 1.5% agarose gel containing ethidium bromide (0.5 μg ml^{-1}). All sequences were deposited in the GenBank and compared with the previously reported sequences available in the NCBI (National Center for Biotechnology Information).

In vitro antifungal activity assay. The ability of *Bacillus* spp. isolates to inhibit the growth of six *Fusarium* spp. isolates was examined using a dual plate assay (Zhao et al., 2010). The 7-day-old mycelial plugs on PDA (6 mm in diameter) of each fungus were aseptically transferred on the same medium, previously streaked with a broth culture of the tested bacteria. After 7 days of incubation at 25°C, the fungal growth in the control (C) and dual cultures (R1) were measured and the percent of growth inhibition (PGI) was calculated according to the formula: PGI (%) = $[(C - R1) / C] \times 100$. Antifungal activity assay was repeated twice, with three replications for each fungal and bacterial isolate.

Polymerase chain reaction (PCR) detection of antimicrobial lipopeptides. Detection of biosynthetic genes for antimicrobial lipopeptides: surfactin (*sfp*), iturin (*itu*), bacillomycin D (*bamC*) and fengycin (*fenD*) in *Bacillus* spp. isolates was carried out using four sets of primers. Primers and corresponding genes for PCR detection of antimicrobial lipopeptides are listed in Table 1. DNA from *Bacillus* spp. was isolated as described earlier. The PCR for the detection of the antimicrobial lipopeptides was programmed as described by Dimkić et al. (2013). Following PCR, the amplified products were electrophoresed as described earlier.

Table 1. Primers and corresponding genes for polymerase chain reaction (PCR) detection of antimicrobial lipopeptides

Lipopeptide	Gene	Primer	Sequence	Product size bp
Surfactin	*sfp*	P17 P18	ATGAAGATTTACGGAATTTA TTATAAAAGCTCTTCGTACG	~675
Iturin	*itu*	ITUP1F ITUP2R	AGCTTAGGGAACAATTGTCATCGGGGCTTC TCAGATAGGCCGCCATATCGGAATGATTCG	~2000
Bacillomycin D	*bamC*	BACC1F BACC1R	GAAGGACACGGCAGAGAGTC CGCTGATGACTGTTCATGCT	~875
Fengycin	*fenD*	FEND1F FEND1R	TTTGGCAGCAGGAGAAGTT GCTGTCCGTTCTGCTTTTTC	~964

In situ antifungal activity assay. Antifungal activity of *Bacillus* spp. on garlic cloves infected with *Fusarium* spp. isolates was examined with a modification of the method described by Palmero et al. (2012). Cloves of garlic cultivar 'Bosut' were surface disinfested in 0.5% sodium hypochlorite (NaOCl) (Sigma-Aldrich) for 1 min, washed with sterile water four times, and then injured to a depth of 4 mm using a 1-mm diameter probe. Each of the 7-day-old fungal isolates on PDA was inoculated into six wounded cloves. Infected wounds were then inoculated with drops of bacterial suspension of selected *Bacillus* isolates (10^8 cfu ml^{-1}). For each fungal isolate, another set of six infected cloves was used as a positive control without bacterial treatment. Clove sets inoculated only with sterile PDA or with bacterial suspensions were used as negative control. Treated cloves were incubated in folded, plastic boxes in a growth chamber at 25°C for 3 weeks, and then monitored for the development of rot symptoms. Disease symptoms were graded into five classes: 1 = <10% rotted cloves, 2 = 11–20% rotted cloves, 3 = 21–50% rotted cloves, 4 = 51–80% rotted cloves, 5 = 81–100% rotted cloves and severe symptoms on cloves. Assay was repeated once, with three replications for each fungal and bacterial isolate. A disease severity index was calculated as the mean of six cloves and three test replicates.

Statistical analysis. Data were processed using a one-way analysis of variance (*ANOVA*). Means were compared using Tukey's multiple comparisons of means test for 95% and 99% confidence levels. All analyses were performed in *STATISTICA 10* (StatSoft Inc., USA).

Results and discussion

Our results confirmed the presence of *Bacillus* spp. in soil samples collected at different locations in Serbia. Species of *Bacillus* are characterized as Gram-positive, motile, aerobic or facultatively anaerobic and usually catalase positive. Cells are straight rods, often arranged in pairs or chains. Colonies are round, with entire or wavy margins, typically cream and dry or pasty looking.

In search of efficient biocontrol agents, 65 indigenous *Bacillus* spp. isolates were primarily characterized according to their ability to produce hydrolytic enzymes. Lytic enzymes such as chitinases, glucanases, cellulases, lipases, and proteases, degrade fungal and bacterial cell wall and prevent plant infection caused by pathogens. According to Raza et al. (2008), isolation and characterization of strains which can produce lytic enzymes should be done to achieve maximal survival of bacteria under detrimental environmental conditions and intrusion of pathogens. In this study, hydrolytic activity, i.e. production of one or more enzymes, was found in 32 isolates, while among them, activity of lipase, phospholipase, cellulase, and pectinase was determined in 43.75, 12.5, 81.2 and 37.5 % of *Bacillus* spp. isolates, respectively (Table 2). It has been found that isolates of *Bacillus* spp. which have the capability to produce lytic enzymes are more effective in the suppression of plant pathogens (Abdallah et al., 2017). Considering the above facts, isolates positive for hydrolytic enzyme production were identified and further screened for their biocontrol potential by examination of antifungal activity and lipopeptide production.

The *Bacillus* species differentiation is difficult because of their large number (over 200 described species and subspecies) and often incomplete descriptions of the newly-reported species. In this study, identification of *Bacillus* isolates based on 16S rDNA homology was performed using PCR with the primers 27F and 1492R (Weisburg et al., 1991). The presence of amplicon of ~1460 bp in size was confirmed in all investigated samples.

Comparison of the sequences with the *Bacillus* ID-database identified seven isolates as *Bacillus megaterium* (B8, B9, B12, B14, B15, B16 and B17), six as *B. cereus* (B6, B24, B27, B29, B30 and B31), five as *B. pumilus* (B3, B11, B21, B22 and B23), four as *Lysinibacillus fusiformis* (B1, B4, B10 and B25) and *B. subtilis* (B5, B7, B13 and B32), three as *B. thuringiensis* (B18, B20 and B28), and one isolate as *B. safensis* (B2), *Lysinibacillus sphaericus* (B19) and

Table 2. Hydrolytic enzyme production of indigenous *Bacillus* spp. isolates

Isolate	Isolation source	Lipase	Phospholipase	Cellulase	Pectinase	Total enzymes
B1	Agricultural soil	+	−	+	−	2
B2	Non-agricultural soil	+	−	+	−	2
B3	Rhizosphere (wheat)	+	−	+	+	3
B4	Non-agricultural soil	−	−	+	−	1
B5	Rhizosphere (sunflower)	+	+	+	+	4
B6	Non-agricultural soil	+	−	−	+	2
B7	Rhizosphere (maize)	+	−	+	+	3
B8	Rhizosphere (pepper)	−	−	+	+	2
B9	Rhizosphere (alfalfa)	−	−	+	−	1
B10	Non-agricultural soil	−	−	+	+	2
B11	Non-agricultural soil	+	+	+	−	3
B12	Agricultural soil	−	−	−	+	1
B13	Rhizosphere (maize)	+	+	−	+	3
B14	Rhizosphere (sunflower)	−	−	+	−	1
B15	Non-agricultural soil	+	−	+	−	2
B16	Rhizosphere (maize)	−	−	+	−	1
B17	Rhizosphere (wheat)	−	−	+	−	1
B18	Agricultural soil	−	−	+	−	1
B19	Non-agricultural soil	−	−	+	−	1
B20	Non-agricultural soil	−	−	+	−	1
B21	Agricultural soil	−	−	+	+	2
B22	Rhizosphere (maize)	+	−	+	+	3
B23	Rhizosphere (wheat)	+	−	+	+	3
B24	Rhizosphere (maize)	−	−	+	−	1
B25	Non-agricultural soil	−	−	+	−	1
B26	Agricultural soil	+	−	−	−	1
B27	Non-agricultural soil	−	+	−	−	1
B28	Agricultural soil	−	−	+	−	1
B29	Non-agricultural soil	+	−	−	−	1
B30	Agricultural soil	−	−	+	−	1
B31	Non-agricultural soil	−	−	+	−	1
B32	Non-agricultural soil	+	−	+	+	3
	Total production (% isolates)	43.75	12.5	81.2	37.5	

B. mycoides (B26). The gene sequences had the values 99% to 100% similar to those deposited in the GenBank: *Bacillus megaterium* BOFC15 (KU851253), *B. cereus* ISE27 (KX035061), *B. pumilus* LX11 (KP192031), *Lysinibacillus fusiformis* VC-1 (HQ610620), *B. subtilis* CR26 (KR780430), *B. thuringiensis* S40 (KY120371), *B. safensis* ZN9 (KJ542766), *Lysinibacillus sphaericus* ARD1 (KX023224) and *B. mycoides* FJAT-44618 (KX767102). Sequences of the isolated *Bacillus* species were deposited in the NCBI GenBank database under a unique accession number (B1–B11: KU953922–KU953931, B12–B26: KX444638–KX444652 and B27–B32: KX766368–KX766373). Similarly, Mandic-Mulec and Prosser (2011) found that up to 95% of the sequences of Gram-positive bacteria from agricultural soils originate from *Bacillus* species, while *B. cereus* and *B. megaterium* were the most commonly found isolates. Also, a high genetic diversity of *Bacillus* species implies that investigation and identification of new strains may increase the number of potential biocontrol agents and improve understanding of mechanisms associated with antagonistic interactions (Mardanova et al., 2017).

According to the morphological characterization of 20 fungal isolates obtained from garlic cloves, 6 representative isolates of *Fusarium* spp. were confirmed as causal agents of rot disease. Although plants did not show symptoms of infection in the field, bulb rot later occurred in storage. Our research results confirm that the postharvest storage conditions greatly influence the intensity of garlic clove rot caused by *Fusarium* (Palmero et al., 2013). *Fusarium* spp. caused similar symptoms on stored garlic and it was difficult to distinguish them based on the symptoms and morphological characteristics. Molecular identification may assist in further epidemiological studies and development of efficient disease control for this pathosystem. In this study, identification of *Fusarium* isolates based on EF-1α gene was performed using PCR with the primers EF1 and EF2 (Geiser et al., 2004) and the presence of amplicon ~700 bp in size was detected in all examined isolates.

In comparison with the sequences from the *Fusarium* ID-database, isolates were identified as *Fusarium tricinctum* (BL12), *F. oxysporum* f. sp. *cepae* (BL13), *F. proliferatum* (BL16 and BL18), *F. acuminatum* (BL20) and *F. verticillioides* (BL21) (Table 3). BLASTn queries of gene sequences showed 99% to 100% identity to *F. tricinctum*, *F. oxysporum* f. sp. *cepae*, *F. proliferatum*, *F. acuminatum* and *F. verticilioides*, to

Table 3. Isolates of *Fusarium* spp. from garlic

Isolate	Isolation source	Species	NCBI account No.
BL12	Garlic genotype JBL12	*Fusarium tricinctum*	KX611146
BL13	Garlic genotype JBL13	*Fusarium oxysporum* f. sp. *cepae*	KX611148
BL16	Garlic genotype JBL531	*Fusarium proliferatum*	KX752415
BL18	Garlic genotype JBL535	*Fusarium proliferatum*	KX752417
BL20	Garlic genotype JBL539	*Fusarium acuminatum*	KX752419
BL21	Garlic genotype R1	*Fusarium verticillioides*	KX752420

accessions EU744838, KP964881, KP964907, KJ194170 and KU372138, respectively. Sequences of the isolated *Fusarium* species were deposited in the NCBI GenBank database (Table 3).

The antifungal activity of *Bacillus* spp. isolates against six isolates of *Fusarium* spp. was examined using a dual plate assay. The results showed that 10 out of the 32 isolates of *Bacillus* spp. from soil were positive for antifungal activity (Table 4). *Bacillus* spp. exhibited the highest antifungal activity against fungal isolate *F. tricinctum* BL12, while they had the least antagonistic effect on *F. proliferatum* BL16 and *F. acuminatum* BL20. On average, percent of growth inhibition (PGI) obtained by confrontation of the active *Bacillus* spp. isolates with tested phytopathogenic fungi ranged from 15.97% to 58.14%. The highest antifungal activity toward *F. oxysporum* f. sp. *cepae* BL13, *F. proliferatum* BL18, *F. acuminatum* BL20 and *F. verticillioides* BL21 was exhibited by isolate *B. subtilis* B5 (from 41.96% to 66.51% reduction in fungal growth), while isolate *B. subtilis* B32 showed the highest antagonistic effect against *F. tricinctum* BL12 and *F. proliferatum* BL16 (from 44.31% to 71.37% reduction in fungal growth). *B. subtilis* isolates B7 and B13 also demonstrated good antifungal potential (PGI up to 69.01% and 63.14%), while the effect of *B. safensis* (B2) and *B. pumilus* (B3, B11, B21, B22 and B23) isolates varied depending on the tested fungi. Also, effective *Bacillus* spp. isolates were better producers of lytic enzymes (Table 2).

Table 4. Mean values and analysis of variance (significance) for antifungal activity of effective *Bacillus* spp. isolates against *Fusarium* spp.

Isolate	*F. tricinctum* BL12	*F. oxysporum* f. sp. *cepae* BL13	*F. proliferatum* BL16	*F. proliferatum* BL18	*F. acuminatum* BL20	*F. verticillioides* BL21
	Percent of growth inhibition %					
B. safensis B2	49.4 de	30.4 d	19.2 de	42.7 d	13.2 d	28.0 d
B. pumilus B3	54.3 cd	12.7 e	19.8 de	42.4 d	12.7 d	30.0 d
B. subtilis B5	65.5 ab	59.6 a	43.1 a	62.6 a	42.0 a	66.5 a
B. subtilis B7	69.0 ab	54.9 ab	34.3 b	53.3 bc	35.5 b	52.2 b
B. pumilus B11	44.1 e	36.7 cd	28.8 bc	40.4 d	–	–
B. subtilis B13	63.1 abc	43.3 c	27.3 c	49.0 c	16.2 d	42.0 c
B. pumilus B21	55.7 cd	32.0 d	16.4 e	–	–	15.2 e
B. pumilus B22	47.7 de	–	4.5 f	–	–	–
B. pumilus B23	61.2 bc	29.6 d	25.7 cd	39.8 d	14.9 d	28.4 d
B. subtilis B32	71.4 a	50.6 b	44.3 a	57.5 b	25.3 c	63.7 a
Average	58.1	35.0	26.3	38.8	16.0	32.6
Isolate	**	**	**	**	**	**

Note. Means with different lowercase letters in the same column within the same fungal isolate and effective *Bacillus* spp. isolates are significantly different ($p < 0.05$, Tukey test); ** – significance at 0.01 probability level.

In our previous study, we observed very strong antifungal activity of *B. subtilis* B5 and B7 against *F. proliferatum* BL5 (KX092461) and *F. oxysporum* f. sp. *cepae* BL7 (KX092466), while the antagonistic effect of *Bacillus* isolates toward *F. verticillioides* BL7 (KX092464) was not detected (Bjelić et al., 2017).

Antifungal compounds involve mainly peptides that are generated ribosomally or non-ribosomally. The most frequent antibiotic and surface-active compounds in *Bacillus* spp. are cyclic lipopeptides of surfactin, iturin and fengycin families, synthesized non-ribosomally by large multienzyme complexes (Cawoy et al., 2015**)**. The surfactin family (surfactin, lichenysins and pumilacidins) comprises heptapeptides containing a β-hydroxy fatty acid with a number of carbon atoms between 13 and 16. Lipopeptides of the iturin family (iturin, mycosubtilin and bacillomycin) are composed of heptapeptides interlinked with a β-amino fatty acid chain, consisting of 14–17 carbons. Members of the fengycin family, represented by plipastatin, are cyclic lipodecapeptides with a β-hydroxy fatty acid having side chain length of 16–19 carbon atoms. In this study, PCR amplifications using specific primers were performed to search the genes involved in the surfactin (*sfp*), iturin (*itu*), bacillomycin D (*bamC*) or fengycin (*fenD*) antibiotics biosynthesis in 32 *Bacillus* spp. isolates. Consequently, only the *sfp* gene (675 bp) was amplified from *B. subtilis* B5, B7, B13 and B32, suggesting that these four isolates could produce surfactin (Fig.).

Previous studies showed that most strains of *Bacillus* produce lipopeptides from one family, while a few were identified as co-producers of lipopeptides belonging to different families (Ongena, Jacques, 2008). Moreover, it was determined that the antifungal activity of *Bacillus* spp. depends on the quantity and diversity in the production of lipopeptides (Nagorska et al., 2007). Four isolates of *B. subtilis* which exhibited the highest antagonistic effect during *in vitro* testing of antifungal

M – PCR marker (Step Ladder, 50–1500 bp), − – negative control

Figure. Polymerase chain reaction (PCR) screening for antibiotic biosynthetic genes in *Bacillus* spp. isolates – detection of sfp gene in B1–B11 (A), B12–B21 (B) and B22–B32 (C) isolates

activity (Table 4), as well as the ability to produce lipopeptide surfactin (Fig.), were selected for *in situ* antifungal activity assay on garlic cloves infected with isolates of *Fusarium* spp.

By assessing the symptoms of rotted cloves in control treatments, the highest disease severity indexes were determined for *Fusarium acuminatum* BL20, followed by *F. proliferatum* BL16 and *F. oxysporum* f. sp. *cepae* BL13, while *F. tricinctum* BL12 and *F. proliferatum* BL18 had the lowest pathogenicity (Table 5). On average, all four *Bacillus* isolates reduced the occurence of rot symptoms (from 28% to 40%). Bacterial isolates had the highest antifungal activity toward *F. oxysporum* f. sp. *cepae* BL13 and *F. proliferatum* BL16, followed by their equally good antagonistic effect on other fungal isolates.

Disease severity index values from garlic cloves inoculated with both fungal and bacterial isolates were significantly lower than in controls inoculated with *Fusarium* spp. isolates. Isolate B5 significantly reduced (up to 58%) the occurrence of rot symptoms on cloves infected with fungal isolates BL13, BL16, BL18, BL20 and BL21. A significant suppression of infection compared to the control treated with isolates BL13, BL18 and BL20 was also achieved by isolate B7 (up to 52%), while isolate B32 led to significant decrease of garlic rot caused by fungal isolates BL12, BL13 and BL21 (up to 45%). Isolate B13 also reduced (up to 27%) the symptom development on infected cloves, although without significant differences compared to control treatments.

Table 5. Mean values and analysis of variance (significance) for antifungal activity of selected *Bacillus* isolates on garlic cloves infected with *Fusarium* spp.

Isolate	*F. tricinctum* BL12	*F. oxysporum* f. sp. *cepae* BL13	*F. proliferatum* BL16	*F. proliferatum* BL18	*F. acuminatum* BL20	*F. verticillioides* BL21	Average
	Disease severity index						
Pathogen	3.7 a	4.2 a	4.3 a	3.8 a	4.8 a	4.0 a	4.1
B.subtilis B5	2.7 ab	2.5 b	1.8 b	2.2 b	2.7 b	2.3 b	2.4
B. subtilis B7	2.7 ab	2.0 b	2.7 ab	2.7 b	3.2 b	3.2 ab	2.7
B. subtilis B13	3.2 ab	3.3 ab	3.2 ab	3.0 ab	4.0 ab	3.5 ab	3.4
B. subtilis B32	2.0 b	2.5 b	2.7 ab	2.8 ab	3.7 ab	2.5 b	2.7
Average	2.6	2.6	2.6	2.7	3.4	2.9	
Isolate	**	**	**	**	**	*	

Note. Means with different lowercase letters in the same column within the same fungal isolate and selected *Bacillus* spp. isolates are significantly different ($p < 0.05$, Tukey test); ** – significance at 0.01 probability level, * – significance at 0.05 probability level.

Unlike the tested fungal species which showed different sensitivity in antifungal activity assays, the antagonistic effect of the particular *Bacillus* isolates via *in vitro* and *in situ* testing largely coincided. Interestingly, newly-isolated *Bacillus* spp. exhibited the least *in vitro* antagonistic effect on fungal isolates which had the highest *in situ* pathogenicity. Similarly, Dimkić et al. (2015) found that *Fusarium* spp. are among the most resistant phytopathogenic fungi to the antagonistic effect of *Bacillus* spp., while within genus there was a higher resistance of *F. verticillioides* and *F. oxysporum* in relation to *F. tricinctum*.

The present study showed that the highest and the most extensive antagonistic effect toward all the tested fungi was achieved by isolates of *B. subtilis*, probably due to the production of lipopeptide surfactin and hydrolytic enzymes. In fact, Stein (2005) showed that *B. subtilis* had strong inhibitory effect against more pathogens as a result of the production of antibiotics. Additionally, Mardanova et al. (2017) established that *B. subtilis* was able to suppress *Fusarium* infection through the production of hydrolytic enzymes and cyclic lipopeptides. Numerous studies revealed that antagonistic strains of *B. subtilis* which inhibit the fungal growth under *in vitro* conditions also reduce the production of mycotoxins, as well as the occurrence and frequency of *Fusarium* caused diseases of various plants in greenhouse and field conditions (Cavaglieri et al., 2005; Baysal et al., 2008; Zalila-Kolsi et al., 2016). However, these are the first data about possible use of *B. subtilis* in biocontrol of *Fusarium* spp. isolated from infected garlic cloves, so further research will be of great importance. In addition to their effect on *Fusarium*, *Bacillus* isolates show antagonistic activities against numerous fungal and bacterial pathogens (Wu et al., 2015). Besides their role in biocontrol, *Bacillus*-based preparations should also have the ability to colonize rhizosphere and promote plant growth (Borriss, 2011). The isolation and characterization of these bacteria is very important from the viewpoint of biocontrol research and could increase the number of strains identified as biocontrol agents of various diseases covering a wider range of plant species.

Conclusions

1. Isolates of *Bacillus subtilis*, *B. safensis* and *B. pumilus* as superior enzyme producers showed antifungal activity against *Fusarium tricinctum*, *F. oxysporum* f. sp. *cepae*, *F. proliferatum*, *F. verticillioides* and *F. acuminatum*.

2. The highest antagonistic effect toward this important pathosystem was observed for *B. subtilis* isolates, and very strong antifungal activity of these isolates was confirmed by significant suppression of common garlic (*Allium sativum* L.) cloves infection.

3. Polymerase chain reaction (PCR) analysis of antimicrobial compounds showed that antifungal activity of *B. subtilis* isolates was merit of lipopeptide surfactin production.

4. Based on our results, *B. subtilis* isolates could be used as biocontrol agents of garlic clove rot caused by *Fusarium* spp., and to our knowledge, this is the first experimental confirmation on using *Bacillus* spp. in biocontrol of the pathogens involved in this disease.

5. This study revealed that the indigenous *Bacillus* isolates from the soil have strong biocontrol potential and could be used for suppression of plant diseases and improvement of plant productivity.

6. Further research of effective *Bacillus* isolates through greenhouse and field trials will be necessary in order to establish their efficiency as biopesticides in different environmental conditions.

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Bacillus izoliatai česnakų skiltelių fuzarinio puvinio biokontrolei

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Santrauka

Skiltelių fuzarinis puvinys yra labai žalinga valgomojo česnako (*Allium sativum* L.) liga, pasitaikanti daugelyje pasaulio regionų, tačiau trūksta žinių apie jos sukėlėjo biologinę kontrolę. *Bacillus* rūšys mokslui yra įdomios dėl galimo jų panaudojimo grybinių ligų biologinei kontrolei. Tyrimo tikslas – atrinkti veiksmingas biokontrolės medžiagas iš vietinių *Bacillus* spp. izoliatų, išskirtų iš dirvožemio. *Fusarium* spp. buvo išskirti iš užkrėstų česnako skiltelių ir su jais buvo atlikti *Bacillus* spp. izoliatų, sintetinančių hidrolitinius fermentus, antibakterinio aktyvumo tyrimai. Polimerazės grandininė reakcija (PGR) buvo taikyta molekuliniams bakterinių (16S rDNR genas) ir grybiniams (EF-1α genas) izoliatams identifikuoti ir antimikrobinių lipopeptidų (surfaktino, iturino, bacilomicino D ir fengicino) biosintezės genams nustatyti *Bacillus* spp. rūšyse.
Tyrimo rezultatai patvirtino, kad *Fusarium tricinctum*, *F. oxysporum* f. sp. *cepae*, *F. proliferatum*, *F. acuminatum* ir *F. verticillioides* yra česnakų skiltelių fuzarinio puvinio sukėlėjai. Keturi bakteriniai izoliatai, identifikuoti kaip *Bacillus subtilis*, pademonstravo didžiausią antagonistinį poveikį, antigrybinį aktyvumą tiriant *in vitro* (grybų augimas sumažintas 71 procentu), ir smarkiai slopino česnakų skiltelių infekciją *in situ* (grybų augimas sumažintas 58 procentais). Antigrybinių junginių, dalyvaujančių tirtų izoliatų antagonistinėje veikloje, analizė atskleidė jų gebą gaminti antibiotinį lipopeptidą surfaktiną. Patys veiksmingiausi *B. subtilis* izoliatai gali būti naudojami kaip potencialios biologinės česnakų skiltelių puvinio kontrolės medžiagos.

Reikšminiai žodžiai: *Allium sativum*, antigrybinis aktyvumas, *Bacillus subtilis*, lipopeptidai.