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ISOLATION AND IDENTIFICATION OF *BACILLUS* SPP. FROM COMPOST MATERIAL, COMPOST AND MUSHROOM CASING SOIL ACTIVE AGAINST *TRICHODERMA* SPP.

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Abstract: The isolation of bacteria was carried out from samples of straw and chicken manure, compost at various stages of the composting process and casing soil used for growing button mushrooms. A preliminary screening of 108 bacterial isolates for antagonistic activity against *Trichoderma aggressivum* f. *europaeum* showed that 23 tested isolates inhibited mycelial growth of the pathogenic fungus. Further screening with four indicator isolates of fungi revealed that all 23 bacterial isolates inhibited the growth of *T. aggressivum* f. *europaeum*, *T. harzianum* and *T. koningii*, while only 13 isolates inhibited the growth of *T. atroviride*. *T. aggressivum* f. *europaeum* proved to be the most sensitive, with many bacterial isolates generating a high percentage of growth inhibition. Only two bacterial isolates (B-129 and B-268) were successful in inhibiting the growth of all 4 tested pathogens. All 23 bacterial isolates were characterized as Gram-positive and catalase-positive and were subjected to molecular identification based on the partial sequence, the hypervariant region of the 16S rDNA. It was shown that the obtained bacterial strains belong to *Bacillus subtilis*, *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus* species.

Key words: *Agaricus bisporus*; *Bacillus*; biocontrol; green mold; hypervariant region

INTRODUCTION

Button mushroom (*Agaricus bisporus* (Lange) Imbach) is one of the most commonly cultivated edible mushrooms worldwide [1]. However, infections during cultivation of the mushrooms frequently occur. Since 1986, the commercial production of *A. bisporus* has been seriously affected by green mold epidemics caused by *Trichoderma* species [2]. Green mold disease is characterized by the occurrence of dense white mycelia on casing soil or compost that changes the color to green after extensive sporulation. Another feature of the disease is the development of brown-colored necrotic lesions on mushroom fruiting bodies, whereas during serious outbreaks of the disease, no fruiting bodies are formed [3].

A common method of pathogen control on mushroom farms is treatment with various fungicides. Only a few fungicides have been officially approved for application in the mushroom industry: prochloraz in Europe and worldwide, and chlorothalonil, thiabendazole and thiophanate-methyl in North America [4]. However, due to problems regarding the growing resistance of pathogens to chemical fungicides, as well as the negative environmental impact of such substances, numerous studies have been conducted to search for alternative methods of pathogen control.

One of the most promising alternatives to synthetic fungicides is biological control of pathogens, which includes the use of biopesticides based on antagonistic microorganisms. In contrast to commonly used fungicides, biopesticides have several advantages: high specificity against target pathogens, rapid degradation in the environment and low mass-production cost. Antagonistic microorganisms operate through various modes of activity such as competition with pathogens for space and nutrients, production of antibiotics and cell-wall degrading enzymes and reduction of pathogen population by hyperparasitism [5].

Most bacterial strains commercially used as biopesticides belong to the genera *Bacillus* and *Pseu-*

Table 1. Indicator strains of pathogenic fungi.

Indicator isolates	Growth medium	Source
Trichoderma aggressivum f. *europaeum* T77	PDA	Collection of the Institute of Pesticides and Environmental Protection
Trichoderma harzianum T54	PDA	
Trichoderma koningii T39	PDA	
Trichoderma atroviride T33	PDA	

domonas [6]. *Bacillus* spp. produces spores that are resistant to various physical and chemical treatments such as heat, desiccation, UV irradiation and organic solvents [7]. Also, they are known to produce an array of secondary metabolites, including antibiotics, cell-wall degrading enzymes and antifungal volatile substances. This indicates that *Bacillus* spp. strains can be efficient biological control agents against a wide range of plant and mushroom pathogens [8,9]. Additionally, *B. pumilus* and species belonging to the *B. subtilis* group are harmless to the environment and human health and are generally recognized as safe (GRAS) organisms [10].

Therefore, the objectives of this study were to (i) isolate antagonistic bacteria from material used in mushroom growing, (ii) investigate their antagonistic potential against *Trichoderma* spp. *in vitro*, and (iii) to identify antagonistic bacterial isolates to the species level.

MATERIALS AND METHODS

Samples

Samples of compost material (straw and chicken manure), compost during various stages of composting and casing soil used for growing button mushrooms were collected from the mushroom compost facility, Uča and Co., Vranovo, Serbia.

Fungal indicators

Pathogenic fungi used as indicator strains for testing of bacterial antagonism were obtained from the culture collection of the Institute of Pesticides and Environmental Protection (Table 1). Previous identification of these isolates was based on the morphological and physiological characteristics and ITS1/ITS4 sequence analyses [3]. A stock culture of each pathogen was maintained on potato dextrose agar (PDA) at 4°C. The working culture was prepared by transferring a stock agar plug containing the mycelium onto PDA plates and incubating for 3 days at 22°C.

Antagonistic bacteria were isolated from straw and manure samples, compost at various stages of composting and casing soil used for growing button mushrooms (Table 2). Five g of each sample were resuspended in 100 mL of sterile distilled water and mixed in a magnetic stirrer for 10 min. The method for isolation of *Bacillus* spp. strains was based on the resistance of their endospores to elevated temperatures. Test tubes containing 2 mL of each sample suspension were placed in a water bath at 80°C for 10 min so that only endospores, not vegetative cells, would be present in the mixture [11]. Concentrated samples, as well as several successive decimal dilutions were spread on the surface of nutrient agar (NA) and PDA plates, and incubated at 30°C for 48 h. Similarly, samples that were not exposed to elevated temperatures and their successive decimal dilutions were spread on NA and PDA plates and incubated at 40˚C for 48 h. The samples for isolation of *Pseudomonas* spp. were not exposed to high temperature and were spread on King's medium B (Proteose peptone No.3, 20 g/L; K_2HPO_4, 1.5 g/L; $MgSO_4$, 1.5 g/L; Agar, 15 g/L; Glycerol, 10 mL/L); PDA plates were incubated at 26°C for 48 h. Bacterial isolates were preliminarily characterized using a rapid method of Gram differentiation without staining [12], and the catalase test.

In vitro antagonistic activity

A preliminary screening of bacterial antagonism against pathogenic *T. aggressivum* f. *europaeum*, as well as screening against all four indicator strains of fungi, were carried out using the dual culture method, in triplicate, as described earlier [13]. The 10-mm agar disks of each tested pathogen were placed on one side of the PDA plates and a loopful of each antagonistic bacterial isolate from an overnight culture was streaked 3 cm away from the edge of the same plate. Plates inoculated with only a pathogen culture served

Table 2. Preliminary screening of antagonistic activity of bacterial isolates against *Trichoderma aggressivum* f. *europaeum* and characterization of isolates.

Isolate	Source	Antagonism	Gram differentiation	Catalase test	Species	Accession number
B-106	Straw	+	+	+	*B. subtilis*	KT692716
B-107	Straw	+	+	+	*B. subtilis*	KT692717
B-108	Straw	+	+	+	*B. subtilis*	KT692718
B-109	Straw	+	+	+	*B. subtilis*	KT692719
B-110	Straw	+	+	+	*B. subtilis*	KT692720
B-111	Straw	+	+	+	*B. subtilis*	KT692721
B-112	Straw	+	+	+	*B. subtilis*	KT692722
B-124	I stage 8. day	+	+	+	*B. subtilis*	KT692723
B-126	I stage 14. day	+	+	+	*B. subtilis*	KT692724
B-128	II stage	+	+	+	*B. subtilis*	KT692725
B-129	II stage	+	+	+	*B. amyloliquefaciens*	KT692726
B-138	Manure	+	+	+	*B. pumilus*	KT692727
B-217	I stage 14. day	+	+	+	*B. pumilus*	KT692728
B-233	I stage 3. day	+	+	+	*B. subtilis*	KT692729
B-241	I stage 14. day	+	+	+	*B. amyloliquefaciens*	KT692730
B-252	Straw	+	+	+	-	-
B-253	Manure	+	+	+	*B. subtilis*	KT692731
B-254	Manure	+	+	+	*B. pumilus*	KT692732
B-256	Manure	+	+	+	*B. pumilus*	KT692733
B-257	Manure	+	+	+	*B. pumilus*	KT692734
B-268	Casing soil	+	+	+	*B. amyloliquefaciens*	KT692735
B-270	III stage	+	+	+	*B. subtilis*	KT692736
B-276	III stage	+	+	+	*B. licheniformis*	KT692737

as controls. After 72-h incubation at 25°C, the size of the growth inhibition zone was measured and the percentage of growth inhibition (PGI) was calculated using the formula:

$$PGI\ (\%) = (KR - R1)/KR \times 100,$$

where KR represents the colony diameter of the pathogen in the control plate, and R1 represents the colony diameter in the treated plate [14].

DNA isolation and purification

Bacterial DNA was isolated using a modified method by Pastrik and Maiss [15]. Cultures were grown overnight in nutrient broth at 30°C, with shaking at 180 rpm. After 5 min of centrifugation at 13000 g at 25°C, the pellet was resuspended in 500 µL of TE buffer with lysozyme and incubated for 30 min at 37°C. The samples were then treated with 100 µL of 5M NaCl and 300 µL of 3% CTAB + PVP buffer and incubated for 20 min at 65°C. In the next step, 900 µL of chloroform was added and after a 10-min centrifugation, the supernatant was transferred to new test tubes and 3 M Na-acetate (1/10 of the volume) and ice-cold isopropanol (up to 1.5 mL of the test tube volume) were added. After a 15-min centrifugation, the samples were rinsed with 96% ice-cold ethanol. In order to further purify the isolated DNA, 200 µL of dH_2O and 100 µL of phenol were added and the mixture was centrifuged for 10 min, after which the supernatant was transferred to new test tubes and 3 M Na-acetate (1/10 of the sample volume) and a 2.5 x volume of cold 96% ethanol were added. The mixture was incubated for 10 min at room temperature and centrifuged for 20 min. In order to precipitate the DNA, 1 mL of 70% cold ethanol was added to the test tubes containing pellet, and the material was centrifuged for 5 min. Finally, the pellet was dried for 10 min at 37°C and resuspended in 50 µL of 10 mM tris, 1 mM EDTA, pH=7.6 (TE) buffer containing 1 µL of RN*ase* (10 mg/mL).

PCR analysis and detection of PCR products

The isolates, characterized as *Bacillus* species, that showed clear antagonism against some of the tested

pathogenic fungi, were subjected to further identification based on analysis of the partial 16S rDNA sequence. Amplification of the hypervariant region (HV region) of the 16S rDNA was carried out using primers 16S-HV-F (5'-TGTAAAACGACGGCCAGTGCCTAATACATGCAAGTCGAGCG-3') and 16S-HV-R (5'-CAGGAAACAGCTATGACCACTGCTGCCTCCCGTAGGAGT-3') [16]. The PCR amplification was performed in a 25-μL reaction mixture containing 12.5 μL of 1xPCR Master Mix (Fermentas, Lithuania) (0.625 U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM of each dNTP), 1 μL of each primer (10 μM), 1 μL of the template DNA. The PCR reaction was performed as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s and extension at 72°C for 90 s, and a final extension step at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis on a 1.5% agarose gel in 1xTE buffer at a constant voltage of 100V for 35 min. The gel was stained with 0.1% ethidium bromide and visualized with a transilluminator. All obtained PCR fragments were sequenced using the Macrogen sequencing service (the Netherlands).

Phylogenetic analysis

In order to identify the bacterial isolates, all obtained sequences were compared to partial 16S rDNA sequences published in the NCBI GenBank database, using a BLAST program [17] and checked manually for nucleotide positions. The closest related type strain sequences were aligned using CLUSTAL W [18] implemented in the BioEdit program. The phylogenetic tree was constructed with MEGA 6.06 using the neighbor-joining method. The obtained sequences were deposited in the NCBI database under the accession numbers given in Table 2.

Statistical analysis

The basic statistical parameters were calculated and the obtained information was presented in histograms as mean values of the mycelial growth inhibition percentage. The data were examined using one-way analysis of variance (ANOVA). The test was used to compare the inhibition of mycelial growth of four fungal pathogens caused by the activity of 23 antagonistic bacterial strains. The mean separation of percentages of mycelial growth inhibition was achieved by Tukey's HSD (honest significance difference) test. The significance was evaluated at $P<0.05$ for all tests. Statistical analyses were conducted by the general procedures of IBM SPSS Statistics v.19 (SPSS Inc.).

RESULTS

Antagonistic bacteria

Out of the total 108 bacterial isolates, 7 belonged to the genus *Pseudomonas* (forming green fluorescent colonies on King's B medium) and the rest were assumed to belong to the genus *Bacillus* based on the specific isolation procedure. In the preliminary investigation of antagonism against the most aggressive pathogen, *T. aggressivum* f. *europaeum*, 23 isolates showed antagonistic effects (Table 2). All isolates were proven to be Gram-positive and catalase-positive. These isolates were selected for further testing against all four *Trichoderma* spp.

In vitro antagonistic activity

The *in vitro* antagonistic potential of the 23 natural isolates of *Bacillus* sp. against several causal agents of green mold disease was evaluated (Fig. 1). The *Bacillus* isolates demonstrated a good antagonistic potential against the tested *Trichoderma* spp., with some exceptions.

The mean values of the growth inhibition percent (PGI) of the tested pathogens are shown in Fig. 2. *T. aggressivum* f. *europaeum* was shown to be the most sensitive to antagonistic bacteria. Next, the activity of 16 bacterial isolates caused significant growth inhibition of this pathogen (PGI>70%). The PGI of *T. harzianum* was slightly lower but nonetheless noteworthy, with the isolates B-112 and B-252 causing the smallest inhibition of mycelial growth (PGI 49.75% and 45.94%, respectively). The most significant inhibition of mycelial growth of *T. koningii* (PGI>70%) was due to the antagonistic potential of 10 isolates (B-106, B-107, B-108, B-109, B-110, B-217, B-233, B-241, B-253 and B-268), while the isolates B-112, B-126 and B-276 caused the lowest growth inhibi-

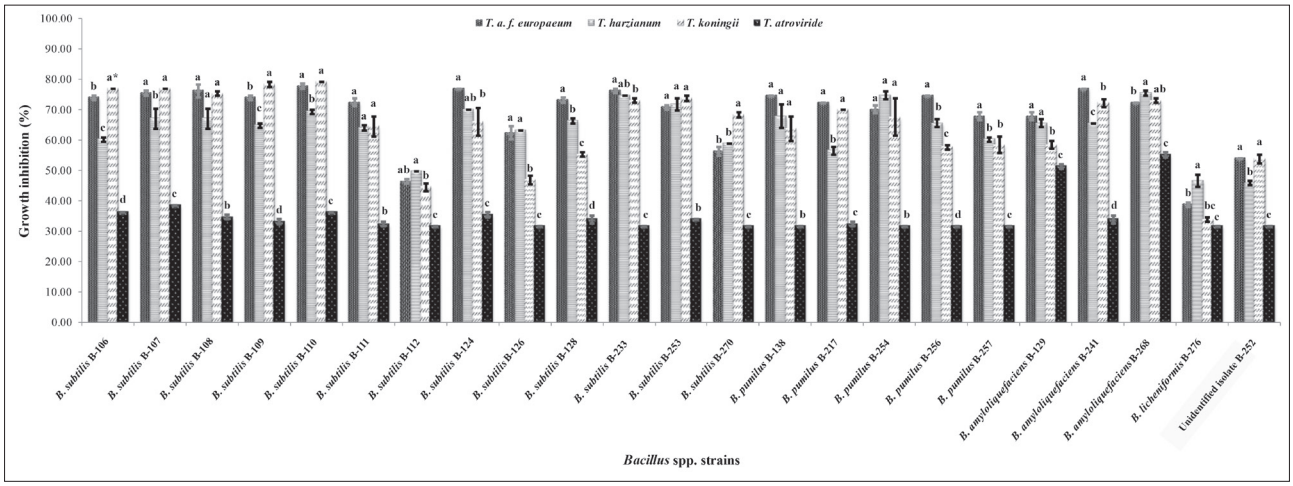

Fig. 1. Dual culture method for determining the antagonistic potential of bacterial isolates. The activity of B-106 isolate against: (a) *Trichoderma aggressivum* **f.** *europaeum***, (b)** *Trichoderma harzianum***, (c)** *Trichoderma koningii* **and (d)** *Trichoderma atroviride*

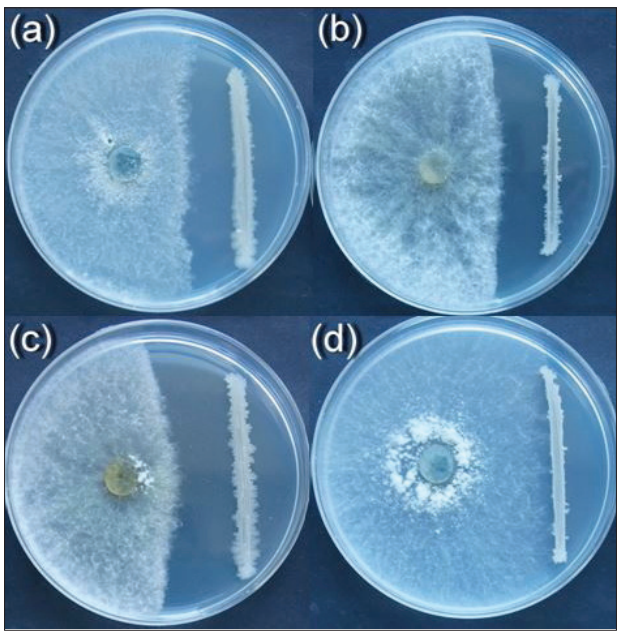

Fig. 2. *In vitro* antifungal activity of *Bacillus* spp. strains against *Trichoderma* spp. strains. Mean values of percent of inhibition (n=3) of fungal growth with standard error for each antagonistic strain are shown. *Values followed by the same letter in columns with all tested fungi for each isolate are not significantly different (P<0.05), according to Tukey's HSD test.

tion (below 50%) of this pathogen. Only two isolates, B-129 and B-268, caused significant inhibition of *T. atroviride* growth (PGI 51.51% and 55.31%, respectively), which makes this fungus the most resistant of all the tested pathogens. Furthermore, the lowest inhibition of the mycelial growth (<50%) of all four tested pathogens was induced by the activity of two isolates, B-112 and B-276. To summarize, most of the isolates demonstrated strong antagonistic activity against at least two tested pathogens, two isolates (B-129 and B-268) exhibited the activity against all four tested pathogens, whereas isolates B-112, B-252 and B-276 had the weakest antagonistic activity.

The ANOVA results confirmed the existence of statistically significant differences in the antagonistic abilities of all *Bacillus* spp. strains against four fungal pathogens. When observing the activity of each strain individually, the PGI values (followed by the same letter) presented in Fig. 2 suggested that some strains were not significantly different in their activity against two or more pathogenic fungi.

Molecular identification of antagonistic bacterial strains

In order to identify *Bacillus* isolates to the species level, amplification and sequencing of the 16S rDNA hypervariant region were carried out. As can be seen in Fig. 3, a single clear PCR product of about 320 bp was detected in all but one of the screened isolates. For isolate B-252, the size of the PCR product was smaller than expected, which indicated that this isolate might not belong to *Bacillus* genera. This bacterial isolate was therefore not sequenced and remained unidentified.

The phylogenetic reconstruction based on the sequence of HV region of 16S rRNA gene showed clustering of the isolates B-138, B-217, B-254, B-256 and B-257 with *Bacillus pumilus* strains. The isolate

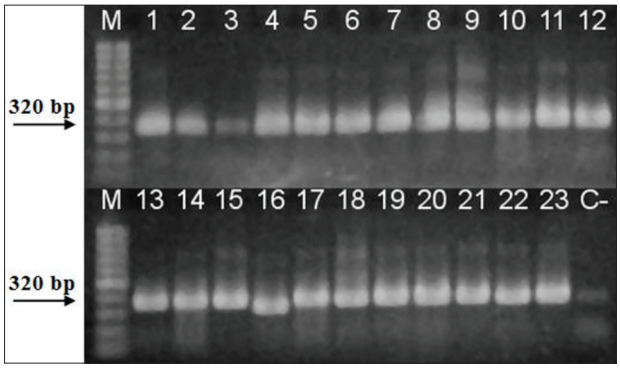

Fig. 3. Detection of amplified partial 16S rDNA (HV region) obtained from *Bacillus* sp. isolates. M – molecular weight marker 100 bp ladder; 1 – B-106, 2 – B-107, 3 – B-108, 4 – B-109, 5 – B-110, 6 – B-111, 7 – B-112, 8 – B-124, 9 – B-126, 10 – B-128, 11 – B-129, 12 – B-138, 13 – B-217, 14 – B-233, 15 – B-241, 16 – B-252, 17 – B-253, 18 – B-254, 19 – B-256, 20 – B-257, 21 – B-268, 22 – B-270, 23 – B-276; C – is a negative control sample without DNA.

B-276 was the closest related to *Bacillus licheniformis* MML2507 (GU084411.1). Isolates B-129, B-241 and B-268 clustered with *Bacillus amyloliquefaciens* strains, while 13 isolates (B-106, B-107, B-108, B-109, B-110, B-111, B-112, B-124, B-126, B-128, B-233, B-253 and B-270) formed a separate branch with several *Bacillus subtilis* strains (Fig. 4).

DISCUSSION

Green mold disease was recently detected in Serbian mushroom farms for the first time. The causal agents of green mold, which is responsible for substantial economic losses in mushroom production, were identified as *Trichoderma aggressivum* f. *europaeum*, *T. harzianum*, *T. koningii*, *T. atroviride* and *T. virens* [3]. The

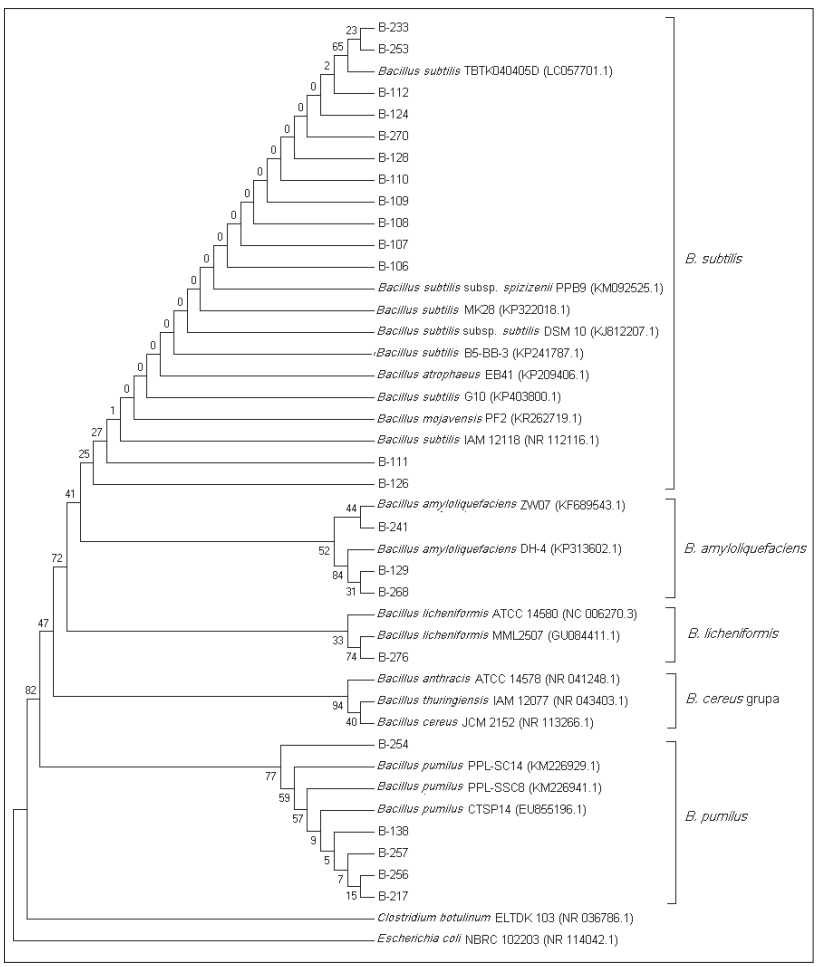

Fig. 4. Phylogenetic tree based on partial 16S rDNA sequences (275bp) of *Bacillus* spp. *Clostridium botulinum* (NR_036786.1) and *Escherichia coli* (NR_114042.1) were used as outgroups. The numbers on branching points indicate bootstrap values from neighbor-joining analysis.

frequent resistance of pathogens to chemical fungicides used in pathogen control along with rising environmental awareness has directed research toward biological control based on antagonistic microorganisms.

Of a total 108 bacterial isolates, 23 showed antagonistic activity against at least two tested fungal pathogens. In previous research, analysis of the partial 16S rRNA gene sequence showed the hypervariant region of this gene to be highly specific for each strain and to be highly conserved within species [16]. Therefore, one of the greatest problems in the identification of *Bacillus* species – the inability to make a clear distinction between *B. subtilis* and *B. amyloliquefaciens* phylogenetic branches – had to be addressed. The identification of antagonistic *Bacillus* spp. strains showed consistency with a number of previously conducted studies that specifically identified these species as effective biocontrol agents against many plant pathogens. For instance, *B. subtilis* isolated from citrus fruit surface was proven to be effective in biocontrol of green and blue mold of these fruits caused by *Penicillium digitatum* and *Penicillium italicum* [19]. *B. licheniformis* showed a great potential in the biocontrol of *Botrytis cinerea*, the causal agent of tomato gray mold disease [20,21], while *B. amyloliquefaciens* strains were proven to produce various antibiotics [22]. Additionally, a study reported that *B. pumilus* antifungal metabolites inhibited mycelial growth of numerous species belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera [23] and clearly showed that some *B. subtilis* and *B. licheniformis* strains could efficiently inhibit the growth of *Trichoderma* species [24]. In the present study, the isolates that were effective in inhibiting the growth of all four pathogens (B-129 and B-268) were identified as *B. amyloliquefaciens*, which is in agreement with the results of a study that showed the great potential of two *B. amyloliquefaciens* strains (SS-12.6 and SS-13.1) in antagonizing a variety of plant pathogens [25].

Screening is a crucial step in the process of identifying suitable candidates for biological control and is responsible for the success of all subsequent stages of the experiment. The method used in the present study proved to be suitable for testing the antagonistic potential of bacterial isolates, with the size of the growth inhibition zones and the PGI being reliable measures for antagonistic activity. Given the results, it can be concluded that a great number of obtained *Bacillus* spp. strains are strong antagonists of green mold disease in button mushroom. Based on the percentage of mycelial growth inhibition, *T. aggressivum* f. *europaeum* proved to be the most sensitive pathogen to antagonistic bacteria, which points to the significant role of the obtained antagonists in the control of this most devastating of molds. Furthermore, two *B. amyloliquefaciens* strains, B-129 and B-268, inhibited the growth of all four pathogenic fungi, thus exhibiting a wider spectrum of activity than other *Bacillus* spp. strains. In addition, the fact that there was at least one *Bacillus* isolate obtained from each sample of the isolation material is of importance when considering the possibility of their application as biopesticides. More precisely, it allows for biopesticide treatment at every stage of composting and mushroom growing, thus providing better control of *Trichoderma* spp., one of the most common and most harmful button mushroom pathogens.

The mode of action of bacterial antagonists against pathogens remains unknown. Given that *Bacillus* species are known to produce a vast amount of antimicrobial secondary metabolites, the most probable mode of action is antibiosis [26]. One of the most studied microorganisms, *B. subtilis*, can utilize 4-5% of the genome for the production of antibiotics [27], and in *B. amyloliquefaciens*, an even greater part of the genome is included in the biosynthesis of these molecules [28]. However, other modes of action, such as competition, could also be responsible for the inhibition of *Trichoderma* spp. growth. With regard to this, determining the exact mode of action for each *Bacillus* spp. strain could be of interest in further research.

As *in vitro* results do not necessarily translate to what occurs *in vivo*, a crucial step in this investigation would be to test the sensitivity of the button mushroom mycelium to the most efficient antagonists and to conduct an *in vivo* experiment in a mushroom-growing room with selected antagonists.

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Authors' contribution: We declare that all authors participated in the research and article preparation. OS participated in the acquisition, analysis and interpretation of the data; the author drafted article. SMM designed the study, participated in the ac-

quisition and analysis of the data and revision of the manuscript. IP and ID participated in the analysis of the data. MS participated in the collection of the data. SS participated in the design of the experiments and revision of the manuscript. TB conceived and designed the study and revised the article. All authors approved the submitted version of the article.

Conflict of interest disclosure: The authors declare that there is no actual or potential conflict of interest, including financial, personal or other relationships with other people or organizations that could inappropriately influence this work.

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