

The activity of native *Bacillus subtilis* strains in control of green mould disease of oyster mushroom (*Pleurotus* spp.)

Ivana Potočnik^{1*}, Svetlana Milijašević-Marčić¹, Olja Stanojević², Tanja Berić², Slaviša Stanković², László Kredics³ and Lóránt Hatvani³

¹*Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Serbia*

²*University of Belgrade, Faculty of Biology, Microbiology Department, Studentski trg 16, 11000 Belgrade, Serbia*

³*University of Szeged, Faculty of Science and Informatics, Department of Microbiology, H-6726 Szeged, Középfasor 52, Hungary*

*Corresponding author: ivana.potocnik@pesting.org.rs

Received: 6 September 2019

Accepted: 24 September 2019

SUMMARY

The study aimed to isolate potential biocontrol agents from mushroom substrate that could serve as an alternative to toxic chemicals commonly used for disease control in mushroom production. The antagonistic potential of ten native *Bacillus subtilis* strains against the causal agents of green mould disease of oyster mushroom, *Trichoderma pleuroti* and *Trichoderma pleuroticola*, was evaluated. The antagonistic potential of *Bacillus* spp. strains was quantified *in vitro* based on dual cultivation with the pathogen. Growth inhibition of *T. pleuroti* ranged from 54.44% to 62.22% and no significant differences in antagonistic activity were found between the tested *B. subtilis* strains. Inhibition of *T. pleuroticola* was slightly higher, ranging from 55.56% to 69.62% and *B. subtilis* strain B-358 induced the highest growth inhibition. This research confirmed mushroom substrate to be a good source of antagonistic microorganisms with potentials for use in biological control of green mould in oyster mushroom production.

Keywords: oyster mushrooms; green mould disease; *Bacillus subtilis*; antagonistic activity; biocontrol

INTRODUCTION

Oyster mushrooms (*Pleurotus* spp.) are the third most commonly cultivated basidiomycetes worldwide. Their production can be negatively affected by different fungi, bacteria, viruses, nematodes and arthropods,

but the most severe crop losses have been caused by green moulds. Initially, severe green mould infections of oyster mushroom, caused by *Trichoderma* spp., have been found in South Korea (Park et al., 2004, 2006). Subsequent reports of green mould outbreaks from other countries, including Italy (Woo et al., 2004),

Hungary (Hatvani et al., 2007, 2008) and Romania (Kredics et al., 2006), indicated a worldwide threat. *Trichoderma* species isolated from substrates used for the cultivation of *Pleurotus* spp. differed from those found on *Agaricus bisporus* (Lange) Imbach, and they have been described as *Trichoderma pleurotum* S.H. Yu & M.S. Park (currently accepted, correct name: *Trichoderma pleuroti*) and *Trichoderma pleuroticola* S.H. Yu & M.S. Park (Park et al., 2006). *T. pleuroticola*, closely related to *T. aggressivum* in damaging *A. bisporus*, is the most serious threat to oyster mushroom cultivation and has been found widely in soils or on decaying wood. Furthermore, it is more efficient in causing inhibitory effects against *P. ostreatus* in the substrate, and in utilizing carbon sources, compared to *T. pleuroti*. So far, *T. pleuroti* has not been found in natural environments, only in association with cultivated oyster mushroom. Nevertheless, *T. pleuroticola* is dominant in Italian *Pleurotus* farms, while the majority of isolates from Hungary belong to the species *T. pleuroti* (Komoń-Zelazowska et al., 2007). Green mould infection of *P. ostreatus* is supposed to be transmitted via the substratum for cultivation (cereal straw, sawdust, waste cotton, etc.).

The usual method of controlling mould diseases in mushroom farms worldwide is based on the use of fungicides (Milijašević-Marčić et al., 2017). However, the development of pathogen resistance to fungicides after frequent applications, and the sensitivity of edible mushrooms to treatment compounds are serious problems. Due to resistance incidence to benzimidazoles in mycopathogenic fungi, and evidence of mutagenic effects of this group of fungicides to mammals, the number of available fungicides has decreased. Prochloraz has been selected as the most effective compound for the control of green mould disease on mushroom beds, although its high concentrations have shown harmful effects on mycelial growth and fruiting body development of *Pleurotus* spp. (Hatvani et al., 2008). The effects of some antagonistic microorganisms have been tested as part of efforts to improve crop protection options. Biopesticides are environment-friendly, have high specificity against target pathogens, and operate through various modes of action. *Bacillus* species are extremely competitive and produce a variety of secondary metabolites: antibiotics, antifungal volatile substances and cell-wall degrading enzymes, which contribute to their antagonistic potential. Additionally, they are safe for the environment, harmless to human health and are generally recognized as safe (GRAS) organisms (Gajbhiye & Kapadnis, 2016). Earlier studies have

shown that strains of *B. subtilis* and *B. licheniformis* efficiently inhibited the growth of *T. harzianum*, *T. viride* and *T. pseudokoningii* in the cultivation of *P. sajor-caju* and *Lentinula edodes* (Chittihunsa et al., 2007). Strains of these two species from mushroom compost were also found effective against *T. pleuroti* on oyster mushroom (Nagy et al., 2012). Remarkable antagonistic activities of *Bacillus* spp. originating from livestock manure and cotton-waste composts were also detected against *T. harzianum*, *T. koningii* and *T. viridescens* in *P. ostreatus* cultivation (Kim et al., 2008).

The objective of this study was to determine and compare the antagonistic activity of *Bacillus* spp. strains isolated from substrates for oyster and button mushroom cultivation (straw, manure, mushroom compost, black peat) against *T. pleuroti* and *T. pleuroticola*, the causal agents of oyster mushroom green mould disease.

MATERIAL AND METHODS

Test organisms and culture conditions

The native isolates of *Bacillus* spp. from the culture collection of the Institute of Pesticides and Environmental Protection which were used in the present study had been isolated from chicken manure samples and compost at various stages of composting, obtained from the composting facility Uča & Co, Vranovo, Serbia (Table 1). The antagonistic *Bacillus* spp. isolates used in the study had been identified previously, based on the partial sequence of their 16S rRNA gene (Stanojević et al., 2019). The isolates were stored in Luria-Bertani broth with 30% glycerol at -20 °C until further use.

Table 1. Antagonistic bacteria tested in the study

Strains	Source	Antagonism to <i>Trichoderma</i> spp.
<i>Bacillus subtilis</i> B-308	Manure	+
<i>Bacillus subtilis</i> B-309	Manure	+
<i>Bacillus subtilis</i> B-310	Manure	+
<i>Bacillus subtilis</i> B-313	Manure	+
<i>Bacillus subtilis</i> B-319	Phase I day 3	+
<i>Bacillus subtilis</i> B-322	Phase I day 3	+
<i>Bacillus subtilis</i> B-325	Phase I day 3	+
<i>Bacillus subtilis</i> B-338	Phase I day 8	+
<i>Bacillus subtilis</i> B-348	Phase II	+
<i>Bacillus subtilis</i> B-358	Phase III	+

The pathogenic fungal isolates *T. pleuroti* SZMC 12454 and *T. pleurotica* SZMC 23033 used as indicator strains for testing bacterial antagonism were obtained from the culture collection of the Department of Microbiology, University of Szeged, Hungary (Szeged Microbiology Collection, <http://szmc.hu/>). Identification of these isolates was performed based on sequence analysis of the ribosomal RNA internal transcribed spacer (ITS) region using the primer set ITS1/ITS4, as described by Andersson et al. (2009). A stock culture of each pathogen was maintained on potato dextrose agar (PDA) (Lab M Limited, Lancashire, UK) at 4°C. The working culture was prepared by transferring stock agar plugs containing the mycelium onto PDA plates and incubated for 3 days at 22°C.

In vitro antagonistic activity

The *in vitro* method for quantification of antagonistic potential of *Bacillus* spp. strains was based on dual cultivation with the pathogen, and was carried out in three replicates. Agar discs with fresh mycelia of each pathogenic test fungus were transferred to PDA media, while bacterial cultures were streaked at 3 cm distance. Plates inoculated only with the fungus served as control plates. After incubation for 72 h at 25 °C, the size of each inhibition zone was measured, along with mycelial growth both in confronted (R1) and control plates (CR). The percentage of growth inhibition (PGI) of the pathogen was calculated according to the formula:

$$\text{PGI}(\%) = [(CR - R1) / CR] \times 100$$

where CR represents the pathogen's colony radius in control plates, and R1 represents the colony radius of the pathogen towards the bacterial isolate in treated plates (Korsten & De Jager, 1995).

Statistical analyses

The basic statistical parameters were calculated and the obtained information was presented in histograms as means of 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 two fungal pathogens caused by the activity of 10 antagonistic bacterial strains. The mean separation of percentages of mycelial growth inhibition was achieved by Tukey's HSD (honest significance difference) test. The significance of differences was evaluated at $P < 0.05$ for all tests.

Statistical analyses were conducted by the general procedures of IBM SPSS Statistics v 2.0 (SPSS Inc.).

RESULTS

In vitro antagonistic activity

Two hundred and twelve bacterial isolates were obtained from substrates used for growing mushrooms. After preliminary screening, ten native *Bacillus* isolates were chosen for *in vitro* tests of their antagonistic potential against two causal agents of green mould disease of oyster mushroom (*T. pleuroti* and *T. pleurotica*). The results of the inhibition potential of these isolates in dual culture are shown in Table 2. The *Bacillus* isolates demonstrated different levels of antagonistic potential against the tested *Trichoderma* spp. Percentage growth inhibition (PGI) means of the test pathogens are shown in Figures 1 and 2. Based on a comparison of PGI values, *T. pleurotica* was shown to be more sensitive to the test bacteria than *T. pleuroti*. Growth inhibition of *T. pleuroti* ranged from 54.44% to 62.22% and no significant differences were found among the PGI values of ten *Bacillus* isolates. The PGI of *T. pleurotica* was slightly higher, ranging from 55.56% to 69.62%, depending on the isolates. Strain B-358 induced the highest inhibition of *T. pleurotica*, while strain B-309 had the least effect on growth of this mushroom mould. The ANOVA results confirmed statistically significant differences in the antagonistic activity of *B. subtilis* strains only against *T. pleurotica*.

Table 2. Inhibition zones of *T. pleuroti* and *T. pleurotica* (mm) in dual culture test with *Bacillus subtilis* isolates

Isolate code	<i>T. pleuroti</i>	<i>T. pleurotica</i>
B-308	11	12
B-309	10	10
B-310	11	14
B-313	12	13
B-319	11.5	12.5
B-322	11.5	14
B-325	10.5	12
B-338	10	11
B-348	10	11.5
B-358	11.5	14.5

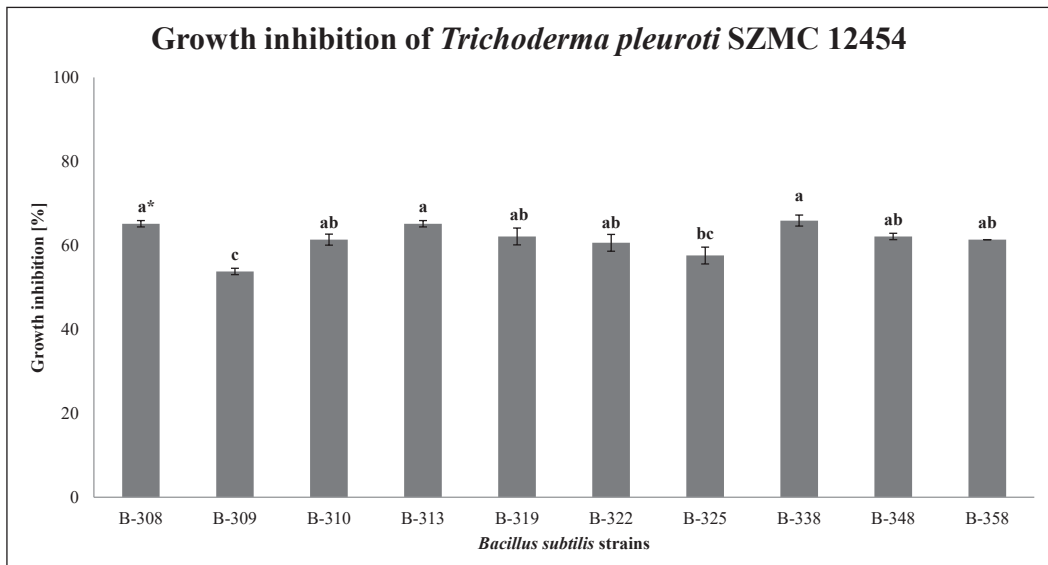


Figure 1. Inhibition of mycelial growth of *T. pleuroti* SZMC 12454 by *B. subtilis* strains. Mean values (n=3) with standard errors are shown. One-factor analysis of variance was performed by Tukey's HSD test. *Values marked by the same letter are not significantly different ($p < 0.05$)

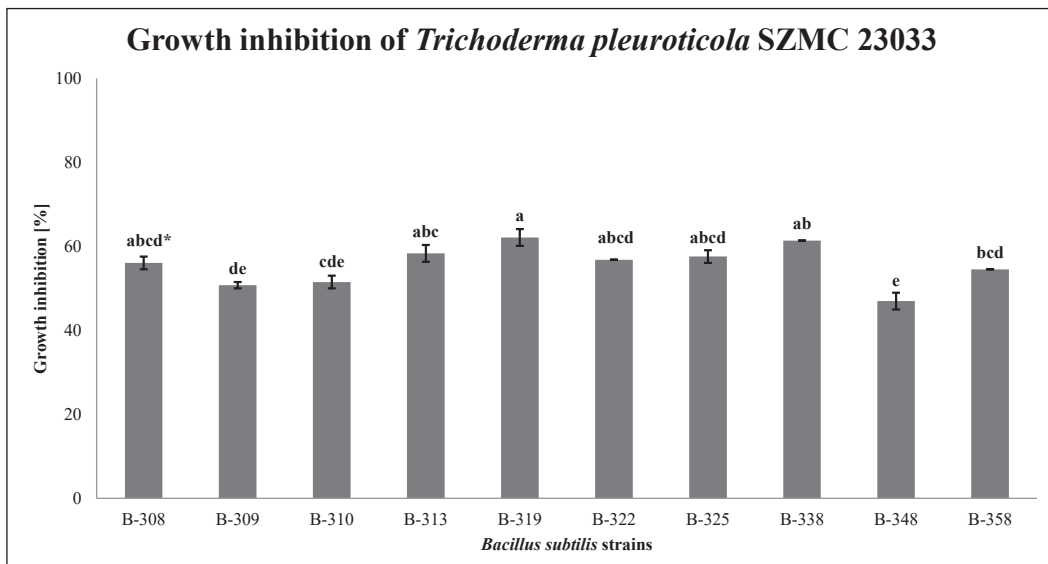


Figure 2. Inhibition of mycelial growth of *T. pleuroticola* SZMC 23033 by *B. subtilis* strains. Mean values (n=3) with standard errors are shown. One-factor analysis of variance was performed by Tukey's HSD test. *Values marked by the same letter are not significantly different ($p < 0.05$)

DISCUSSION

It is well-known that microbial inoculants with antagonistic properties towards pathogens of agricultural crops have the potential to replace chemical pesticides which can be harmful to the environment and human health (Solanki et al., 2015). As reported before (Walker

et al. 1998, Yoshida et al., 2001; Stanojević et al., 2016), the technique of dual culture on agar plates has proved to be an efficient and easy way to select antagonistic bacteria from a random group of bacterial isolates based on their ability to inhibit fungal growth. Several previous studies had shown that *Bacillus* spp. recovered from mushroom compost exhibited strong activity against causal agents

of green mould of *A. bisporus*, such as *T. aggressivum* f. *europaeum*, *T. harzianum*, *T. koningii* and *T. atroviride* (Stanojević et al., 2016, Milijašević-Marčić et al., 2017). Additionally, Kim et al. (2008) tested 20 *Bacillus* spp. from livestock manure and cotton-waste composts. They reported inhibition zones of 9, 12 and 6 mm against *T. harzianum*, *T. koningii* and *T. viridescens*, respectively, similar to the effect that *B. subtilis* strains had in the present study against *T. pleuroti* (10 to 12 mm) and *T. pleuroticola* (10 to 14.5 mm). Furthermore, an evaluation of biocontrol agents against three *Trichoderma* species (*T. harzianum*, *T. viride* and *T. pseudokoningii*) occurring in *P. sajor-caju* cultivation showed that the best percentage of inhibition was produced by three *Bacillus* spp. (35.1%-51.1%) (Shah & Nasreen, 2011). The bacterial strain CNU LI-1 and a range of *Bacillus* spp. were able to inhibit the mycelial growth of *Trichoderma* and *Hypocrea* species *in vitro*, and their inoculation into pre-sterile substrate was effective for preventing the occurrence of green mould without significantly affecting *Pleurotus* (Hatvani et al., 2008). Nagy et al. (2012) found that *B. subtilis* and *B. amyloliquefaciens* strains were very effective against *T. pleuroti* *in vivo* and improved crop yield by 10%. These authors found that treatment with *B. amyloliquefaciens* strain 27 in the amount of 10^7 bacteria g^{-1} substrate proved to be the most efficient, increasing crop yield by 15-21%. In the current study, the highest growth inhibition of *T. pleuroti* induced by *B. subtilis* strains was 62.22%, while the highest PGI of *T. pleuroticola* was 69.62%. Strain B-358 induced the highest inhibition of *T. pleuroticola* among the *B. subtilis* strains tested in the study, while all examined strains had good antifungal effect against *T. pleuroti*. This research also confirmed composting material as a good source of antagonistic microorganisms with a potential for use in biological control of green mould in oyster mushroom production. However, to find an adequate biocontrol candidate, it is necessary to test the sensitivity of *P. ostreatus* to *B. subtilis* strains, and to conduct *in vivo* trials under growing conditions.

ACKNOWLEDGEMENT

This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia [grants TR31043 and OI 173026], and by the Hungarian Government and European Union Széchenyi 2020 Programme [grant GINOP-2.3.2-15-2016-00052] and the Hungarian National Research, Development and Innovation Office [grant NKFI K-116475]. László Kredics was supported by the János Bolyai Research Scholarship.

REFERENCES

- Andersson, M.A., Mikkola, R., Raulio, M., Kredics, L., Maijala, P., & Salkinoja-Salonen M.S. (2009). Acrebol, a novel toxic peptaibol produced by an *Acremonium exuviarum* indoor isolate. *Journal of Applied Microbiology*, 106, 909-923. <https://doi.org/10.1111/j.1365-2672.2008.04062.x>
- Chittihunsa, T., Bangeekhan, E., Wongsamitkul, N., & Subsomboon, T. (2007). Screening of *Bacillus* spp. suppressing the infection of *Trichoderma* sp. in mushroom cultivation. *KMITL Science and Technology Journal* 7(S1), 19-27.
- Gajbhiye, M.H., & Kapadnis, B.P. (2016). Antifungal-activity-producing lactic acid bacteria as biocontrol agents in plants. *Biocontrol Science and Technology* 26 (11), 1451-1470. <https://doi.org/10.1080/09583157.2016.1213793>
- Hatvani, L., Antal, Z., Manczinger, L., Szekeres, A., Druzhinina, I.S., Kubicek ... Kredics, L. (2007). Green mould diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* species. *Phytopathology* 97(4), 532-537. doi: 10.1094/phyto-97-4-0532
- Hatvani, L., Kocsubé, S., Manczinger, L., Antal, Z., Szekeres, A., Druzhinina, I.S ... Kredics, L. (2008). The green mould disease global threat to the cultivation of oyster mushroom (*Pleurotus ostreatus*): a review. In M. Van Greuning (Ed.), *Science and cultivation of edible and medicinal fungi: Mushroom science XVII, Proceedings of XVII Congress of the International Society for Mushroom Science*, Cape Town, South Africa (pp 485-495). Pretoria, SA: South African Mushroom Farmers Association (CD-ROM).
- Kim, W.G., Weon, H.Y., Seok, S.J., & Lee, K.H. (2008). *In vitro* antagonistic characteristics of Bacilli isolates against *Trichoderma* spp. and three species of mushrooms. *Mycobiology* 36(4), 266-269. doi: 10.4489/MYCO.2008.36.4.266
- Komoń-Zelazowska, M., Bissett, J., Zafari, D., Hatvani, L., Manczinger, L., Woo, S. ... Druzhinina, I.S. (2007). Genetically closely related but phenotypically divergent *Trichoderma* species cause green mold disease in oyster mushroom farms worldwide. *Applied and Environmental Microbiology*, 73(22), 7415-7426.
- Korsten, L., & De Jager, E.E. (1995). Mode of action of *Bacillus subtilis* for control of avocado postharvest pathogens. *South African Avocado Growers' Association Yearbook*, 18, 124-130.
- Kredics, L., Hatvani, L., Antal, Z., Manczinger, L., Druzhinina, I.S., Kubicek, C.P.... Nagy, E. (2006). Green mould disease of oyster mushroom in Hungary and Transylvania. *Acta Microbiologica et Immunologica Hungarica* 53, 306-307.

- Nagy, A., Manczinger, L., Tombácz, D., Hatvani, L., Györfi, J., Antal, Z. ... Kredics, L. (2012). Biological control of oyster mushroom green mould disease by antagonistic *Bacillus* species. *IOBC-WPRS Bulletin* 78, 289-293.
- Milijašević-Marčić, S., Stepanović, M., Todorović, B., Duduk, B., Stepanović, J., Rekanović, E., & Potočnik, I. (2017). Biological control of green mould on *Agaricus bisporus* by a native *Bacillus subtilis* strain from mushroom compost. *European Journal of Plant Pathology* 148(3), 509-519.
- Park, M.S., Bae, K.S., & Yu, S.H. (2004). Molecular and morphological analysis of *Trichoderma* isolates associated with green mold epidemic of oyster mushroom in Korea. *Journal of Huazhong Agricultural University* 23, 157-164.
- Park, M.S., Bae, K.S., & Yu, S.H. (2006). Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. *Mycobiology* 34, 111-113. doi:10.4489/myco.2006.34.3.111
- Shah, S., & Nasreen, S. (2011). Evaluation of bioagents against the infection of green mould (*Trichoderma* spp.) in *Pleurotus sajor-caju* cultivation. *International Journal of Plant Pathology* 2(2), 81-88. doi:10.3923/ijpp.2011.81.88
- Solanki, M.K., Singh R.K., Srivastava, S., Kumar, S., Kashyap, P.L., & Srivastava, A.K. (2015). Characterization of antagonistic-potential of two *Bacillus* strains and their biocontrol activity against *Rhizoctonia solani* in tomato. *Journal of Basic Microbiology* 53, 1-9.
- Stanojević, O., Berić, T., Potočnik, I., Rekanović, E., Stanković, S., & Milijašević-Marčić, S. (2019). Biological control of green mould and dry bubble diseases of cultivated mushroom (*Agaricus bisporus* L.) by *Bacillus* spp. *Crop Protection*, (in press) online 1 September 2019, 104944. <https://doi.org/10.1016/j.cropro.2019.104944>
- Stanojević, O., Milijašević-Marčić, S., Potočnik, I., Stepanović, M., Dimkić, I., Stanković, S., & Berić, T. (2016). Isolation and identification of *Bacillus* spp. from compost material, compost and mushroom casing soil active against *Trichoderma* spp. *Archives of Biological Sciences* 68(4), 845-852. doi: 10.2298/abs151104073s
- Walker, R., Powell, A.A., & Seddon, B. (1998). *Bacillus* isolates from the spermosphere of peas and dwarf French beans with antifungal activity against *Botrytis cinerea* and *Pythium* species. *Journal of Applied Microbiology* 84, 791-800. doi: 10.1046/j.1365-2672.1998.00411.x
- Woo, S.L., Di Benedetto, P., Senatore, M., Abadi, K., Gigante, S., Soriente, I., ... Lorito, M. (2004). Identification and characterization of *Trichoderma* species aggressive to *Pleurotus* in Italy. *Journal of Zhejiang University (Agriculture and Life Sciences)* 30, 469-470.
- Yoshida, S., Hiradate, S., Tsukamoto, T., Hatakeda, K., & Shirata A. (2001). Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. *Phytopathology* 91(2), 181-187. doi: 10.1094/PHYTO.2001.91.2.181

Delovanje domaćih sojeva *Bacillus subtilis* u suzbijanju zelene plesni bukovače (*Pleurotus* spp.)

REZIME

Cilj ovog rada je bio pronalaženje potencijalnih agenasa biološke kontrole, izolovanih iz supstrata za gajenje šampinjona, koji bi predstavljali alternativu toksičnim hemikalijama koje se koriste u suzbijanju prouzrokovača bolesti u proizvodnji gajenih gljiva. Ispitivan je antagonistički potencijal deset domaćih sojeva *Bacillus subtilis* protiv prouzrokovača zelene plesni bukovače, *Trichoderma pleuroti* i *Trichoderma pleuroticola*. Korišćen je *in vitro* metod za kvantifikaciju antagonističkog potencijala sojeva *Bacillus* spp. zasnovan na dvojnoj kultivaciji s patogenom. Inhibicija porasta *T. pleuroti* varirala je između 54,44% i 62,22% i nisu utvrđene statistički značajne razlike između antagonističke aktivnosti sojeva *B. subtilis* testiranih u radu. Inhibicija *T. pleuroticola* je bila neznatno veća, (55,56% do 69,62%), a soj *B. subtilis* B-358 je ispoljio najveći procenat inhibicije. Ovo istraživanje je potvrdilo da je supstrat za gajenje gljiva dobar izvor antagonističkih mikroorganizama sa potencijalom korišćenja u biološkom suzbijanju zelene plesni bukovače.

Ključne reči: bukovača; zelena plesan; *Bacillus subtilis*, antagonistička aktivnost; biološko suzbijanje