



## BIOLOGICAL CONTROL OF GREEN MOULD DISEASE AND MUSHROOM FLY USING BIOFUNGICIDE *BACILLUS SUBTILIS* CH-13 AND BOTANICAL INSECTICIDE AZADIRACHTIN (TECHNICAL SOLUTION)

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### Abstract

The result of this study was accepted technical solution of disease/pest control of cultivated mushroom (*Agaricus bisporus* L.) based on biological pesticides (Biogenesis d.o.o., Serbia): microbial biofungicide *Bacillus subtilis* Ch-13 (Ekstrasol  $1 \times 10^8$  CFU/cm<sup>3</sup>) and botanical bioinsecticide azadirachtin (Ozoneem trishul 1 %). The efficiency of bio/pesticides in disease/pest control and impact on mushroom yield were evaluated in a large and small-scale experiments. The efficacy of biofungicide *Bacillus subtilis* Ch-13 to control of *Trichoderma aggressivum* Samuels & W. Gams (green mould disease) was evaluated in comparison with chemical fungicide prochloraz ( $2 \times 1.5$  mL/m<sup>2</sup>). Biofungicide was applied in different procedures, in two ( $2 \times 30$  mL/m<sup>2</sup>), three ( $30 + 2 \times 15$  mL/m<sup>2</sup>), and six split doses ( $6 \times 10$  mL/m<sup>2</sup>). The highest statistically significant effectiveness in pathogen control was shown when three (53.57-58.43%) or six doses (63.05%), were used. Biofungicide significantly improved yield in all different procedures, compared with untreated control in small-scale experiments 6.11-12.12% and in large-scale 5.07-8.41%. The impact of the bioinsecticide azadirachtin ( $4 \times 0.5$  mL/m<sup>2</sup>) on the density of the mushroom fly *Lycoriella ingenua* Dufour (Diptera: Sciaridae) was compared to the effects of the chemical insecticide malathion ( $2 \times 0.3$  mL/m<sup>2</sup>). The average number of the mushroom fly adults on yellow sticky traps per each mushroom row was significantly lower in the test chamber in comparison with two controlled chambers in commercial mushroom facility. The results of our study suggest that biofungicide *Bacillus subtilis* Ch-13 ( $30 + 2 \times 15$  mL/m<sup>2</sup> or  $6 \times 10$  mL/m<sup>2</sup>) and bioinsecticide azadirachtin ( $4 \times 0.5$  mL/m<sup>2</sup>) may provide a good alternative to conventional chemicals.

**Keywords:** *Agaricus bisporus*, biopesticides, *Lycoriella ingenua*; *Trichoderma aggressivum*.

### INTRODUCTION

The most important technological process in production of white button mushroom [*Agaricus bisporus* (Lange) Imbach] is the substrate preparation (Royse, 2010). Pesticide residues in straw and poultry manure obtained from the conventional agriculture production affect the quality of the substrates. Consequently, the genuine microbiome of the substrate is disturbed and no longer able to compete with harmful organisms. The major fungal disease of cultivated mushrooms is *Trichoderma aggressivum* Samuels & W. Gams causing green

mould disease and crop losses exceeding 60% (Kosanović et al., 2013). The most significant pest in mushroom production is the mushroom fly, *Lycoriella ingenua* Dufour (Diptera: Sciaridae), which causes great economic losses and further dissemination of the conidia of the fungal infections (Mazin et al., 2019).

Disease and pest control in mushroom farms includes strict hygiene measures and application of disinfectants and pesticides. Considerable problems for mushroom growers are lack of effective pesticides and resistance development

of pests and pathogens to pesticides (Bartlett and Keil, 1997; Grogan, 2008). Pesticides may induce harmful effects on mushroom mycelia, causing loss of quality and yield, furthermore the residues present in the harvested mushrooms reduce the quality of the products (Navarro & Gea, 2006). In the recent years, a number of pesticides have been banned for use, by the European Commission. Currently, only fungicides such as prochloraz and metrafenone and some bioinsecticides have the approval to be used in mushroom cultivation by OEPP (Luković et al., 2021).

Application of beneficial bacterium *Bacillus subtilis* (Ehrenberg) Cohn, which produces extra cellular metabolites for competition and antibiosis, is a good alternative to chemical fungicides (Milijašević-Marčić et al., 2017). The bacterium is harmless to the environment and human health and is generally recognized as safe (GRAS) organism (FDA, U. S., 1999). Some metabolites and remains of *B. subtilis* could serve as a nutrient source for edible mushrooms and promote the yield (Carrasco and Preston, 2000). Pyrethrin-based products, which showed good efficacy in the mushroom fly suppression, have not been registered for edible mushrooms in Serbia, and the latest researches has revealed their high toxicity to non-target organisms (Drobnjaković et al., 2019). Besides, the use

of entomopathogenic nematodes has shown instable efficacy against the mushroom flies. The triterpenoid azadirachtin from the class of limonoids is the primary active ingredient in plant extracts, oils and other derivatives obtained from seeds of the Indian neem tree [*Azadirachta indica* A. Juss. (Meliaceae)]. Neem based products have been shown to be active against more than 200 species of insects, including many dipterans and may act as repellents, feeding inhibitors, oviposition deterrents and insect growth regulators (Chaudhary et al., 2017). Neem-based products can be used together with other bioinsecticides based on living organisms, and in contrast to synthetic pesticides, the low persistence of azadirachtin products make them considerably safer for most beneficial arthropod species (Raguraman and Kannan, 2014). Due to the complex mode of action of azadirachtin, there is no risk of cross-resistance (Siegwart et al., 2015). Available literature data on azadirachtin activity against the mushroom flies are scarce and refer only to the mushroom phorid fly *Megaselia halterata* (Wood) (Diptera: Phoridae), and the first research on *L. ingenua* (Drobnjaković et al., 2019). In our study, biofungicide based on *B. subtilis* Ch-13 (Extrasol F) and bioinsecticide based on azadirachtin (Ozoneem trishul 1%) was tested in control of the green mould disease agent and the mushroom sciarid flies.

### EXPERIMENTAL CONDITIONS

New strain *B. subtilis* Ch-13, recently available in Serbia, was compared with chemical fungicide prochloraz and biofungicide *Bacillus amyloliquefaciens* QST713 (formerly known as *B. subtilis*) (Priest et al., 1987) (Tab. 1). The impact on yield and efficacy in green mould disease control were evaluated on white button mushroom (*A. bisporus*) using artificial infection of *T. aggressivum* f. *europaeum* T77 ( $10^6/m^2$ ) in the small-scale experiment and natural infection (*Trichoderma* spp.) in the commercial mushroom producing facility (large-scale). For preparing 0.7% spawned substrate of *A. bisporus* A15 (Sylvan, Hungária zRt) substrate was placed in: a) plastic boxes sized 0.340 x 0.215 x 0.130 m (*l* x *w* x *h*) contained 1.5 kg of compost, six replicates per treatment (small scale experiment), and b) plastic bags sized 0.6 x 0.4 x 0.2 m (*l* x *w* x *h*) contained 18 kg of compost, 224 replicates per treatment (large scale experiment). After 14 days

of incubation, substrate was cased with casing soil Terahum (Treset d.o.o., Veliko Gradište, Serbia) in the height of 3-5 cm. The harvested mushrooms were weighed and divided into two groups, with or without symptoms of green mould disease. The effect of fungicides on mushroom productivity was evaluated as biological efficiency (BE), calculated as the ratio of fresh weight of total fruiting body yield and the weight of dry spawned substrate, and expressed as %:  $BE = (\text{fresh total fruiting body yield} / \text{dry spawned substrate mass}) \times 100$ . Fungicide efficacy in disease control was calculated: % efficacy =  $[(I_c - I_t) / I_c] \times 100$ , where  $I_c$  - disease incidence in inoculated control;  $I_t$  - disease incidence in treated samples. Disease incidence was recorded as the percentage of fruiting bodies with symptoms compared to those without symptoms. Biofungicide *B. subtilis* Ch-13 was applied in different procedures, in

two (2×30 mL/m<sup>2</sup>), three (30 + 2×15 mL/m<sup>2</sup>), and six split doses (6×10 mL/m<sup>2</sup>). Biofungicide *B. amyloliquefaciens* QST713 was applied 2×0.5 mL/m<sup>2</sup>, and fungicide based on prochloraz 2×1.5 mL/m<sup>2</sup>.

Furthermore, the impact of an azadirachtin-based bioinsecticide (applied 4×0.5 mL/m<sup>2</sup>)

on regulation of the abundance of sciarid mushroom fly adults, was investigated compared to a conventional insecticide based on malathion (2×0.3 mL/m<sup>2</sup>) (Tab. 1). The density of mushroom flies was observed using yellow sticky traps. All traps were inspected under a binocular microscope to count mushroom flies caught.

**Table 1.** Pesticide products used in the study

Trade name	Active ingredient	Concentration of active ingredient	Manufacturer
Ekstrasol F SC	<i>Bacillus subtilis</i> Ch-13	1 x 10 <sup>8</sup> CFU/mL	BioGenesis d.o.o., Serbia
Serenade <sup>®</sup> ASO	<i>Bacillus amyloliquefaciens</i> QST713	5.13x10 <sup>9</sup> CFU/g	Bayer CropScience, Serbia
Mirage <sup>®</sup> EC	Prochloraz	450 mL/L	ADAMA Agricultural Solutions UK Ltd., UK
Ozoneem trishul	Azadirachtin	1% (10 g/L)	BioGenesis d.o.o., Serbia
Etiol tečni	Malathion	600 g/L	Galenika-Fitofarmacija a.d., Serbia

Data were examined by using the one-way analysis of variance (ANOVA), including the comparison of means by *F* test. The test was used to compare the significance of differences among data on the average biological efficiency and efficacy in disease control of different

biopesticide/pesticide treatments in the mushroom growing room. In all analyses, the level of significance was at least *P*<0.05. Statistical data analysis was performed by the software Statistica for Windows 6.0 (StatSoft Inc., 2004).

### EFFICACY OF FUNGICIDES

The efficacy of biofungicides/fungicide in suppression of symptoms of green mould disease is shown in Fig. 1 and 2. Impact on yield is shown in Fig. 3. Preliminary small-scale experiment showed that *B. subtilis* Ch-13, applied at a concentration of 2 or 3×10<sup>8</sup> CFU/m<sup>2</sup>, achieved respective better efficacy than strain QST713 (23%) formulated at higher concentration (5 × 10<sup>9</sup> CFU/m<sup>2</sup>) (Potočnik et al., 2019). Despite the same final concentration of 60 mL/m<sup>2</sup>, the efficacy of *B. subtilis* Ch-13 in green mould disease control was significantly higher when it was applied in three split doses (30 + 2×15 mL/m<sup>2</sup>) than in two applications (2×30 mL/m<sup>2</sup>) in the large-scale evaluation (Potočnik et al., 2021). Fungicide prochloraz showed

the highest efficacy in disease control in all experiments. Efficacy of the prochloraz was 71% in the small-scale study after artificial infection (*T. aggressivum* 10<sup>6</sup> conidia/m<sup>2</sup>), and 77% in the large-scale assay after natural infection (Potočnik et al., 2019; 2021). In the extended small-scale study, efficacy of the biofungicide was similar, 58.43%, when applied three times (30 + 2 × 15 mL m<sup>2</sup>), and 63.05% after six split doses (6×10 mL/m<sup>2</sup>) applied to suppress green mould disease symptoms (Potočnik et al., 2022). No statistically significant difference was found in the efficacy in disease control of the biofungicide, applied in three (58.43%) or six (63.05%) split doses, and the fungicide prochloraz (71.08%) against *T. aggressivum* (Fig. 1).

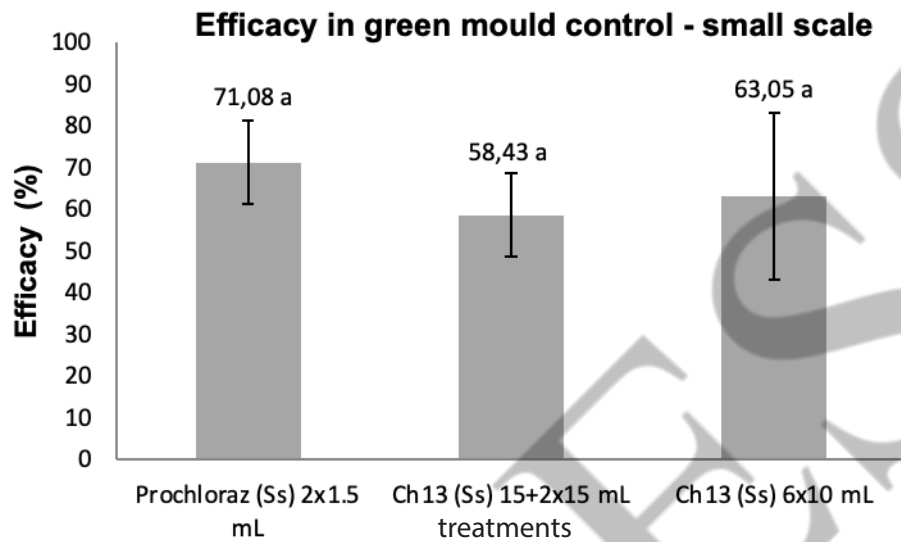
### IMPACT OF FUNGICIDES ON MUSHROOM YIELD

A statistically significant increase of mushroom yield was noted when the biofungicide *B. subtilis* Ch-13 was used in two and three split doses in comparison with the untreated control and prochloraz fungicide (Potočnik et al., 2021). The mushroom yield was enhanced in larger

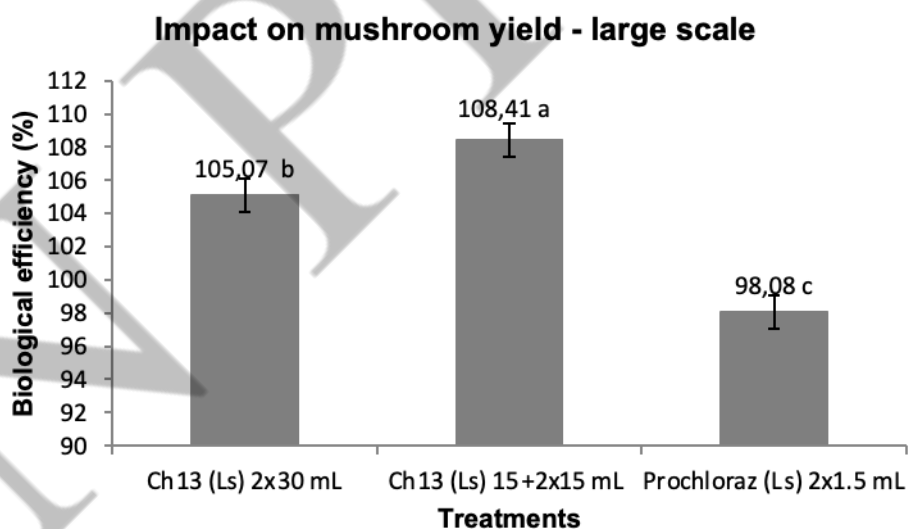
extent by *B. subtilis* Ch-13 when the biofungicide was used more frequently. Biofungicide *B. subtilis* Ch-13 considerably improved mushroom yield compared to uninoculated treatments: control plots (5-15%), fungicide prochloraz treated plots (7-12%), and plots with biofungicide *B.*

*amyloliquefaciens* QST713 (24-31%) (Potočnik et al., 2019; 2021). In extended small-scale experiment, the similar enhancement of mushroom yield by strain Ch-13 was recorded

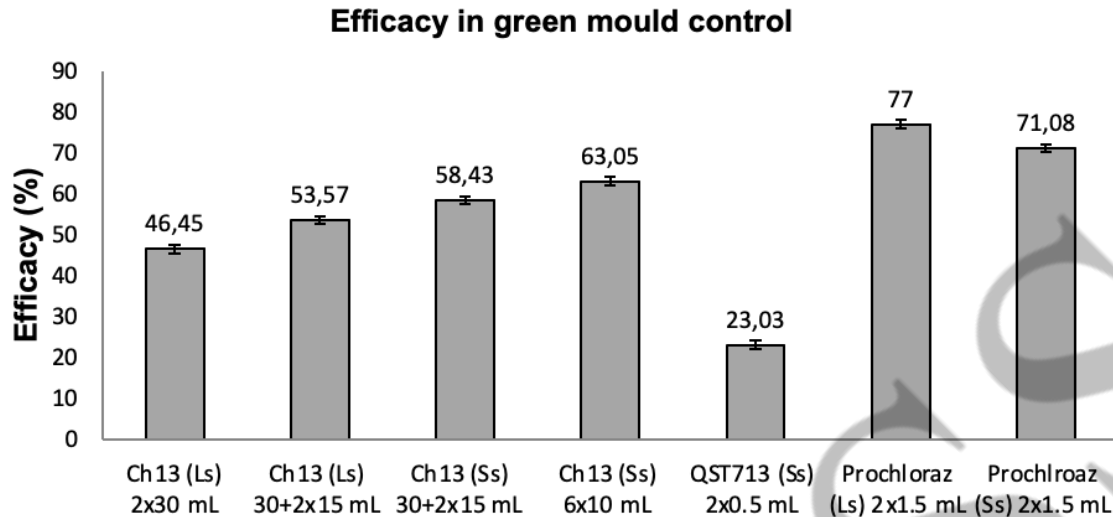
after three or six split doses (Potočnik et al., 2022). Some variation in yield promotion was noted depending of the mushroom compost quality and the seasonal changes.



**Figure 1.** The efficacy of bio/fungicides against *Trichoderma aggressivum* T77 on white button mushroom (*Agaricus bisporus*). (Ss) – small scale experiment: SEDs, standard error of differences = 14.66; df, degree of freedom = 2;  $F = 0.26$ ;  $P$ -value = 0.77. Values within series marked with the same letter are not significantly different according to  $F$  test ( $P < 0.05$ ).



**Figure 2.** The efficacy of bio/fungicides against *Trichoderma aggressivum* T77 on white button mushroom (*Agaricus bisporus*). (Ls) – large scale experiment: SEDs, standard error of differences=9.41; df, degrees of freedom=3;  $F=70.22$ ;  $P$ -value=0.001. Values with series marked with same letters are not significantly different according to  $F$  test ( $P < 0.05$ ). (Ss) – small scale experiment: SEDs, standard error of differences = 14.66; df, degree of freedom = 2;  $F = 0.26$ ;  $P$ -value = 0.77. Values within series marked with the same letter are not significantly different according to  $F$  test ( $P < 0.05$ ).

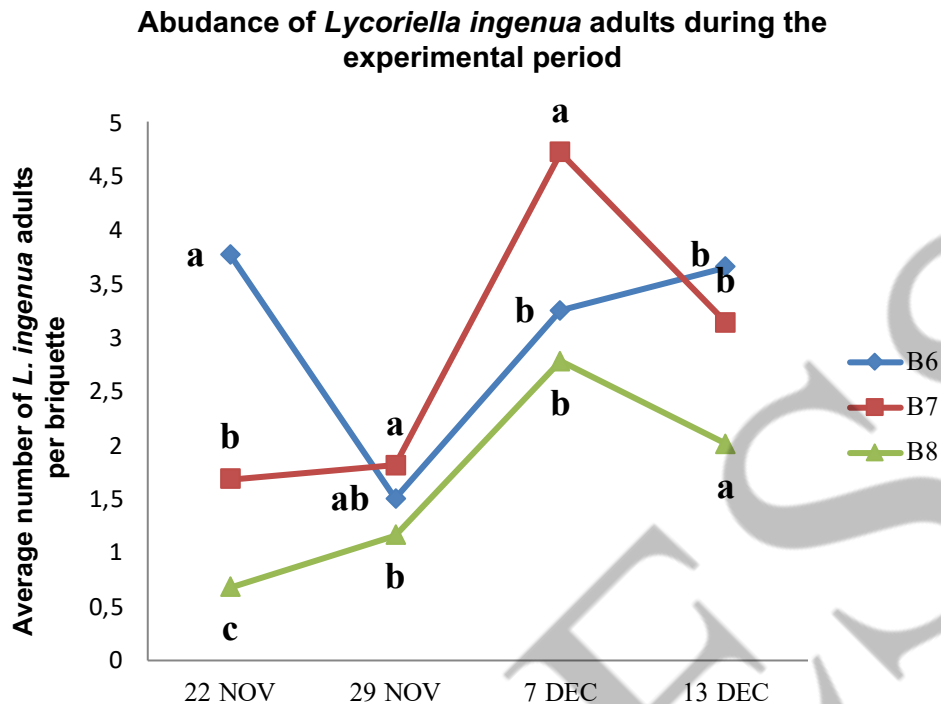


**Figure 3.** Impact on mushroom yield (*Agaricus bisporus*) of different treatments of bio/fungicides. (Ls) – large scale experiment: SEDs, standard error of differences=48; df, degrees of freedom=3;  $F=25$ ;  $P$ -value=0.001. Values with series marked with the same letters are not significantly different according to  $F$  test ( $P<0.05$ ).

Results regarding the management of the mushroom flies showed that, in comparison to the positive control chambers in the commercial facility (large scale), where the traditional malathion-based insecticide was used, the average number of sciarid fly adults over the entire test period was significantly lower in the test chambers (15, 22, 30 and 36 Days After Treatment - DAT), where the biorational azadirachtin-based product was tested (Fig. 4) (Drobnjaković et al., 2019).

The number of adult *L. ingenua* caught on yellow sticky traps was significantly lower in all three test chambers near the chamber entrance than in the chambers at the opposite end, suggesting that spatial isolation of fungus flies is a key element in preventing damage. The highest average number of adults of the mushroom fly was recorded at the third inspection (30 DAT), at the peak of mushroom production, corresponding to the time when the first harvest

ended. In the fourth inspection period, 36 DAT, the average number of the mushroom fly adults started to decrease (Drobnjaković et al., 2019). No negative effect of azadirachtin on the growth of *A. bisporus* yield or quality was observed compared to the positive control. These are the first data on the efficacy of an azadirachtin-based product in reducing *L. ingenua* populations. Similarly to the results of our research, Erler et al. (2009) proposed that the neem-based products Neemazal (10 g/L azadirachtin EC) and Greeneem oil (3 g/L azadirachtin in the 100% pure natural cold-pressed neem oil) may suppress populations of the mushroom phorid fly *M. halterata* and may provide an alternative to the standard chemical chlorpyrifos-ethyl. The researchers discovered that phorid larvae treated with neem showed aberrant growth and remained at the immature stage of development before dying (Erler et al., 2009).



**Figure 4.** The average number of adults of *Lycoriella ingenua* (the mushroom fly) adults per briquette during the experimental period in the commercial facility (large scale) (B6, B7 – chambers treated with chemical insecticide malathion; B8 – chamber treated with bioinsecticide azadirachtin).

#### CONCLUDING REMARKS

The biofungicide based on *B. subtilis* Ch-13 showed better efficacy in green mould disease control and the highest positive impact on mushroom production when used in three or six split applications, rather than two in comparison with the chemical fungicide prochloraz. It suggests that the biofungicide should be applied three times (30 mL/m<sup>2</sup> on the second day after casing + 15 mL/m<sup>2</sup> two weeks after casing + 15 mL/m<sup>2</sup> after the first flush) or six times (10 mL/m<sup>2</sup> on the second day after casing + 5 × 10 mL/m<sup>2</sup> at seven-day intervals). Azadirachtin-based bioinsecticide applied four times at the

rate of 4×0.5 mL/m<sup>2</sup> (starting with casing time and proceeding with successive treatments at seven-day intervals), significantly reduced the density of sciarid fly *L. ingenua*, compared with the conventional insecticide. Using those two biopesticides reduces the use of chemical pesticides in cultivated mushrooms, enabling the processing and export of the substrate and mushrooms according to the required standards for product safety and quality. It would further increase the competitiveness of domestic mushroom producers in a regional market.

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## БИОЛОШКА КОНТРОЛА НА БОЛЕСТА ЗЕЛЕНА МУВЛА И ГАБНА МУВА СО КОРИСТЕЊЕ НА БИОФУНГИЦИД *BACILLUS SUBTILIS* CH-13 И БОТАНИЧКИ ИНСЕКТИЦИД АЗАДИРАХТИН (ТЕХНИЧКО РЕШЕНИЕ)

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### Резиме

Резултатот од ова истражување беше прифатено техничко решение за контрола на болести/штетници на култивирана печурка (*Agaricus bisporus* L.) базирано на биолошки пестициди (Биогенесис Д.О.О., Србија): микробен биофунгицид *Bacillus subtilis* Ch-13 (Ekstrasol 1×10<sup>8</sup> CFU/cm<sup>3</sup>) и ботанички биоинсектицид азадирахтин (Ozoneem trishul 1 %). Ефикасноста на био/пестицидите во контролата на болести/штетници и влијанието врз приносот на печурките беа оценети во експерименти од големи и мали размери. Ефикасноста на биофунгицидот *Bacillus subtilis* Ch-13 за контрола на *Trichoderma aggressivum* Samuels & W. Gams (болеста зелена мувла) беше оценета во споредба со хемискиот фунгицид прохлораз (2×1,5 mL/m<sup>2</sup>). Биофунгицидот беше применет во различни постапки, во две (2×30 mL/m<sup>2</sup>), три (30 + 2×15 mL/m<sup>2</sup>) и шест поделени дози (6×10 mL/m<sup>2</sup>). Статистички највисока значајна ефективност во контролата на патогенот беше достигната кога беа користени три (53,57-58,43 %) или шест дози (63,05 %). Биофунгицидот значително го подобри приносот во сите различни процедури, во споредба со нетретираната контрола во експериментите од мал 6,11-12,12 % и во експериментите од голем размер 5,07-8,41 %. Влијанието на биоинсектицидот азадирахтин (4×0,5 mL/m<sup>2</sup>) врз густината на габната мува *Lycoriella ingenua* Dufour (Diptera: Sciaridae) беше споредено со ефектите на хемискиот инсектицид малатион (2×0,3 mL/m<sup>2</sup>). Просечниот број на возрасни габни муви на жолти лепливи стапици на секој ред од печурки беше значително помал во комората за тестирање во споредба со две контролирани комори во комерцијалните капацитети за печурки. Резултатите од нашата студија сугерираат дека биофунгицидот *Bacillus subtilis* Ch-13 (30 + 2×15 mL/m<sup>2</sup> или 6×10 mL/m<sup>2</sup>) и биоинсектицидот азадирахтин (4×0,5 mL/m<sup>2</sup>) можат да обезбедат добра алтернатива на конвенционалните хемикалии.

**Клучни зборови:** *Agaricus bisporus*, биопестициди, *Lycoriella ingenua*, *Trichoderma aggressivum*.