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Original scientific paper

Control of mushroom sciarid fly *Lycoriella inguena* (Dufour) with an azadirachtin-based insecticide

Tanja Drobnjaković*, Dejan Marčić, Ivana Potočnik, Emil Rekanović, Mirjana Prijović, Svetlana Milijašević-Marčić and Miloš Stepanović

Institute of Pesticides and Environmental Protection, Banatska 31B, 11080, Belgrade, Serbia
**Corresponding author: tanjadrobnjakovic@gmail.com*

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SUMMARY

The impact of a bioinsecticide based on azadirachtin (Ozoneem trishul 1 %) on the abudance of mushroom flies (Sciaridae: Diptera) was compared to the effect of a commercial formulation of the malathion-based chemical insecticide Etiol tečni. Experiments were conducted in three growing chambers (B6, B7 and B8) of a commercial mushrom farm "Delta Danube" d.o.o., Kovin. Casing treatments were performed in eight replications in a random block design. The azadirachtin-based bioinsecticide was applied in chamber B8 four times (0.5 ml/m^2): during casing and later at seven-day intervals. The standard chemical insecticide based on malathion was applied in chambers B6 and B7 twice (2 x 0.3 ml/m^2), on the third and sixth day after casing. In all three chambers, the abudance of mushroom flies was monitored by using yellow sticky traps, which were collected weekly and replaced with new ones four times at seven days intervals. The yellow sticky traps were examined in the laboratory under a binocular microscope to determine the presence and density of mushroom flies. Only one species of mushroom fly, *Lycoriella ingenua* (Dufour), was found on the yellow sticky traps throughout the experimental period. The average number of sciarid flies per mushroom block 15 and 22 days after treatment (DAT) was significantly lower in the test chamber B8 than in chambers B7 and B6, while there was no significant difference 30 and 36 DAT, compared to the control chamber B6. The average number of sciarid flies per mushroom row throughout the experiment was significantly lower in the test chamber B8 than in chambers B6 and B7. The results of our study suggest that the azadirachtin-based bioinsecticide can suppress populations of the mushroom fly *L. ingenua* and may provide a good alternative to conventional chemical insecticide.

Keywords: mushroom sciarid flies; *Lycoriella inguena*; *Agaricus bisporus*; azadirachtin; biopesticides

INTRODUCTION

Commercial production of the cultivated mushroom, *Agaricus bisporus* (Lange) Imbach, is affected by several pests wherever the crop is grown (Hussey et al., 1974). Three groups of pests that are most commonly encountered are the cecid flies [*Heteropeza pygmaea* Winnertz and *Mycophila speyeri* (Barnes)], phorid flies [*Megaselia halterata* (Wood)], and sciarid flies (*Lycoriella* spp.), and these pests are a consistent problem for growers

throughout the world (White, 1985). Great economic losses caused by sciarid pests (known as dark-winged fungus gnats, sciarid flies, big flies or mushroom flies) in the mushroom industry have been reported from Australia (Greenslade & Clift, 2004), to the United States (White, 1985), South Korea (Kim & Hwang, 1996), the United Kingdom (White, 1985), and to some other countries of Western Europe (Scheepmaker et al., 1995). The sciarid fly, *Lycoriella ingenua* (Dufour) (syn. *L. mali*, *L. solani*) (Diptera: Sciaridae) is a major pest species of commercial mushrooms throughout the world, which causes crop damage and reductions in yield (Erler et al., 2011). *L. ingenua* damages mushrooms by direct larval feeding on developing mushroom mycelia (Lewandowski et al., 2004), by larval competition with developing mushroom mycelia for compost nutrients (Binns, 1980), and negative effects of larval frass on mycelial growth (Hussey & Gurney, 1968). Furthermore, *L. ingenua* adults and larvae are associated with the vectoring of *Trichoderma aggressivum* (Samuels & Gams) (Hypocreales: Hypocreaceae), which causes severe epidemics of "green mold" and consequently leads to additional crop losses (Shamshad, 2010). The economic threshold for sciarid larvae is virtually zero, necessitating chemical control at very low larval densities (Kielbasa & Snetsinger, 1980; White, 1986).

Traditionally, sciarid flies in mushroom crops have been controlled by insecticides. Other methods for controlling sciarid flies are based on the use of physical barriers to exclude adults from growing in farms (Rinker, 2017); biocontrol organisms, such as mites, bacteria and entomopathogenic nematodes (Scheepmaker et al., 1995, 1997; Keil, 2002; Jess & Bingham, 2004); and, more recently, plant extracts (Park et al., 2006). Literature shows varying results with regards to the efficacy against sciarids (Cantelo, 1983; Geels & Rutjens, 1992; Keil & Bartlett, 1995). However, the application of phytosanitary products may give rise to two further problems: detrimental effects on mushroom mycelia, leading to yield loss or quality degradation (Shamshad, 2010), and the presence of residues in harvested mushrooms (Navarro & Gea, 2006). Furthermore, resistance to certain products has been recorded (Bartlett & Keil, 1997; Smith, 2002).

Annual button mushroom production of 9,000 t in Serbia is five times lower than it is in the Republic of Ireland – which has the same area and half as many inhabitants (Balaz Erdey, Sylvan, Hungary, personal communication) – and the fact is indicative of some unused capacities. The current state of the mushroom industry in Serbia results from the presence and spreading of mushroom pests and pathogens, and their inadequate control (Milijašević-Marčić et al., 2012). The synthetic conventional insecticide diflubenzuron has been registered in Serbia for mushroom protection from flies, even though only bioinsecticides are allowed to be applied to edible mushrooms under OEPP rules (Helen Grogan, personal communication). Pyrethrin-based products have not been registed on the Serbian market, and the latest research has revealed their high toxicity to nontarget organisms. The use of bioinsecticides based on nematodes has so far shown variable success results in controlling flies, and no such formulated products have been registered in Serbia. Our investigation therefore focused on testing an available bioinsecticide based on azadirachtin, Ozoneem trishul 1 % (Ozone Biotech, Harayana, India), for controlling mushroom sciarid flies. The limonoid-based azadirahtin is the primary active ingredient in extracts, oils and other derivatives obtained from seeds of the tropical plant *Azadirachta indica* A. Juss. (Meliaceae) (known as the Indian neem tree). Neem-based products have been shown to be active against more than 200 species of insects, including many dipterans, and they may act as repellents, feeding inhibitors, oviposition deterrents and insect growth regulators (Stark et al., 1990; Isman, 1993; Naumann & Isman, 1995; Liang et al., 2003; Greenberg et al., 2005; Erler & Cetin, 2007). In addition, Ignatowicz et al. (1998) reported that neem-based products can be used together with entomopathogenic fungi and entomopathogenic nematodes. The low persistence of azadirachtin products generally make them considerably safer for most beneficial arthropod species, in contrast to synthetic pesticides (Raguraman & Kannan, 2014). Due to the specific and complex mode of action of azadirachtin, there is no risk of cross-resistance (Siegwart et al., 2015), so that this product is potentially a good alternative for successful control of resistant mushroom fly populations.

Available research-based literature data on azadirachnin activity against mushroom flies refer only to the mushroom phorid fly *Megaselia halterata* (Wood) (Diptera: Phoridae). The focus of this study was to investigate the impact of an azadirachtin-based bioinsecticide on regulation of the abundance of sciarid fly adults, compared to a conventional insecticide based on malathion (positive control). The data obtained in this study are discussed in terms of improvement of mushroom flies integrated management strategy.

MATERIALS AND METHODS

Bioinsecticide

The commercial product Ozoneem trishul 1 % (manufactured by Ozone Biotech, Harayana, India) is a formulation of concentrated and standardized extract of *A. indica*, containing 10 g/l of azadirachtin (azadirachtin-A and azadirachtin-B) as its leading active ingredient. The content of the azadirachtin-A and azadirachtin-B mixture in the product is standardised to 1% w/v (10 g/l).

Trials

Experiments for testing the efficacy of the botanical bioinsecticide based on azadirachtin in controlling mushroom flies were conducted in three separate growing chambers (B6, B7 and B8) in the commercial mushroom farm "Delta Danube", Kovin. Compost blocks weighing 18 kg, sized 0.60 x 0.40 x 0.25 m with 0.24 m^2 surface area, were covered with casing soil ("Terahum", "Treset" d.o.o., Veliko Gradište) and seeded with botton mushroom micelia (*Agaricus bisporus* strain F56 Italspawn, Onigo di Pederobba, Italy). Compost was incubated for 16 days at the temperature of 24 °C. Each experimental plot was covered with 3-5 cm of black peat casing soil and incubated at 21 °C for eight days. After incubation, temperature in the growing chamber was lowered to 17 °C.

Treatments of casing soil in the mushroom growing chambers were carried out according to standard PP 1/167 (2) methodology (EPPO, 2004), using the azadirachtin-based bioinsecticide (Ozoneem trishul 1 %), which was compared to treatments in the other chambers with a liquid formulation of the commercial malathion-based chemical insecticide Etiol tečni (Galenika Fitofarmacija, Zemun). All treatments had eight replications in a randomized block design. The azadirachtin bioinsecticide and the proposed standard treatments were tested by split (multiple) application using an automatic sprayer with 10 full cone nozzles (using 1 l/m^2 liquid).

The tested bioinsecticide based on azadirachtin was applied in the B8 experimental chamber after casing at intervals of seven days in four treatments: 4 x 50 ml/100 l water per 100 m^2 (0.5 ml/m^2 or 0.5 ml/1 l water). Mushroom was treated at the following growth stages:

I treatment – coincided with casing, the product Ozoneem trishul 1 % while micelia was peeking out of casing (0.5 ml/m^2);

II treatment – one week after casing (6-7 days), Ozoneem trishul 1 %) while micelia was peeking out of casing (0.5 ml/m^2);

III treatment – two weeks after casing (13-15 days), Ozoneem trishul 1 % during the formation of primordial mushroom fruit bodies (0.5 ml/m^2);

IV treatment – after the first flush (20-22 days after casing) and final yield of all mushrooms regardless of their size - Ozoneem trishul 1 %) (0.5 ml/m^2).

The standard malathion insecticide, intended for controlling mushroom flies, was applied in the control chambers B6 and B7 after casing until first symptoms of damage caused by flies appeared to such a degree as to allow clear distinction between positive control (malathion) and azadirachtin-treated plots. The standard insecticide Etiol tečni (malathion) was applied in the control chambers B6 and B7 in two treatments with 30 ml/10 l (0.3 ml/m^2) on the third and sixth day after casing. It was impracticable to include negative control (water) in the experiments because the owners of the commercial mushroom facility declined to provide it.

In all three experimental chambers, the density of mushroom flies was observed using yellow sticky traps. The traps were positioned to enable their early observation, covering the area in which they appeared during the three flushes of mushroom bearing fruit. Each chamber (containing mushroom compost blocks arranged in 8 rows at three levels) had 16 yellow sticky traps at the beginning and the end of each row. The yellow sticky traps were collected once a week and replaced with new ones. All traps were inspected under a binocular microscope in the laboratory to count mushroom flies caught, i.e. their density. The species of the collected flies were identified based on the identification key given by Menzel and Mohrig (1999). The observed treatment parameter was the number of adult flies on yellow sticky traps per row and per block (compost in plastic bags). The trial included eight replications in block design with casing area of 56 m^2 per block, which is equivalent to an area of 224 blocks (Figure 1).

Four inspections of yellow sticky traps for fly catch were conducted to assess impact:

I assessment – 22 November 2018 (9 November – 22 November),

II assessment – 29 November 2018 (22 November – 29 November),

III assessment – 7 December 2018 (29 November – 7 December),

IV assessment – 13 December 2018 (7 December – 13 December).

Data were processed by one-way ANOVA, while the significance of differences between means per block and row between the chambers was determined by Duncun's test, p< 0,05) (StatSoft, 2004).

RESULTS

A single mushroom fly species, the sciarid fly *L. ingenua,* was observed on yellow sticky traps throughout the experiment in all three chambers. To our knowledge, this is the first documented report of this species in Serbia.

a)

b)

Figure 1 (a, b). Yellow sticky traps in rows in mushroom growing chamber during trial

Fifteen days after treatment, a significantly lower number of *L. ingenua* adults was found in chamber B8, where the azadirachtin-based bioinsecticide was applied three times (3 x 0.5 ml/m^2), compared to both control chambers in which the conventional malathion insecticide was applied (Figure 2a).

In the second assessment interval 22 DAT, the average number of pest adults in test chamber B8 was significantly lower than it was in control chamber B7, while no significant difference was found in comparison with the average number of sciarid adult flies in control chamber B6 at $p < 0.05$ significance (Figure 2b).

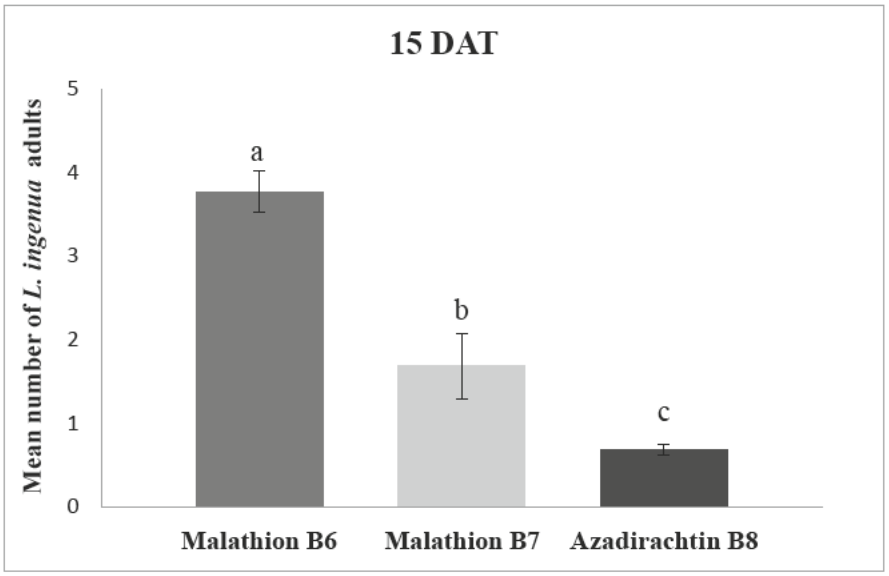

Figure 2a. Abudance of *Lycoriella ingenua* adults per compost block in growing chambers (± SE*) 15 days after treatment (15 DAT); B8 – test chamber (azadirachtin); B6 and B7 - control chambers (malathion); F=38.21; $p < 0.001$; *Means marked by different letters are significantly different (Duncun's test, $p < 0,05$)

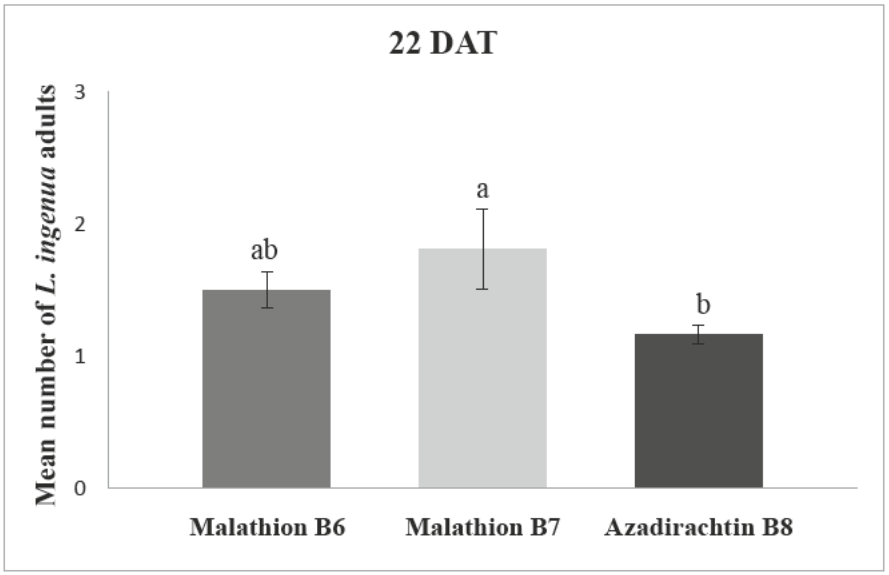

Figure 2b. Abudance of *Lycoriella ingenua* adults per compost block in growing chambers (± SE*), 22 days after treatment (22 DAT); B8 – test chamber (azadirachtin); B6 and B7 - control chambers (malathion); F = 2.97; $p < 0.073$; *Means marked by different letters are significantly different (Duncun's test, $p < 0,05$)

During the third assessment period 30 DAT, a significantly lower number of ciar d fly adu ts was recorded in test chamber B8, compared to control chamber B7, while there was no significant di ference at p < 0 05 ompared to control chamber B6 Figure 2c).

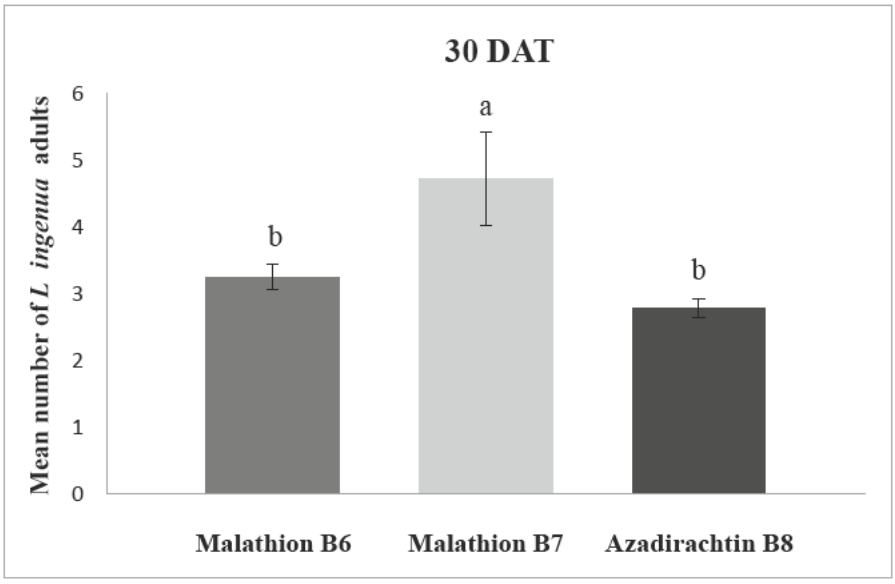

Figure 2c. Abudance of *Lycoriella ingenua* adults per compost block in growing chambers (± SE*), 30 days after treatment (30 DAT); B8 – test chamber (azadirachtin); B6 and B7 - control chambers (malathion); F = 6.58; p < 0.006; *Means marked by different letters are significantly different (Duncun's test, p < 0,05)

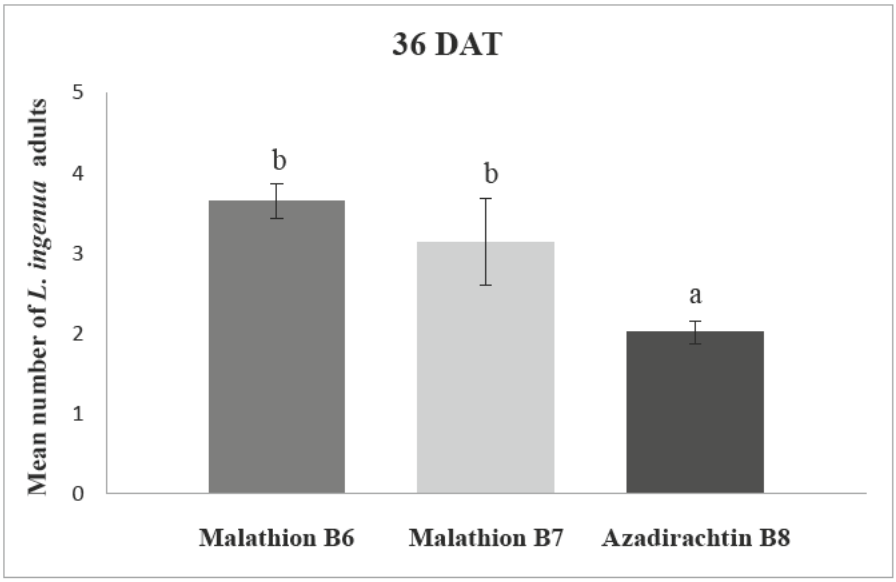

Figure 2d. Abudance of *Lycoriella ingenua* adults per compost block in growing chambers (± SE*), 36 days after treatment (36 DAT); B8 – test chamber (azadirachtin); B6 and B7 - control chambers (malathion); F = 6.95; p < 0.004; *Means marked by different letters are significantly different (Duncun's test, p < 0,05)

During the fourth assessment period, the number of sciarid fly adults per block in test chamber B8 was significantly lower than in both control chambers B6 and B7 (Figure 2d).

Duncan's test showed that the average abudance of sciarid fly adults per mushroom row throughout the test period was significantly lower in test chamber B8, where the biorational azadirachtin product was tested, than it was in control chambers B6 and B7 were the conventional malathion insecticide was used ($F = 23{,}456$, $p < 0{,}05$).

In all three experimental chambers, the number of *L. ingenua* adults caught on yellow sticky traps was far lower in the vicinity of chamber entrance than it was on traps positioned at the oposite side, which indicates that spatial isolation is an important factor in damage prevention from mushroom flies. Also, a general tendency was observed of finding more flies in rows positioned closer to the light source as flies tend to group together and are attracted to light.

DISCUSSION

The global availability of crop protection products for use in the mushroom growing industry is limited. Legislation and consumer pressure groups discourage their use on the basis of human health and environmental concerns. Consequently, future crop protection strategies in mushroom cultivation should primarily focus on pest prevention. If insect infestations persist, alternatives to chemical crop protection products will be required. There are currently no registered biopesticides available for the control of mushroom pests, and most mushroom growers use products registered for other agricultural pests. Current dependence on chemicals for the control of sciarid flies (mushroom gnats) and improved hygiene reflect a need for a more sustainable approach to crop protection.

The results of our study suggest that the tested azadirachtin-based product may suppress populations of sciarid fly *L. ingenua* and may provide an alternative to the conventional chemical insecticide malathion. The presented results show that the average number of sciarid fly adults per mushroom row over the entire test period was significantly lower in the test chamber B8, in which the biorational azadirachtin-based product was tested, compared to the positive control chambers B6 and B7, where the conventional malathion-based insecticide was applied. The average number of sciarid fly adults per compost block grew with time after casing, and the highest average number was recorded at the third inspection period (30 DAT). That was the peak of mushroom production, the time when the first harvest ended (25-26 days after casing), the chambers had much fewer compost blocks and mushrooms bodies, and the dispersal of sciarid fly adults was significant, which is probably the reasons why fly adults were recorded in the greatest average number at that time. During the fourth inspection period (36 DAT), the average number of recorded sciarid fly adults began to decline. Although neem extracts have been reported to have fungitoxic activity (Khan et al., 1974; Steinhauer, 1996; Amadioha & Obi, 1998), no adverse effect on mushroom formation, yield or quality of fruiting bodies, compared to positive control, was observed in the present study. Also, the density of *L. ingenua* flies caught by yellow sticky traps was much lower in the vicinity of chamber entrance than at the opposite side, which clearly shows that spatial isolation is an important factor for prevention of damage by sciarid flies. Another general tendency was also observed that fly numbers grew in rows closer to the source of light as they are attracted to light in groups (Wuest & Bengston, 1982).

There are no public data to our knowledge that show independent activity of an azadirachtin-based product in reducing *L. ingenua* populations. Rovesti et al. (1996) concluded that integrated application of the nematode *Steinernema feltiae* (Filipjev), the bacterium *Bacillus thuringiensis* subsp. *israelensis* and azadirachtin reduced the eclosion of *Lycoriella* spp. adults with effectiveness extending over three weeks. Similar to our results, the results reported by Erler et al. (2009) suggest that the neem-based products Neemazal (an emulsifiable concentrate containing 10 g/l azadirachtin) and Greeneem oil (100% pure natural cold-pressed neem oil with 3 g/l azadirachtin) may suppress populations of the mushroom phorid fly *M. halterata* and may provide an alternative to conventional chemicals. Their results have demonstrated that the reduction in adult emergence under both neem treatments was greater than it was in the positive control (chlorpyrifos-ethyl). All botanical extracts and the negative control (water) had significantly greater yields in each flush and total yield (overall in two flushes) than those subjected to the positive control treatment. In a study by Erler et al. (2009), inspection of neem-treated phorid larvae under the microscope showed that the affected larvae had abnormal growth and remained at the immature stage they had been at when they were exposed to the products, and then died. Larvae surviving neem treatments were

small and weak, compared with those in the negative control which developed normally and were active. These findings suggest that the main effect of neem-based products in that study was interruption in larval development, as they failed to develop normally and soon died. This depressed development and mortality is consistent with findings reported from earlier studies on various dipterans (Stark et al. 1990; Okumu et al., 2007). Similarly, in a study with greenhouse-grown ornamentals, Ludwig and Oetting (2001) evaluated the impact of medium drenches of insect growth regulators (among them the azadirachtin-based product Azatin XL, 0.04 g a.i./l) and conventional insecticides intended to reduce the emergence of adults of mushroom gnut *Bradysia coprophila* (Lintner) from the medium. Fenoxycarb, pyriproxyfen and azadirachtin resulted in the most significant reduction of mushroom gnut emergence.

The results of our present study give ground for a conclusion that the azadirachtin-based bioinsecticide (Ozoneem trishul 1%) applied four times at the rate of 4 x 0.5 ml/m^2 (starting with casing and proceeding with successive treatments at 7 day intervals), significantly reduced the density of sciarid fly *L. ingenua*, compared to the standard (conventional) insecticide based on malathion (Etiol tečni, Galenika Fitofarmacija). Protection of mushrooms from mushroom sciarid flies in Serbia is based on treatments with chemical pesticides, which are toxic to nontarget organisms, humans and the environment. As the European Commission Regulation 1107/2009 stimulates the use of low-risk active ingredients and renders alternatives to chemical pesticides a special status (Villaverde et al., 2014), the results of this study are important in supporting the introduction of azadirachtin-based biopesticides for mushroom sciarid flies control.

The use of azadirachtin in controlling mushroom sciarid flies contributes to reducing the use of chemical pesticides in cultivated mushroom production, and enables processing and exports of compost and mushrooms in conformity with required standards for product safety and quality. It would further boost the competitiveness of domestic bottom mushroom producers on a regional market.

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Suzbijanje šampinjonske mušice *Lycoriella inguena* (Dufour) bioinsekticidom na bazi azadirahtina

REZIME

Ispitivan je uticaj botaničkog bioinsekticida na bazi azadirahtina (Ozoneem trishul 1 %) u regulaciju brojnosti šampinjonskih mušica iz fam. Sciaridae, u odnosu na komercijalnu formulaciju sintetičkog insekticida na bazi malationa (Etiol tečni). Eksperimenti su izvedeni u komercijalnom uzgajalištu šampinjona „Delta Danube" d.o.o., Kovin, u tri odvojene komore za uzgajanje (B6, B7 i B8). Tretmani pokrivke su izvedeni u osam ponavljanja po potpuno slučajnom blok sistemu. Ispitivani bioinsekticid na bazi azadirahtina je primenjen u eksperimentalnoj komori B8, nakon postavljanja pokrivke i u intervalima od po sedam dana, četvorokratno (4 x 0,5 ml/m^2). U komorama B6 i B7 su izvedena tretiranja pokrivke standardnim insekticidom na bazi malationa, dvokratno (2 x 0,3 ml/m2), trećeg i šetog dana od postavljanja pokrivke. U sve tri eksperimentalne komore za uzgajanje šampinjona, brojnost šampinjonske mušice je praćena pomoću žutih lepljivih klopki, koje su sakupljane svake nedelje i zamenjivane novim. Nakon toga, pregledane su u laboratoriji, gde je pod binokularom utvrđivano prisustvo i brojnost šampinjonskih mušica. U sve tri posmatrane komore, na žutim lepljivim klopkama zabeležena je samo jedna vrsta šampinjonske mušice, *Lycoriella ingenua* (Dufour)(Diptera: Sciaridae). Dobijeni rezultati su pokazali da je prosečan broj mušica po briketu šampinjona, 15 i 22 dana nakon tretmana (DNT), bio statistički značajno manji u eksperimentalnoj komori B8, u odnosu na B7 i B6, dok se 30 i 36 DNT nije značajno razlikovao od prosečnog broja šampinjonskih mušica u kontrolnoj komori B6.

Prosečan broj mušica po redu šampinjona, tokom čitavog eksperimentalnog perioda, bio je statistički značajno manji u eksperimentalnoj komori B8, u odnosu na kontrolne komore B6 i B7. Rezultati našeg istraživanja su pokazali da bioinsekticid na bazi azadirahtina, Ozoneem trishul 1 %, može smanjiti brojnost šampinjonske mušice *L. ingenua*, predstavljajući dobru alternativu konvencionalnom insekticidu, na bazi malationa.

Ključne reči: šampinjonska mušica; *Lycoriella inguena*; *Agaricus bisporus*; azadirahtin; biopesticidi