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Interactions among biocontrol agents in the management of *Lycoriella ingenua* and *Trichoderma aggressivum* on white button mushrooms

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SUMMARY

Relationships (synergistic/antagonistic/additive) among three biocontrol agents – the native antagonistic bacterium *Bacillus amyloliquefaciens* B-241, the yield-stimulating actinobacterium *Streptomyces flavovirens* A06, and a commercial strain of the entomopathogenic nematode *Steinernema feltiae* – were investigated for the purpose of evaluating their effects on the suppression of artificially inoculated green mould disease agent *Trichoderma aggressivum* f. *europaeum* T77, as well as the suppression of natural infestation of the fungus gnat, *Lycoriella ingenua*, in an experimental growing chamber of cultivated white button mushroom *Agaricus bisporus*. Biocontrol agents were applied at standard application rates, or reduced rates of 40% or 20%. The impact of biocontrol agents and their interactions on mushroom productivity was calculated as the ratio of the fresh weight of the total mushroom yield to the weight of the dry spawned substrate. The density of fungus gnat flies was monitored by using yellow sticky traps placed inside each insect-rearing cage with mushroom substrate. The evaluation of disease and pest control efficacy was based on disease and pest incidence in the inoculated control and treatment groups. Simultaneous application of three biocontrol agents revealed mild antagonistic interactions in their efficacy in green mould disease control, an antagonistic relationship in the control of the fungus gnat, while synergy was detected regarding their impact on mushroom yield. The results of this study suggest that each biological agent should be applied three times every seven days to provide efficient pest and disease control: entomopathogenic nematodes used individually at the first day after the casing time (*S. feltiae* 0.75×10^6 IJ m^{-2}, total amount 2.25×10^6 IJ m^{-2}), and beneficial microorganisms used combined a few days later (*B. amyloliquefaciens* 1×10^9 CFU ml^{-1} m^{-2}, total amount 3×10^9 CFU ml^{-1} m^{-2}, and *S. flavovirens* 1×10^8 CFU ml^{-1} m^{-2}, total amount 3×10^8 CFU ml^{-1} m^{-2}).

Keywords: edible mushrooms; entomopathogenic nematodes; beneficial microorganisms; pest control; disease control

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INTRODUCTION

The substrate for cultivation of the white button mushroom [*Agaricus bisporus* (Lange) Imbach] is composed of compost and casing soil. Thus, microbial communities occupying such a substrate play a significant role in *A. bisporus* production. Compost represents a complex environment, inhabited by both beneficial and pathogenic micro/organisms. During compost fermentation and pasteurization, plant material (e.g., wheat straw in Europe) is decomposed by a myriad of microorganisms from the added chicken manure (Coles & Berber, 2002), providing nitrogen and carbon sources for the crop and playing a crucial role in generating *A. bisporus* fruiting bodies (McGee, 2017). In the beginning of the composting process, most of the microbial activity is attributed to diverse species of bacteria (*Bacillus* spp. and *Pseudomonas* spp.), while during the final stage, actinobacteria (*Termomonospora* spp. and *Streptomyces* spp.) predominate (Sharma et al., 2000). After the composting process is completed, bacteria and actinobacteria from the genera *Bacillus* and *Streptomyces* persist and are capable of producing various metabolites that have antibiotic properties and/or promote fructification (Stein, 2005; Milijašević-Marčić et al., 2017; Šantrić et al., 2018). Casing soil, mainly composed of sphagnum black peat, is abundant with aerobic bacterial, actinobacterial, and fungal populations, as well (Clarke et al., 2022; Milijašević-Marčić et al., 2024). Insect-predaceous nematodes (*Steinernema* spp.) are also present in the substrate. Some of these entomopathogenic species are currently used in commercial formulations against sciarid flies (Jess et al., 2005; Rinker et al., 1995). However, industrial-scale production of cereals contaminated with pesticides, as well as poultry polluted with antibiotics, results in disturbed microbial balance, often in the favour of pathogenic organisms and pests (Milijašević-Marčić et al., 2024). Among mycopathogenic fungi, the most problematic pathogen of cultivated mushrooms in Serbia and worldwide has lately been the competitor fungus *Trichoderma aggressivum* Samuels & W. Gams (green mould disease), which causes substantial mushroom yield losses (Kosanović et al., 2013). In addition, the sciarid fungus gnat, *Lycoriella ingenua* (Dufour), is the major pest that causes serious crop reduction (Rinker et al., 1995; Drobnjaković et al., 2019; 2025; Rijal et al., 2021). Mushroom production throughout the world is facing a serious problem – the lack of effective chemicals for disease/pest control. The development of pathogen and pest resistance to certain chemicals, along with their negative impact on the environment, has led to the withdrawal of such chemicals from the market. As a consequence, there has been an increase of interest in new alternative methods, such as the use of biocontrol agents (BCAs) and biopesticides against pests and pathogens. Furthermore, interest in entomopathogenic nematodes and other microbial pest control agents has arisen from the need to find alternatives to chemical insecticides, which can cause secondary pest resurgence, host resistance, environmental contamination, and health concerns for non-target organisms, including humans. In complex agricultural environments, interactions among simultaneously applied biocontrol agents may be more nuanced, resulting in antagonistic effects on some pests, and additive or synergistic effects on others, for example. Harmful properties of chemical pesticides underscore the importance of carefully selecting and testing microbial combinations in organic or integrated mushroom production management programmes (Keil, 2002; Fatimah et al., 2025; Marčić et al., 2025).

Therefore, the main goal of the current study was to investigate mutual relationships (synergistic/antagonistic/additive) among biocontrol agents, which may potentially be effective against both pests and pathogens in cultivated *A. bisporus* production. Tripartite interactions among the beneficial bacterium *Bacillus amyloliquefaciens*, the entomopathogenic nematode *Steinernema feltiae* (Filipjev), and yield-stimulating actinobacterium *Streptomyces flavovirens*, and their combined effects were evaluated in terms of the following: *a)* suppression of *T. aggressivum*, *b)* control of the mushroom gnat *L. ingenua*, and *c)* impact on mushroom yield in an experimental growing chamber.

MATERIAL AND METHODS

Mycopathogenic fungus

Half of the experimental plots were artificially inoculated with a conidial suspension of the green mould disease agent, *Trichoderma aggressivum* f. *europaeum* T77 [Accession number KC555186 in Genbank (https://ncbi.nlm.nih.gov)], from the collection of the Institute of Pesticides and Environmental Protection, Belgrade-Zemun, Serbia. The inoculum was prepared according to the protocol described by Milijašević-Marčić et al. (2024) and applied in the amount of 10^6 conidia per m^2 of casing soil one day after spawning.

Biocontrol agents: bacterial strains and entomopathogenic nematodes

The bacterium *Bacillus amyloliquefaciens* B-241 and actinobacterium *Streptomyces flavovirens* A06, isolated from mushroom compost by Stanojević et al. (2016; 2019) and Šantrić et al. (2018), respectively, were used in this study. The compost was produced in the compost factory Uča d.o.o. (Vranovo, Serbia), and microorganisms were obtained from the culture collection of the Institute of Pesticides and Environmental Protection, Belgrade-Zemun, Serbia. Treatments with these two biocontrol agents were performed as described previously by Milijašević-Marčić et al. (2024). Microbial concentrations of bacterial and actinobacterial suspensions were adjusted to 10^8 and 10^9 CFU ml^{-1}, respectively, and confirmed using the plate count technique.

A commercial population of the entomopathogenic nematode *S. feltiae* (Nemaplus, E-nema GmbH, Germany) was used in the mushroom growing chamber experiments. Fresh infective juveniles (IJs) were produced *in vivo* using the last larval instar of the greater wax moth *Galleria mellonella* (Lepidoptera: *Pyralidae*) (Drobnjaković et al., 2025). Infective juveniles not older than 4-5 days (99% viability confirmed before treatment) were used at a concentration of 0.75×10^6 IJ m^{-2} in 450 ml H_2O and applied three times – at the casing time, and then seven and 14 days later (total amount 2.25×10^6 IJ m^{-2}).

Mushroom growing room experiments

The experiments were carried out in an environmentally controlled mushroom growing chamber (Institute of Pesticides and Environmental Protection, Belgrade-Zemun, Serbia) during October and November 2024. Plastic containers ($l \times w \times h$ dimensions of 0.285 m × 0.2 m × 0.140 m) were filled with 1.5 kg of compost and spawned with 1% mycelium of *A. bisporus* A15 (Sylvan, Hungária, zRt, Hungary). Spawned compost was incubated at 24°C for 15 days, and then covered with a 40 mm-thick layer (1.3 kg) of black peat-based mushroom casing soil (Terahum d.o.o., Veliko Gradište, Serbia). The casing layer was sterilized with 0.02% peracetic acid (15% Peral S, MidraEko, Belgrade, Serbia) and enriched with 1.4% limestone (Tara Stil d.o.o., Serbia) prior to covering. The casing time was regarded as day one. After substrate incubation at 21°C for 8 days (case run), the air temperature was gradually reduced to 17°C to stimulate the development of mushroom fruiting bodies.

Singular and combined treatments with biocontrol agents were conducted in three split applications, starting from the casing time in a seven-day interval. Biocontrol agent treatments applied at full (100%) or reduced rates (20 or 40%), used in *T. aggressivum* f. *europaeum* inoculated and uninoculated briquettes, are shown in Table 1. Both the uninoculated and inoculated control plots were sprayed with tap water. All plots were arranged in a randomized block system with six replicates for each treatment. The experimental units were placed in insect-rearing cages (one plot per cage). The fungus gnat, *L. ingenua*, was monitored under conditions of natural compost infestation. Ecological interactions (synergistic/antagonistic/additive) among the three biological agents were evaluated based on their efficacy in controlling *T. aggressivum* f. *europaeum*, and suppressing the fungus gnat *L. ingenua* fourth instar (L_4) larvae. The influence of these biocontrol agents on mushroom yield (biological efficiency) was also evaluated.

During two flushes, hand-picked mushrooms were categorized as healthy or diseased, and then their weight was measured. Disease incidence was defined as the ratio of diseased mushrooms to the total number of fruiting bodies, expressed as a percentage. The efficacy (E%) of microbial agents in pathogen control was calculated using Abbott's formula (Abbott, 1925). The efficacy (E%) of entomopathogenic nematodes (EPNs) in pest control was evaluated by counting the mushroom fly adults captured in yellow sticky traps inside each insect-rearing cage. A binocular microscope was used to identify the fungus gnat adults and larvae, according to the identification key provided by Menzel and Mohrig (1999). The efficacy of EPN populations in sciarid pest larvae control was calculated using Abbott's formula (Abbott, 1925), based on adult pest incidence in treatments compared to the control (Gea et al., 2005). The harvesting period lasted 42 days. The influence of individual or combined treatments of the three biological agents on mushroom yield (biological efficiency, BE%) was calculated (Chrysayi-Tokousbalides et al., 2007). The synergy factor (Sf) was determined as the ratio of observed effects to expected effects on pest/disease control or yield. Limpel's formula was used to calculate expected values of efficacy in pest/disease control (E%) and impact on yield (BE%) in dual [x+y-(xy)/100] or tripartite interactions [x+y+z-(xy+xz-yz)/100+xyz/10000] (Richer, 1987). Accordingly, Sf > 1, Sf < 1, and Sf = 1 indicate a synergistic interaction, antagonistic interaction, and additive interaction among biocontrol agents, respectively.

Table 1. Treatments in the experimental growing chamber and their abbrevations

Treatments of uninoculated briquettes		Treatments of inoculated briquettes with *Trichoderma aggressivum*	
BA[a]100[f]	*Bacillus amyloliquefaciens* B-241 1×10^9 CFU ml^{-1} in 1 l H_2O m^{-2}	BA100 I[e]	*Bacillus amyloliquefaciens* B-241 1×10^9 CFU ml^{-1} in 1 l H_2O m^{-2} + TA[d]
BA40	*Bacillus amyloliquefaciens* B-241 4×10^8 CFU ml^{-1} in 1 l H_2O m^{-2}	BA40 I	*Bacillus amyloliquefaciens* B-241 4×10^8 CFU ml^{-1} in 1 l H_2O m^{-2} + TA
SF[b]100	*Streptomyces flavovirens* A06 1×10^8 CFU ml^{-1} in 1 l H_2O m^{-2}	SF100 I	*Streptomyces flavovirens* A06 1×10^8 CFU ml^{-1} in 1 l H_2O m^{-2} + TA
SF40	*Streptomyces flavovirens* A06 4×10^7 CFU ml^{-1} in 1 l H_2O m^{-2}	SF40 I	*Streptomyces flavovirens* A06 4×10^7 CFU ml^{-1} in 1 l H_2O m^{-2} + TA
EPN[c]100	*Steinernema feltiae* 0.75×10^6 IJ in 450 ml H_2O m^{-2}	EPN100 I	*Steinernema feltiae* 0.75×10^6 IJ in 450 ml H_2O m^{-2} + TA
EPN20	*Steinernema feltiae* 0.15×10^6 IJ in 450 ml H_2O m^{-2}	EPN20 I	*Steinernema feltiae* 0.15×10^6 IJ in 450 ml H_2O m^{-2} + TA
BA40 + SF40	*Bacillus amyloliquefaciens* B-241 4×10^8 CFU ml^{-1} in 1 l H_2O m^{-2} + *Streptomyces flavovirens* A06 4×10^7 CFU ml^{-1} in 1 l H_2O m^{-2}	BA40 + SF40 I	*Bacillus amyloliquefaciens* B-241 4×10^8 CFU ml^{-1} in 1 l H_2O m^{-2} + *Streptomyces flavovirens* A06 4×10^7 CFU ml^{-1} in 1 l H_2O m^{-2} + TA
BA40 + SF40 + EPN20	*Bacillus amyloliquefaciens* B-241 4×10^8 CFU ml^{-1} in 1 l H_2O m^{-2} + *Streptomyces flavovirens* A06 4×10^7 CFU ml^{-1} in 1 l H_2O m^{-2} + *Steinernema feltiae* 0.15×10^6 IJ in 450 ml H_2O m^{-2}	BA40 + SF40 + EPN20 I	*Bacillus amyloliquefaciens* B-241 4×10^8 CFU ml^{-1} in 1 l H_2O m^{-2} + *Streptomyces flavovirens* A06 4×10^7 CFU ml^{-1} in 1 l H_2O m^{-2} + *Steinernema feltiae* 0.15×10^6 IJ in 450 ml H_2O m^{-2} + TA
C	Control uninoculated	CI	Control inoculated with TA

[a]BA – bacterium *Bacillus amyloliquefaciens* B-241,[b]SF – actinobacterium *Streptomyces flavovirens* A06, [c]EPN – entomopathogenic nematode *Steinernema feltiae*, [e]I – artificial inoculation with [d]TA – *Trichoderma aggressivum* f. *europaeum* T77. [f]The numbers following the abbreviations indicate application rates.

Statistical analyses

The data transformation $\sqrt{(x + 0.1)}$ was applied to normalize and eliminate zero values associated with fungus gnat adults, while the percentage efficacy of the biocontrol agents was transformed with $arcsin\sqrt{(x/100)}$. Data were processed using one-way ANOVA (treatment as a factor); the significance of differences between the mean values associated with each treatment used in pest/disease control, as well as the impact of treatments on mushroom yield compared to the control, was determined with Fisher's post hoc test ($p<0.05$) (StatSoft Inc., 2004).

RESULTS AND DISCUSSION

Disease control

The first symptoms of green mould disease were observed on mushroom caps 17 days after casing in plots infested with the pathogen *T. aggressivum* f. *europaeum*. Large necrotic lesions appeared on mushroom caps and stems a few days later. Green colonies, a few centimetres in diameter, developed on the casing soil 28 days after casing.

The efficacy of single or combined biocontrol agents in suppressing the green mould pathogen is shown in Figure 1 ($F_{7,32}=1.93$; $p<0.09$). No statistically significant differences were found among the three biocontrol agents when applied individually at the full standard rates, or in combination (BA40 + SF40; BA40 + SF40 + EPN20). Comparable efficacies were recorded for the tripartite (BA40 + SF40 + EPN20; E=70.8%) and dual (BA40 + SF40; E=69.2%) applications of biocontrol agents in *T. aggressivum* control. The lowest efficacy in disease control (E=37.32%) was observed for the individual application of entomopathogenic nematodes at a reduced rate (EPN20). Previously, Milijašević-Marčić et al. (2024) reported comparable efficacy (E=62-69%) in disease control by using the same two beneficial microorganisms at different ratios (BA80 + SF20) in six split applications. However, through the combined use of an entomopathogenic nematode and each microbial strain

(BA80 + EPN20; E=75-81%; or SF80 + EPN20; E=65–78%), Potočnik et al. (2025) achieved higher efficacy in disease control than found in this study (BA40 + SF40; E=69%), and by Milijašević-Marčić et al. (2024).

A dual combination of beneficial microorganisms (BA40 + SF40) in this study showed an additive relationship in efficacy of pathogen suppression, as the synergy factor was approximately equal to 1 (Table 2).

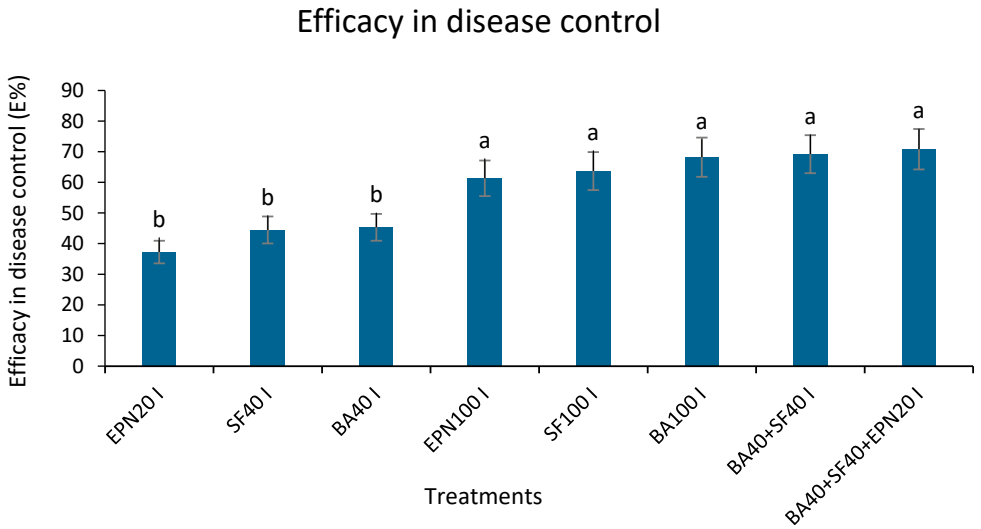

Figure 1. Efficacy of single or combined biological agents (BA – bacterium *Bacillus amyloliquefaciens* B-241, SF – actinobacterium *Streptomyces flavovirens* A06, EPN – entomopathogenic nematode *Steinernema feltiae*) in the control of green mould disease agent (I – artificial inoculation with *Trichoderma aggressivum* f. *europaeum* T77) on *Agaricus bisporus*. The numbers following the abbreviations indicate application rates. Data represent means of six replicates ± SE, standard error of the mean. Values within a repetition series marked with the same letter are not significantly different according to the *F*-test ($p<0.05$).

Table 2. Dual interaction between biological agents *Bacillus amyloliquefaciens* B-241 and *Streptomyces flavovirens* A06 (40%:40%): impact on efficacy of disease/pest control and mushroom yield

Effects		Treatment	Value
Efficacy (E%) in suppressing the pathogenic fungus (*Trichoderma aggressivum* f. *europaeum* T77)	Observed E% (Mean ± SE[d])	Inoculated[c]	69.2 ± 6.2[f]
	Expected E%[a]	Inoculated	69.62
	Synergy Factor (Sf)[e]	Inoculated	0.99 (0.98-1.12)[f]
Efficacy (E%) in suppressing the mushroom gnat *Lycoriella ingenua*	Observed E% (Mean ± SE)	Uninoculated[b]	19.2 ± 2.4
		Inoculated	17.4 ± 1.48
	Expected E%	Uninoculated	32.76
		Inoculated	37.11
	Synergy Factor (Sf)	Uninoculated	0.59 (0.45-1.29)
		Inoculated	0.47 (0.3-0.93)
Biological efficiency (BE%) in mushroom productivity (*Agaricus bisporus*)	Observed BE% (Mean ± SE)	Uninoculated	119.33 ± 4.4
		Inoculated	123.29 ± 4.7
	Expected BE%	Uninoculated	94.66
		Inoculated	93.8
	Synergy Factor (Sf)	Uninoculated	1.26 (1.21-1.4)
		Inoculated	1.31 (1.24-1.37)

[a]Expected (Exp) E% and BE% values, calculated as Exp = X+Y-(XY)/100, represent the percentage of an effect obtained from the additive responses of two combined biocontrol agents (X and Y) in treatments of briquettes [b]uninoculated and [c]inoculated with *Trichoderma aggressivum* f. *europaeum* T77. [d]Data represent means of six replicates ± SE, standard error of the mean for observed E% and BE% values. [e]Sf – the synergy factor represents a ratio of observed to expected effects, associated with a [f]confidence interval.

The obtained values for efficacy were consistent with the findings of Milijašević-Marčić et al. (2024) concerning the dual use of the same beneficial strains in different proportions (BA80 + SF20). Similarly, a previous study by Potočnik et al. (2025) regarding a dual combination of an entomopathogenic nematode with each microorganism (BA80 + EPN20 or SF80 + EPN20) in pathogen control, also revealed an additive interaction among biocontrol agents. In contrast, the tripartite application of biocontrol agents (BA40 + SF40 + EPN20) indicated a mild antagonistic interaction (Sf=0.83) impacting the efficacy in green mould disease control (Table 3). Accordingly, the majority of published studies on the combined use of biocontrol agents in plant disease management confirm the prevalence of antagonistic interactions (Xu et al., 2011).

Table 3. Tripartite interaction of biological agents *Bacillus amyloliquefaciens* B-241, *Streptomyces flavovirens* A06, and *Steinernema feltiae* (40%:40%:20%): impact on efficacy of disease/pest control and mushroom yield

Effects		Treatment	Value
Efficacy (E%) in suppressing the pathogenic fungus (*Trichoderma aggressivum* f. *europaeum* T77)	Observed E% (Mean ± SE[d])	Inoculated[c]	70.8 ± 6.6
	Expected E%[a]	Inoculated	85.79
	Synergy Factor (Sf)[e]	Inoculated	0.83 (0.82-1.17)[f]
Efficacy (E%) in suppressing the mushroom gnat *Lycoriella ingenua*	Observed E% (Mean ± SE)	Uninoculated[b]	46.4 ± 2.97
		Inoculated	47.2 ± 1.52
	Expected E%	Uninoculated	60.06
		Inoculated	60.24
	Synergy Factor (Sf)	Uninoculated	0.77 (0.63-1.39)
		Inoculated	0.78 (0.61-1.24)
Biological efficiency (BE%) in mushroom productivity (*Agaricus bisporus*)	Observed BE% (Mean ± SE)	Uninoculated	146.58 ± 5.5
		Inoculated	134.76 ± 9.2
	Expected BE%	Uninoculated	101.48
		Inoculated	100.41
	Synergy Factor (Sf)	Uninoculated	1.44 (1.43-1.8)
		Inoculated	1.34 (1.31-1.59)

[a]Expected (Exp) E% and BE% values, calculated as Exp = (X+Y+Z)-[(XY+XZ+YZ)/100]+[XYZ/10000], represent the percentage of effect obtained from the additive responses of three combined biocontrol agents (X, Y, and Z) in treatments of briquettes [b]uninoculated and [c]inoculated with *Trichoderma aggressivum* f. *europaeum* T77. [d]Data represent means of six replicates ± SE, standard error of the mean for observed E% and BE% values. [e]Sf – the synergy factor represents a ratio of observed to expected effects, associated with a [f]confidence interval.

Table 4. Effects of biocontrol agents on *Lycoriella ingenua* fourth instar larvae

Treatments	Number of emerged adult flies from treated fungus gnat L_4[d] larvae	
	Inoculated[e]	Uninoculated[f]
BA[a]100[g]	111.00[h] ± 7 c[i]	64.8 ± 3.9 c
BA40	128.4 ± 6.2 b	75 ± 2.91 b
SF[b]100	105.4 ± 6.76 d	61.6 ± 3.35 c
SF40	127 ± 7.59 bc	75 ± 3.35 b
EPN[c]100	17.6 ± 1.08 g	11 ± 0.55 e
EPN20	92.2 ± 2.31 e	53.8 ± 1.39 d
BA40 + SF40	124.8 ± 3.26 bc	73.8 ± 7.07 b
BA40 + SF40 + EPN20	81.2 ± 1.9 f	48.6 ± 1.29 d
Control	156.2 ± 8.8 a	91.6 ± 4.13 a

Biocontrol agents ([a]BA – bacterium *Bacillus amyloliquefaciens* B-241, [b]SF – actinobacterium *Streptomyces flavovirens* A06, [c]EPN – entomopathogenic nematode *Steinernema feltiae)* and their effects on *Lycoriella ingenua* fourth instar larvae [d](L_4) on *Agaricus bisporus*, in briquettes [e]inoculated or [f]uninoculated with *Trichoderma aggressivum* f. *europaeum* T77. [g]The numbers following the abbreviations indicate application rates. [h]Data represent means of six replicates ± SE, standard error of the mean. [i]Values within columns marked with same letter are not significantly different according to the *F*-test ($p<0.05$).

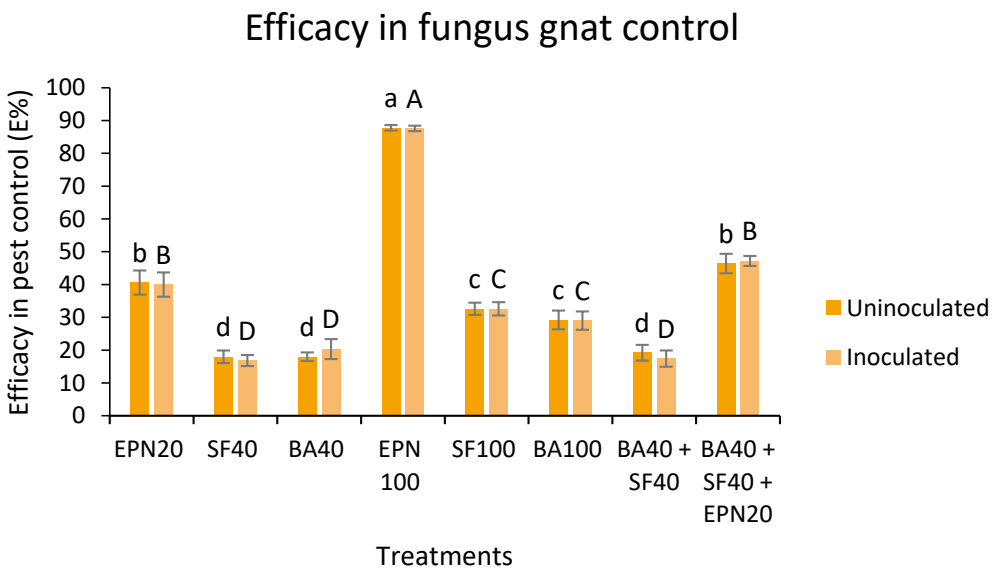

Figure 2. Efficacy of single or combined biological agents (BA – bacterium *Bacillus amyloliquefaciens* B-241, SF – actinobacterium *Streptomyces flavovirens* A06, EPN – entomopathogenic nematode *Steinernema feltiae*) in the control of fungus gnat *Lycoriella ingenua* fourth instar larvae on *Agaricus bisporus* over the entire test period; briquettes were uninoculated or artificially inoculated with *Trichoderma aggressivum* f. *europaeum* T77. The numbers following the abbreviations indicate application rates. Data represent means of six replicates ± SE, standard error of the mean. Values within a repetition series marked with the same letter are not significantly different according to the *F*-test ($p<0.05$).

Pest control

Over the entire test period, the biocontrol agents significantly reduced the emergence of *L. ingenua* adults; the mean number of pest flies that emerged from treated fungus gnat fourth instar (L_4) larvae was significantly lower in all treatments with beneficial organisms compared to the control in substrates inoculated ($F_{8,36}$ = 87.58, $p<0.001$) and uninoculated with *T. aggressivum* f. *europaeum* T77 ($F_{8,36}$ = 115.34, $p<0.001$) (Table 4).

In plots inoculated with *T. aggressivum*, Fisher's post-hoc test showed that treatment with the commercial EPN population applied at the standard rate in three split applications achieved the best overall pest control performance ($F_{8,36}$=122.68, $p<0.001$), with efficacy over 87%. The efficacy recorded for the single reduced application rate of beneficial nematodes (EPN20; E=40%) was not significantly different from that achieved by the tripartite combination of beneficial organisms (BA40 + SF40 + EPN20; E=47.2%) (Figure 2). The lowest efficacy was recorded for the individual applications of beneficial microorganisms at their respective reduced rates (SF40 and BA40), as well as their combined application (BA40 + SF40) (Figure 2). Drobnjaković et al. (2025) recorded lower efficacy (over 75%) for the commercial EPN population applied at the standard rate in two split applications, than was observed in the current study. In addition, Potočnik et al. (2025) recorded efficacy (over 94%) for the standard rate of the commercial EPN population, also used in three split applications, that was similar to the efficacy obtained in the current study.

Similar to the findings in inoculated plots, the highest efficacy in controlling fungus gnat larvae in uninoculated plots (87.8%) was recorded after treatment with the commercial EPN population applied at the standard application rate ($F_{8,36}$=127.57, $p<0.001$) (Figure 2). The lowest efficacy in pest larvae control (below 18%) was observed after individual treatments with beneficial microorganisms at their reduced rates (BA40 or SF40) (Figure 2). In addition, Fisher's test showed no statistically significant difference in the efficacy of EPN used at the reduced application rate, either individually (EPN20; E=40.6%) or combined with microorganisms (BA40 + SF40 + EPN20; E=46.4%) (Figure 2).

Potočnik et al. (2025) previously recorded higher efficacy of the commercial EPN in dual combinations with each microbial strain (BA80 + EPN20 or SF80 + EPN20; $E_{unicoluated}$≈84%; $E_{inoculated}$≈90%), than in the tripartite combination (BA40 + SF40 + EPN20).

Similarly, Potočnik et al. (2025) reported higher efficacy of EPN in pest control when applied in three split applications – either when used individually (EPN100; E≈94%) or combined with each microorganism (BA80+EPN20 or SF80 + EPN20; E≈90%) – in comparison to the efficacy of two split applications as recorded by Drobnjaković et al. (2025) (EPN100; E=83%). Likewise, in the current study, EPN applied individually at the standard application rate showed higher efficacy (EPN100; E≈88%), than when applied in the tripartite combination (BA40 + SF40 + EPN20; E≈47%).

The values of synergy factors revealed an antagonistic relationship among three biocontrol agents (BA40 + SF40 + EPN20) ($Sf_{uninoculated}$=0.59; $Sf_{inoculated}$=0.47), when combined to control *L. ingenua* fourth instar larvae (Table 3). In the present study, combined microorganisms (BA40 + SF40) showed antagonistic interactions against pest larvae either in the presence (Sf=0.47) or absence of *T. aggressivum* (Sf=0.59). In contrast, the dual combination of EPN with each microbial strain (BA80 + EPN20 or SF80 + EPN20) resulted in a mild antagonistic relationship among the biocontrol agents in the suppression of pest larvae in briquettes without the green mould disease agent (Sf≈0.9), and additive relationships (Sf≈1) in plots infested with *T. aggressivum* (Potočnik et al., 2025).

Few studies have confirmed antagonistic interactions between EPNs and microorganisms as biocontrol agents in mushroom fly control (Burns, 1999; Potočnik et al., 2025). The relationship between *Steinernema* and *Bacillus* species may be antagonistic, particularly when they compete directly for the same insect host or produce compounds that harm the other organism, potentially reducing the overall efficacy of one or both biocontrol agents. However, the interaction between EPNs and *Bacillus* spp. is not uniformly antagonistic. Interaction among biocontrol agents is highly dependent on the specific *Bacillus* strain and the target organism. For example, a study of Burns (1999) involving *Steinernema carpocapsae* and *Bacillus* spp. showed that some *Bacillus* spp. strains supported the development of EPNs, whereas EPN population growth was adversely affected by the presence of other *Bacillus* spp. strains. Such findings suggest that antagonistic interactions between *S. feltiae* and microorganisms may result from antibiotic activities of symbiotic bacteria in EPNs towards diverse gram-positive (which also include species of the genera *Bacillus* and *Streptomyces*) and gram-negative bacteria (Furgani et al., 2008; Shi et al., 2017; Caldas et al., 2002). Furthermore, many studies detected inhibitory or toxic effects of *Bacillus* spp. on the development of some EPNs (Grewal & Hand, 1992; El-Ashry & El-Marzoky, 2018). Conversely, additive or synergistic effects are common in the co-application of EPNs and entomopathogenic fungi for pest control in various cultivated plants, while antagonistic effects are rare (Půža & Tarasco, 2023).

Impact on mushroom yield

The highest mushroom production on substrate uninoculated with *T. aggressivum* f. *europaeum* ($F_{8,36}$=0.91; p<0.52) was recorded after combined treatment with three biocontrol agents (BA40 + SF40 + EPN20; BE=146.6%), and the lowest with BA40 alone (BE=119.3%) (Figure 3). The highest mushroom production in *T. aggressivum* f. *europaeum* inoculated plots ($F_{8,36}$=2.21; p<0.05) was also observed with the tripartite (BA40 + SF40 + EPN20) and dual (BA40 + SF40) application of biocontrol agents ($BE_{tripartite}$=134.8%; BE_{dual}=123.29%), while the lowest production was noticed in the control plots and those treated with EPN alone (BE=107%) (Figure 3). Greater mushroom yields were promoted by the association of two microorganisms with an entomopathogenic nematode (BA40 + SF40 + EPN20), than with the combination of microorganisms alone (BA40 + SF40).

Statistically significant differences were not found among any other treatments, either in inoculated or uninoculated plots. The combined use of three biocontrol agents (BA40 + SF40 + EPN20) increased mushroom yield up to 25% in inoculated plots compared to the control, while the yield increased up to 10% in uninoculated plots. Single use of the actinobacterium *S. flavovirens* increased mushroom production in inoculated plots up to 19% at the full application rate (SF100), and up to 17.5% at the reduced rate (SF40). Potočnik et al. (2025) found that the actinobacterium applied at a higher rate (three split applications) in inoculated plots enhanced mushroom yield up to 26-41%, while its combination with an entomopathogenic nematode (SF80 + EPN20) improved the yield up to 39%.

Regarding the impact of biocontrol agents on mushroom yield, synergy was observed in their combined use, both in dual (BA40 + SF40) or tripartite combinations (BA40 + SF40 + EPN20), with respective values of $Sf_{uninoculated}$ = 1.26; 1.44, and $Sf_{inoculated}$ = 1.31; 1.34 (Tables 2 and 3). Previous studies that also recorded synergistic effects of biocontrol agents on yield pertained to the dual use of microorganisms in different proportions, such as BA80 + SF20 (Milijašević-Marčić et al., 2024), and the dual use of EPN with each microorganism (BA80 + EPN20 or SF80 + EPN20) (Potočnik et al., 2025).

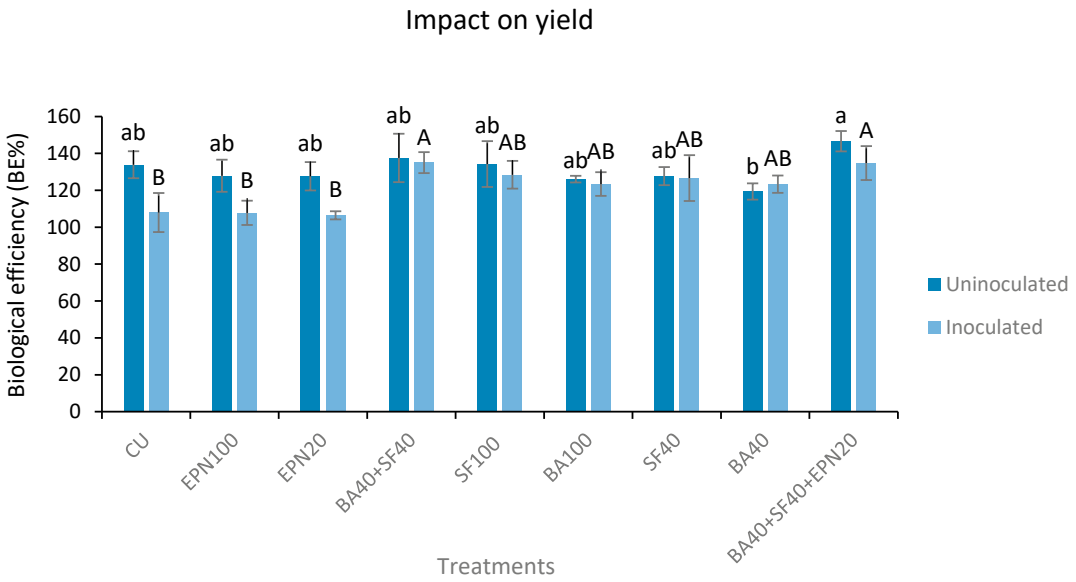

Figure 3. Impact of single or combined biological agents (BA – bacterium *Bacillus amyloliquefaciens* B-241, SF – actinobacterium *Streptomyces flavovirens* A06, EPN – entomopathogenic nematode *Steinernema feltiae*) on the yield (BE%) of *Agaricus bisporus* uninoculated or artificially inoculated with *Trichoderma aggressivum* f. *europaeum* T77. The numbers following the abbreviations indicate application rates. Data represent means of six replicates ± SE, standard error of the mean. Values within a repetition series marked with the same letter are not significantly different according to the *F*-test ($p<0.05$).

CONCLUSION

The combined use of three biocontrol agents (*B. amyloliquefaciens*, *S. flavovirens*, and *S. feltiae*) in the ratio of 40%:40%:20%, resulted in their antagonistic interaction in disease (*T. aggressivum* f. *europaeum*) and pest (*L. ingenua*) control in white button mushrooms, while a synergistic effect on overall mushroom production was recorded for these biocontrol agents. For most efficient disease and pest control, and greatest mushroom production, current findings support that the entomopathogenic nematode *S. feltiae* (0.75×10^6 IJ m^{-2} per application, total amount 2.25×10^6 IJ m^{-2}) may be applied individually at the beginning of the cultivation cycle (on the first day after casing), three times rather than twice, at weekly intervals, while beneficial microorganisms may be applied in combination a few days later (after one to four days) (*B. amyloliquefaciens* 1×10^9 CFU ml^{-1} m^{-2} per application, total amount 3×10^9 CFU ml^{-1} m^{-2}; and *S. flavovirens* 1×10^8 CFU ml^{-1} m^{-2} per application, total amount 3×10^8 CFU ml^{-1} m^{-2}). The development of new application procedures for these three beneficial organisms contributes to the improvement of mushroom production technology. The implementation of an appropriate and well-timed novel microbial combination may likely provide biorational protection for edible mushrooms against pests and diseases, resulting in improved mushroom yield and quality.

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Interakcije između bioloških agenasa u zaštiti šampinjona od najznačajnijih bolesti i štetočina

REZIME

Ispitivani su međusobni odnosi (sinergistički/antagonistički/aditivni) između tri biološka agensa: domaćih sojeva antagonističke bakterije *Bacillus amyloliquefaciens* B-241 i stimulatora prinosa, aktinobakterije *Streptomyces flavovirens* A06, kao i komercijalnog soja entomopatogene nematode *Steinernema feltiae*. Posmatran je uticaj interakcija datih bioloških agenasa na efikasnost u suzbijanju prouzrokovača bolesti zelene plesni *Trichoderma aggressivum* f. *europaeum* T77 (veštačka inokulacija), kao i u suzbijanju šampinjonske mušice *Lycoriella ingenua* (prirodna infestacija) u oglednom gajilištu šampinjona, *Agaricus bisporus*. Biološki agensi su primenjeni u standardnoj dozi, kao i u redukovanim dozama primene od 40% ili 20%. Uticaj na prinos šampinjona je izračunat kao odnos sveže mase ubranih šampinjona i suve mase komposta zasejanog micelijom šampinjona. Efikasnost u suzbijanju prouzrokovača bolesti šampinjona je procenjena na osnovu odnosa pojave i intenziteta bolesti, u inokulisanoj kontroli i tretmanima. Brojnost šampinjonske mušice je praćena korišćenjem žutih lepljivih traka koje su postavljene u kaveze za gajenje insekata sa supstratom za gajenje šampinjona. Prilikom trostruke primene bioloških agenasa, utvrđena je blaga antagonistička reakcija u njihovoj efikasnosti u suzbijanju prouzrokovača bolesti zelene plesni, antagonistička u suzbijanju šampinjonske mušice, i sinergistička u povećanju prinosa. Na osnovu dobijenih rezultata, biološke agense bi trebalo primeniti tri puta na sedam dana: entomopatogene nematode pojedinačno na početku ciklusa gajenja šampinjona (*S. feltiae* $0{,}75 \times 10^6$ IJ m^{-2}; ukupna količina $2{,}25 \times 10^6$ IJ m^{-2}), korisne mikroorganizme kombinovano nakon nekoliko dana (*B. amyloliquefaciens* 1×10^9 CFU ml^{-1} m^{-2}; ukupna količina 3×10^9 CFU ml^{-1} m^{-2}) i *S. flavovirens* 1×10^8 CFU ml^{-1} m^{-2}; ukupna količina 3×10^8 CFU ml^{-1} m^{-2}).

Ključne reči: jestive gljive; entomopatogene nematode; korisni mikroorganizmi; zaštita od štetočina; zaštita od bolesti