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Penicillium expansum, *P. crustosum*, and *P. paneum* cause blue mold of sugar beet roots in Serbia

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SUMMARY

In this study, three *Penicillium* species, *P. expansum*, *P. crustosum*, and *P. paneum*, were identified in sugar beet roots with blue mold collected at harvest and from overwintering roots, marking a novel finding for Serbia. Notably, our study is the first to document *P. crustosum* as the causal agent of blue mold in sugar beet in the world. Pathogenicity tests on artificially inoculated sugar beet roots confirmed that all *Penicillium* isolates can induce rot, with *P. expansum* demonstrating the highest virulence, followed by *P. crustosum* and *P. paneum*. As *Penicillium* species are important postharvest pathogens linked to decay, economic losses and mycotoxin contamination, further research into their postharvest presence and impact is essential. This study presents the first analysis of *Penicillium* spp. on sugar beet in Serbia, aimed to characterize isolates both molecularly and morphologically, and to evaluate their pathogenic potential as postharvest pathogens. The results contribute to the current knowledge of *Penicillium* species capable of colonizing sugar beet roots, and expand our understanding of *Penicillium* spp. and their diversity and distribution in Serbia.

Keywords: sugar beet, blue mold, characterization, phylogeny, virulence

INTRODUCTION

The genus *Penicillium* Link comprises a diverse group of species that are widely distributed across a broad range of habitats worldwide. Many *Penicillium* species are commonly present in soil as saprobes, where their primary ecological role is decomposition of organic matter. Some species, however, are capable of infecting healthy plant tissue as wound pathogens, typically causing disease postharvest. These *Penicillium* spp. are characterized by the ability to produce pectolytic enzymes, which efficiently degrade plant cell walls and induce decay. They are also known for their abundant sporulation, ability to grow at low temperatures, and capacity to colonize various substrates, including plant products (Frisvad & Samson, 2004; Pitt & Hocking, 2009). The primary inoculum source of *Penicillium* spp. for cultivated plants is the soil, where these fungi can survive on organic debris. Increased plant susceptibility at harvest, inoculum present in soil adhering to plants, combined with wounds caused by harvest operations or other fungal infections, may provide entry points and

favor *Penicillium* infection leading to development of blue mold or Penicillium rot (Dugan & Strausbaugh, 2019; Fugate & Campbell, 2009).

In sugar beet (*Beta vulgaris* L.), which is an important industrial crop, *Penicillium* species have been frequently found to be associated with postharvest decay of roots. Liebe and Varrelmann (2016) found *Penicillium* only on stored sugar beet roots, while it was absent from freshly harvested roots. Kusstatscher et al. (2019) showed that species belonging to *Penicillium*, along with *Candida* and *Fusarium*, were the main disease indicators in the microbiome of decaying sugar beets. The reported *Penicillium* species on sugar beet include: *P. vulpinum* (Cooke & Massee) Seifert & Samson, *P. cellarum* C.A. Strausb. & Dugan, *P. cyclopium* Westling, *P. expansum* Link, *P. polonicum* K. Zaleski, *P. tulipae* Overy & Frisvad, and *P. paneum* Frisvad. Additionally, members of the closely related genus *Talaromyces* C.R. Benj. (formerly classified under *Penicillium*) reported on sugar beet include: *T. funiculosus* (Thom) Samson, Yilmaz, Frisvad & Seifert (syn. *P. funiculosum*), *T. variabilis* (Sopp) Samson, Yilmaz, Frisvad & Seifert (syn. *P. variabilis*), and *T. rugulosus* (Thom) Samson, N. Yilmaz, Frisvad & Seifert (Bugbee, 1975; Bugbee & Nielsen, 1978; Dugan & Strausbaugh, 2019; Fugate & Campbell, 2009; Liebe et al., 2016; Strausbaugh, 2018; Strausbaugh & Dugan, 2017). In addition, *P. roqueforti* Thom and *P. paneum* have been isolated from sugar beet fiber silage (Boysen et al., 2000). *P. vulpinum* (syn. *P. claviforme*) has been the most prevalent among *Penicillium* species in various regions of the USA (Bugbee, 1975; Fugate & Campbell, 2009). Recent studies have shown that *P. expansum* has emerged as the dominant species in Idaho and Japan (Strausbaugh, 2018; Uchino, 2001). The presence and prevalence of *Penicillium* species on stored sugar beet roots may be the result of environmental factors, such as temperature. For example, Liebe et al. (2016) described *P. paneum* as the dominant *Penicillium* species on roots stored at 20 °C, while Strausbaugh and Dugan (2017) observed the prevalence of *P. cellarum* on sugar beet roots stored at higher temperatures.

In Serbia, several *Penicillium* species that cause blue mold on pome fruits (apple, pear, quince and medlar), lemon, mandarin, nectarine and tomato, as well as on onion and garlic bulbs, have been described (Duduk et al., 2017, 2021; Stošić et al., 2021a, 2021b, 2025; Vico et al., 2014; Žebeljan et al., 2021a; Živković et al., 2021), yet there is no data available on their presence on sugar beet. The aim of this study was to identify and characterize *Penicillium* isolates obtained from sugar beet at harvest, as well as from overwintering roots, both molecularly and morphologically, according to Visagie et al. (2014). Additionally, in view of the fact that *Penicillium* species have been documented as postharvest pathogens, the pathogenic potential of the isolates was evaluated on sugar beet.

MATERIAL AND METHODS

Sample collection and isolation

Sugar beet roots with blue mold symptoms were collected in November 2020 and February 2021 from a field at Rimski Šančevi, Novi Sad, Serbia. Fungal isolation was performed by placing fragments of decayed sugar beet tissue on Potato Dextrose Agar (PDA) in Petri plates. Following incubation at room temperature for five days, the plates were examined and fragments of fungal colonies were transferred to sterile PDA to obtain pure cultures. Single spore cultures were obtained and preserved as conidial suspensions in 30% glycerol, 0.05% agar, and 0.05% Tween 20 (Sigma-Aldrich, USA) at -80 °C.

Molecular identification and characterization

DNA extraction was performed from 7-day-old cultures of 15 obtained *Penicillium* spp. isolates grown on PDA using the CTAB protocol of Day and Shattock (1997). Using Bt2a/Bt2b primers, amplification of partial β tubulin region (*BenA*) was performed for all isolates (Glass & Donaldson, 1995). In nine selected isolates, nuclear ribosomal internal transcribed spacer region (ITS), partial gene for calmodulin (*CaM*), and RNA polymerase II second largest subunit (*RPB2*) were amplified using ITS1/ITS4, CMD5/CMD6, and 5F/7CR primers, respectively (Hong et al., 2006; Liu et al., 1999; Visagie et al., 2014; White et al., 1990). PCR reaction mix (25 µl) contained 1 µl of template DNA, 1xPCR Master Mix (Thermo Scientific, Vilnius, Lithuania) and 0.4 µM of each primer. Samples lacking DNA were used as negative controls. *BenA* and *CaM* amplifications were performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 60 s, and final elongation at 72 °C for 7 min. Conditions for amplification of the ITS region were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, and final elongation at 72 °C for 10 min. Conditions for *RPB2* amplification were as follows:

initial denaturation at 94 °C for 5 min, followed by 5 cycles at 94 °C for 45 s, 50 °C for 45 s and 72 °C for 60 s, then 5 cycles at 94 °C for 45 s, 52 °C for 45 s and 72 °C for 60 s and 30 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 60 s. The final elongation occurred for 7 min at 72 °C (Visagie et al. 2014). PCR products (5 µl) were analyzed on a 1.5% agarose gel, which was stained in ethidium bromide and visualized with a UV transilluminator. Amplified products were purified and sequenced in both directions with the primers applied in amplification. Sequences were assembled using Pregap4 from the Staden program package (Staden et al. 2000), manually inspected and compared to reference sequences available in the NCBI GenBank databases, then deposited in GenBank.

Multilocus sequence analysis and phylogeny

The obtained fungal DNA sequences were compared with those publicly available using BLAST (http://www.ncbi.nlm.nih.gov/). Related sequences and those of the closest species were retrieved from GenBank and aligned with sequences obtained in this study using ClustalX (Thompson et al., 1997), under MEGA version X (Kumar et al., 2018). Evolutionary history was inferred based on individual and combined analyses of four loci (ITS, *BenA*, *CaM*, and *RPB2*) of nine isolates from this study, reference isolates, and *P. expansum* CBS 325.48 or *P. paneum* CBS 101032 as outgroup for the two datasets, using the Maximum Likelihood (ML) method (MEGA X). The best nucleotide substitution model for ML was determined using the "find best model" option in MEGA X. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. To estimate the statistical significance of the inferred clades, 1,000 bootstraps were performed.

Morphological characterization

Colony morphology (appearance, presence of exudate, reverse color) and growth of eight selected *Penicillium* spp. isolates were analyzed on Malt Extract Agar (MEA), Czapek Yeast Extract Agar (CYA), and Yeast Extract Sucrose Agar (YES) according to Visagie et al. (2014), and additionally on PDA. Plates were inoculated with 1 µl of spore suspension (10^6 conidia/ml) of each isolate at three points, and three plates were used per medium. All inoculated plates were incubated in the dark at 24±2 °C. The morphology of conidiophores and conidia was evaluated from 7-10 day-old-cultures grown on MEA at 24±2 °C (Frisvad & Samson, 2004; Visagie et al., 2014), using the compound microscope Zeiss Axio Lab. Photographs of conidiophores and conidia were obtained using: Axiocam ERc 5s, Zeiss and conidia diameters were measured using ZEN 2 (blue edition) software.

Ehrlich test

The isolates were examined for the production of cyclopiazonic acid and other alkaloids according to Lund (1995). Filter paper (20 x 20 mm) was immersed in Ehrlich's reagent (2 g of 4-dimethylaminobenzaldehyde in 85 ml of 96% ethanol, with 15 ml of 10 N HCl) and placed on top of the mycelial side of agar plugs (three plugs per isolate, 8 mm in diameter) from 7-day-old cultures grown on CYA at 24±2 °C. After incubation for 2 to 10 minutes, color change was recorded. A positive reaction was indicated by the appearance of a violet ring, signifying the presence of cyclopiazonic acid or related alkaloids, while a pink, red or yellow ring indicated the production of other alkaloids (Frisvad & Samson, 2004).

Pathogenicity and virulence test

Fully developed healthy sugar beet roots were washed and surface-sanitized with 70% ethanol, then cut crosswise into 2 cm wide slices, sanitized with 70% ethanol and dried. Two holes per slice were made with the point of a finishing nail (10 mm deep and 3 mm in diameter), and inoculated by pipetting 40 µl of spore suspension into each hole. Seven-day-old cultures grown on PDA were used to prepare conidial suspensions (~10^6 spores/ml) in sterile distilled water with Tween 20 (0.05%). Four slices were used per isolate and control. Control slices were inoculated with 40 µl of Tween-treated sterile distilled water. Inoculated and control slices were placed in plastic containers and incubated under high humidity, at 24±2 °C in natural light/dark cycles. After 7 days of incubation, symptoms were recorded and lesion diameters measured. Fungal re-isolation was performed from lesions that developed on inoculated slices.

Statistical analysis

One-way ANOVA was used to determine whether there were significant differences in colony diameters among isolates of the same species, among different

species and/or among culture media, as well as to determine the difference in lesion size among isolates and species in the pathogenicity test. Post hoc Tukey's HSD test was used to evaluate differences that occurred in testing multiple groups. Statistical significance for all tests was defined at $p<0.05$. The statistical analyses were conducted with STATGRAPHICS (Centurion StatPoint 2005).

RESULTS

Penicillium identification and phylogeny

A total of 15 *Penicillium* isolates were obtained from sugar beet roots: five from samples collected at harvest in November 2020, and 10 from samples collected from overwintering roots in February 2021 (Table 1). *BenA* amplicons of the expected size (~500 bp) were obtained from all isolates, yielding 405-416 nt long sequences. Based on multiple sequence comparison and BLAST analyses of generated *BenA* gene sequences, three species were identified from sugar beet roots: *P. expansum* (seven isolates), *P. crustosum* Thom (three isolates), and *P. paneum* (five isolates). The *BenA* sequences of all *P. paneum* and *P. crustosum* isolates were identical to AY674387 of *P. paneum* ex-type CBS 101032 and AY674353 of *P. crustosum* ex-type CBS 115503, respectively, whereas *BenA* sequences of *P. expansum* isolates were 99-100% similar to AY674400 of *P. expansum* ex-type CBS 325.48 (four isolates were identical, and three isolates differed by 2 nt) (Visagie et al. 2014).

ITS, *CaM*, and *RPB2* amplicons of expected sizes (~600, 580, 1000 bp, respectively) were obtained from nine selected *Penicillium* isolates, resulting in sequences 508-528 nt long for ITS, 435-480 nt long for *CaM*, and 918-1056 nt long for *RPB2*. The obtained sequences were deposited in NCBI GenBank under accession numbers provided in Table 1. The multilocus phylogeny confirmed the assignment of *Penicillium* isolates obtained in this study to *P. expansum*, *P. crustosum* and *P. paneum,* which formed well-supported clusters (Figure 1 a, b). Intraspecific variability within all three species was observed. Three well-supported subclades were formed within *P. expansum* and two within *P. paneum*, while separation within *P. crustosum* clade was supported with lower bootstrap values.

Table 1. *Penicillium* spp. from sugar beet roots.

Species	Isolate	Date of isolation	Accession numbers			
			BenA	ITS	*CaM*	*RPB2*
P. expansum	PSR464	February 2021	PQ614149	–	–	–
	PSR465/2*	February 2021	PQ614140	PQ606579	PQ614155	–
	PSR466/3P*	February 2021	PQ614141	PQ606580	PQ614156	PQ614164
	PSR467	February 2021	PQ614150	–	–	–
	PSR468/5*	February 2021	PQ614142	PQ606581	PQ614157	PQ614165
	PSR469	February 2021	PQ614151	–	–	–
	PSR471/8*	February 2021	PQ614143	PQ606582	PQ614158	PQ614166
P. crustosum	PSR1748*	November 2020	PQ614145	PQ606587	PQ614160	PQ614168
	PSR400/1z*	November 2020	PQ614144	PQ606586	PQ614159	PQ614167
	PSR470	February 2021	PQ614152	–	–	–
P. paneum	PSR401/1i*	November 2020	PQ614146	PQ606591	PQ614161	–
	PSR402	November 2020	PQ614147	PQ606592	PQ614162	–
	PSR403/3*	November 2020	PQ614148	PQ606593	PQ614163	PQ614169
	PSR463	February 2021	PQ614153	–	–	–
	PSR472	February 2021	PQ614154	–	–	–

Legend: *Isolates used for morphological characterization

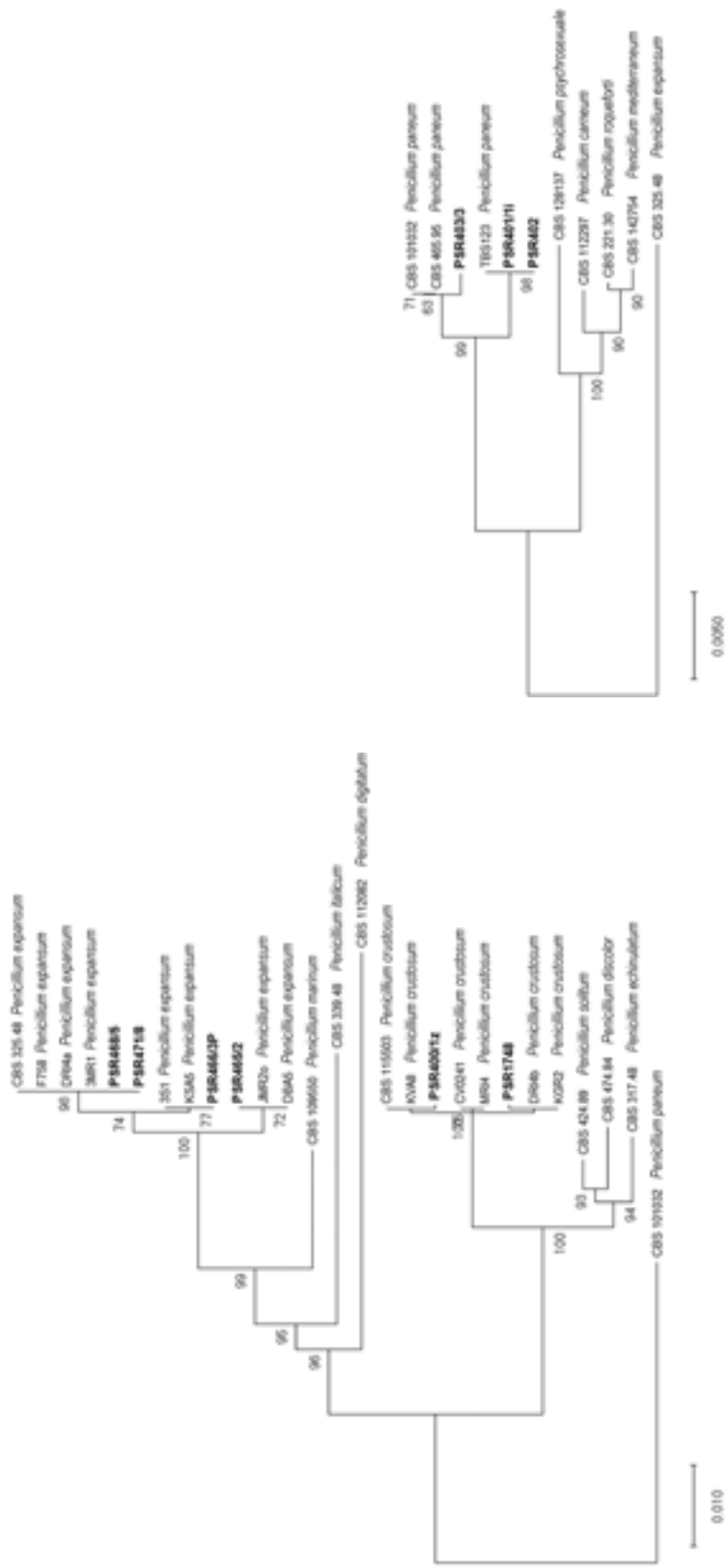

Figure 1. Phylogenetic relationships between *Penicillium expansum* and *P. crustosum* (a), and *P. paneum* (b), based on analysis of concatenated ITS, *BenA*, *CaM*, and *RPB2* sequences; inferred using the Maximum Likelihood method under the K2 + G model (a) and T92 model (b). The tree is rooted to *P. paneum* (a) and *P. expansum* (b). Numbers on the branches represent bootstrap values above 60% obtained from 1,000 replicates. Isolates obtained in this study are shown in bold. Scales show substitutions per site.

Colony characteristics and micromorphology

Penicillium expansum isolates formed blue-green to green colonies with white margins on all media. On PDA, colonies were mostly fasciculate, with or without concentric zones, and radially sulcate. Exudate was clear (PSR468/5 and PSR471/8) or yellow (PSR465/2 and PSR466/3P). Colony reverse was cream to pale yellow (PSR468/5 and PSR471/8) or intense yellow with a brown ring around the centre (PSR465/2 and PSR466/3P). On MEA, *P. expansum* formed fasciculate colonies, some with concentric zones and radially sulcate. Abundant clear exudate droplets were present in all colonies. Colony reverse was cream (PSR468/5 and PSR471/8) or yellow with a light brown ring (PSR465/2 and PSR466/3P). On CYA, colonies were concentrically fasciculate and weakly radially sulcate, rarely velutinous, and exudate was absent. Colony reverse was salmon pink (PSR468/5 and PSR471/8) or salmon cream (PSR465/2 and PSR466/3P). On YES, colonies were mostly fasciculate, radially sulcate, and exudate was clear to yellow. Colony reverse was pale yellow to intense yellow. Isolates formed terverticillata condiophores with smooth walled stipes. Conidia were blue-green in color, globose (mean diam 3.23±0.37 µm), with smooth walls, and formed in chains on ampulliform phialides (Figure 2; Table 2).

Penicillium crustosum isolates formed dull green colonies with white margins on all media. Colonies on PDA and MEA were velutinous to slightly fasciculate and radially sulcate, and became crustose after 10 days. Clear exudate was present. Colony reverse was yellow. On CYA, *P. crustosum* formed velutinous colonies with clear exudate. Colony reverse was

Table 2. Conidial dimensions and colony diameters of *Penicillium expansum*, *P. crustosum*, and *P. paneum* isolates on different media, 7 dpi, in the dark at 24±2 °C

Isolate	Conidia (µm ± SD, n=50)	Colony diameter on different culture media (mm ± SD)			
		PDA	MEA	CYA	YES
Penicillium expansum					
PSR465/2	3.18±0.34	18.83±0.49a*	20.76±0.54b	45±0.82a	35.94±1.25a
PSR466/3P	3.45±0.28	21.36±0.49b	19.83±0.51a	46.11±0.78ab	44.31±3.31c
PSR468/5	3.11±0.40	27.18±0.40c	29.10±0.57c	46.73±0.90b	42.37±4.19bc
PSR471/8	3.18±0.33	27.27±0.47c	29.22±0.44c	47.11±0.78b	40.44±3.78b
Mean	**3.23±0.37**	**22.42±3.55a****	**23.22±4.19a**	**46.45±1.02a**	**40.69±4.48a**
Penicillium crustosum					
PSR1748	3.08±0.23	34.23±0.97b	28.53±0.82b	44.00±1.48a	41.89±1.47a
PSR400/1z	3.06±0.31	30.27±1.05a	26.03±0.89a	44.33±0.52a	40.54±3.14a
Mean	**3.07±0.27**	**32.25±2,22b**	**27.28±1.51b**	**44.11±1.20a**	**41.30±2.4a**
Penicillium paneum					
PSR401/1i	3.92±0.18	45.33±2.72a	45.37±1.99a	41.54±0.88a	59.40±2.16b
PSR403/3	3.82±0.18	53.8±1.81b	52.93±1.68b	61.82±1.08b	47.14±8.11a
Mean	**3.88±0.19**	**49.57±4.80c**	**49.15±4.16c**	**50.83±10.15b**	**53.48±8.39b**

* Numbers labelled with different letters represent means of colony diameters that are significantly different among isolates of the same species according to Tukey's HSD test ($p<0.05$).

** Bolded numbers labelled with different letters represent means of colony diameters that are significantly different among species according to Tukey's HSD test ($p<0.05$).

cream yellow. On YES, colonies were fasciculate, radially sulcate and dense. Exudate was absent. Colony reverse was yellow to intense yellow. Isolates formed terverticillata conidiophores with rough walled stipes. Conidia were dull green to blue-green, globose (mean diam 3.07±0.27 µm), with smooth walls, and formed in chains on ampulliform phialides (Figure 3, Table 2).

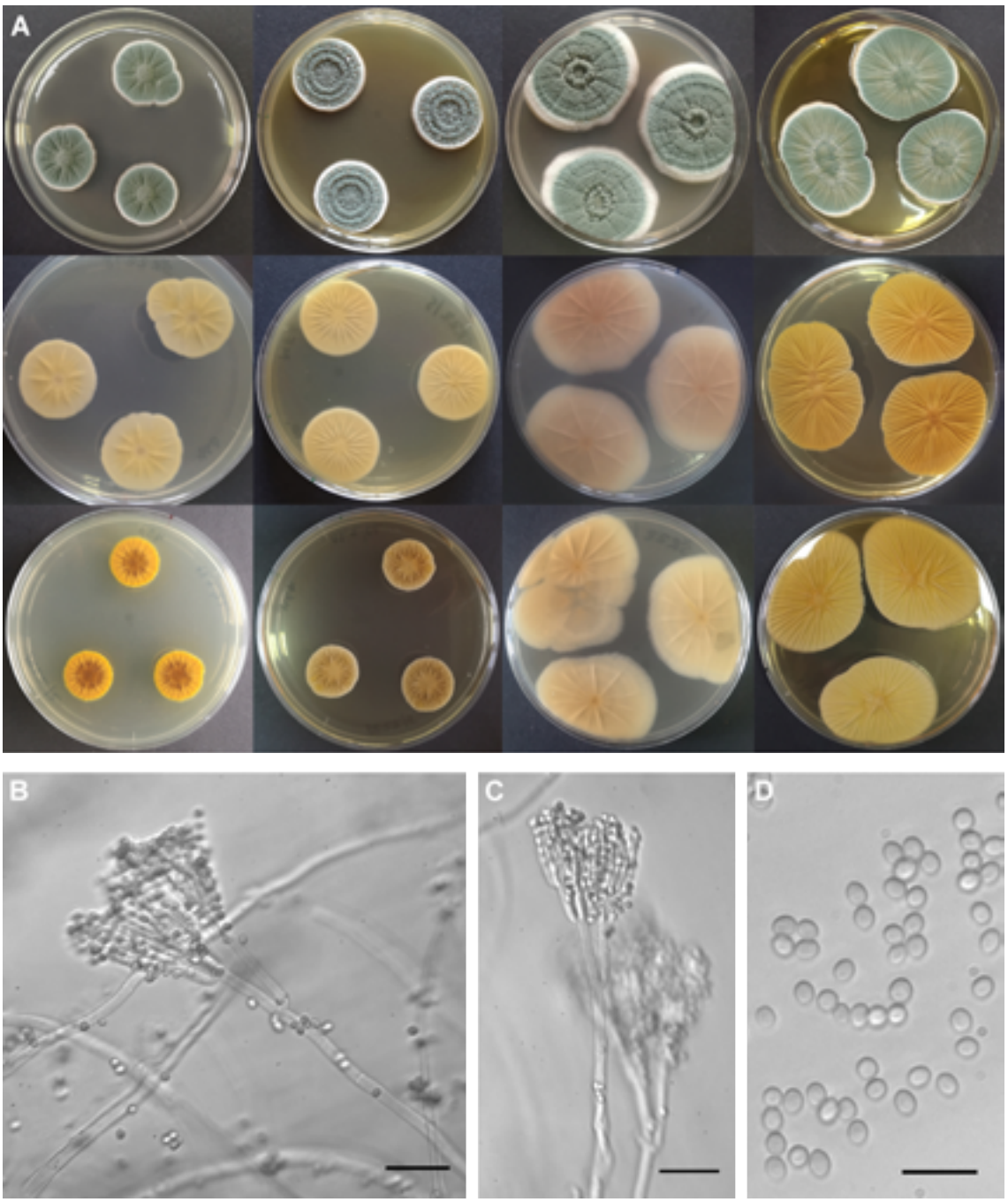

Figure 2. *Penicillium expansum*. A. Colonies on PDA, MEA, CYA and YES (left to right): obverse (top row) and reverse (middle row) of isolate PSR471/8; reverse of isolate PSR466/3P (bottom row). B, C. Conidiophores (Scale bars = 20 µm). D. Conidia (Scale bar = 10 µm).

Penicillium paneum formed blue-green to green colonies with olive brown centre on CYA and YES. On PDA and MEA, colonies were velutinous, slightly radially sulcate with white margins, with clear exudate droplets present. Colony reverse was cream. On CYA, colonies were velutinous with white margins. Yellow to brown exudate was present, and reverse was cream. On YES, colonies were radially sulcate and dense, and exudate was absent. Colony reverse was cream orange. The isolates formed terverticillata and, occasionally, biverticillata conidiophores with very rough walled stipes. Conidia were blue-green to green, globose (mean diam 3.88±0.19 µm), with smooth walls, and formed in chains on ampulliform phialides (Figure 4, Table 2).

Colony growth and Ehrlich test

Colony growth of *P. expansum*, *P. crustosum,* and *P. paneum* on four media is presented in Table 2. *P. paneum* had the fastest colony growth on all media ($p<0.05$). YES was the most favourable growth media for

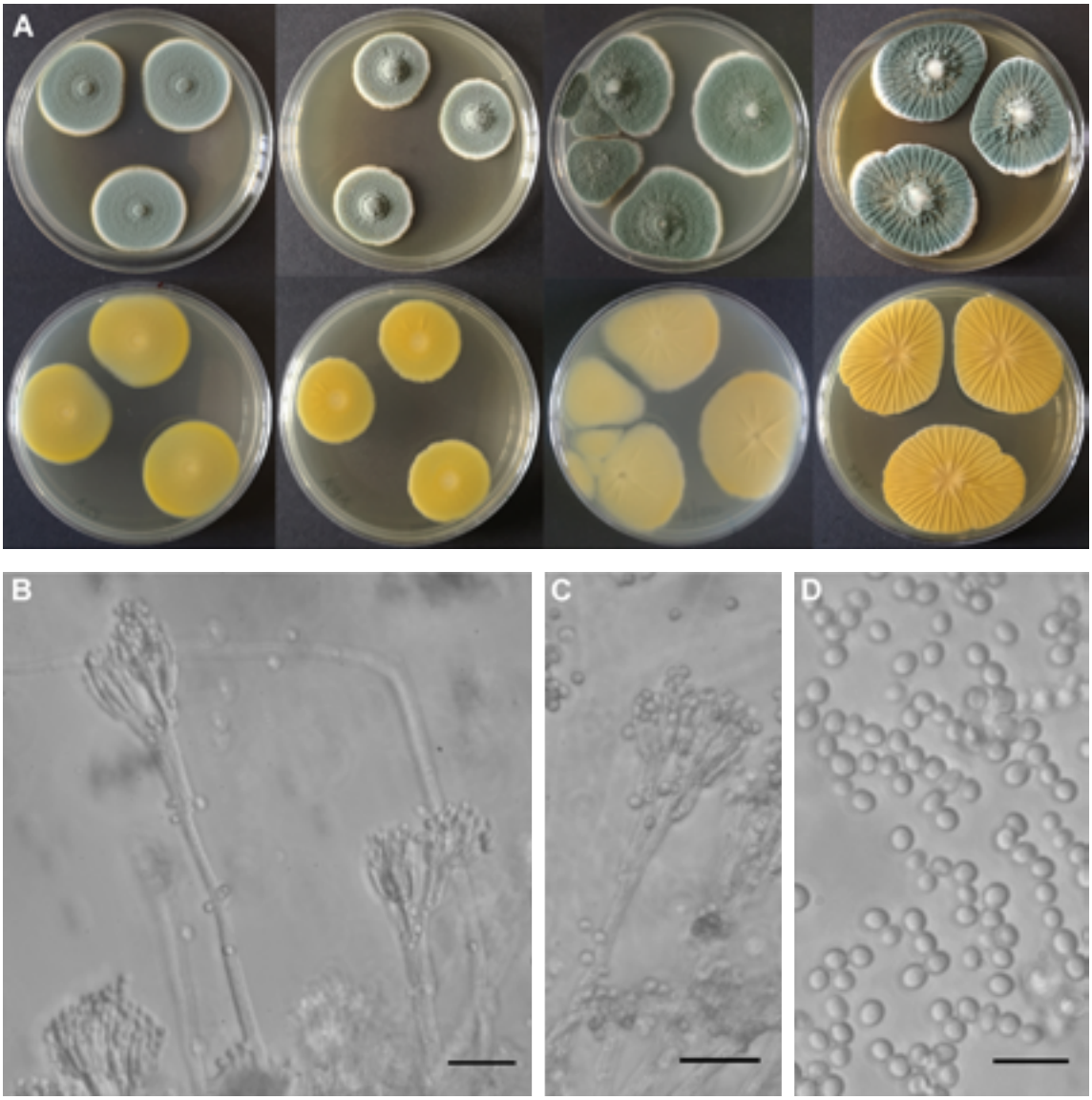

Figure 3. *Penicillium crustosum*. A. Colonies on PDA, MEA, CYA and YES (left to right): obverse (top row) and reverse (bottom row) of isolate PSR1748. B, C. Conidiophores (Scale bars = 20 µm). D. Conidia (Scale bar = 10 µm).

P. paneum, and CYA for *P. expansum* and *P. crustosum*. On PDA and MEA, *P. crustosum* growth was faster than that of *P. expansum* ($p<0.05$), while the growth of these two species on CYA and YES was comparable. Variability in colony growth was observed among isolates of *P. expansum* and *P. paneum* on all media ($p<0.05$), but it was found only on PDA and MEA among *P. crustosum* isolates ($p<0.05$). *P. expansum* isolates, which differed in reverse color on PDA, MEA, and CYA, also differed in colony growth on these media (PSR468/5 and PSR471/8 had faster growth than PSR465/2 and PSR466/3P).

In the Ehrlich test, *P. crustosum* and *P. expansum* isolates formed a yellow ring, while no color change was

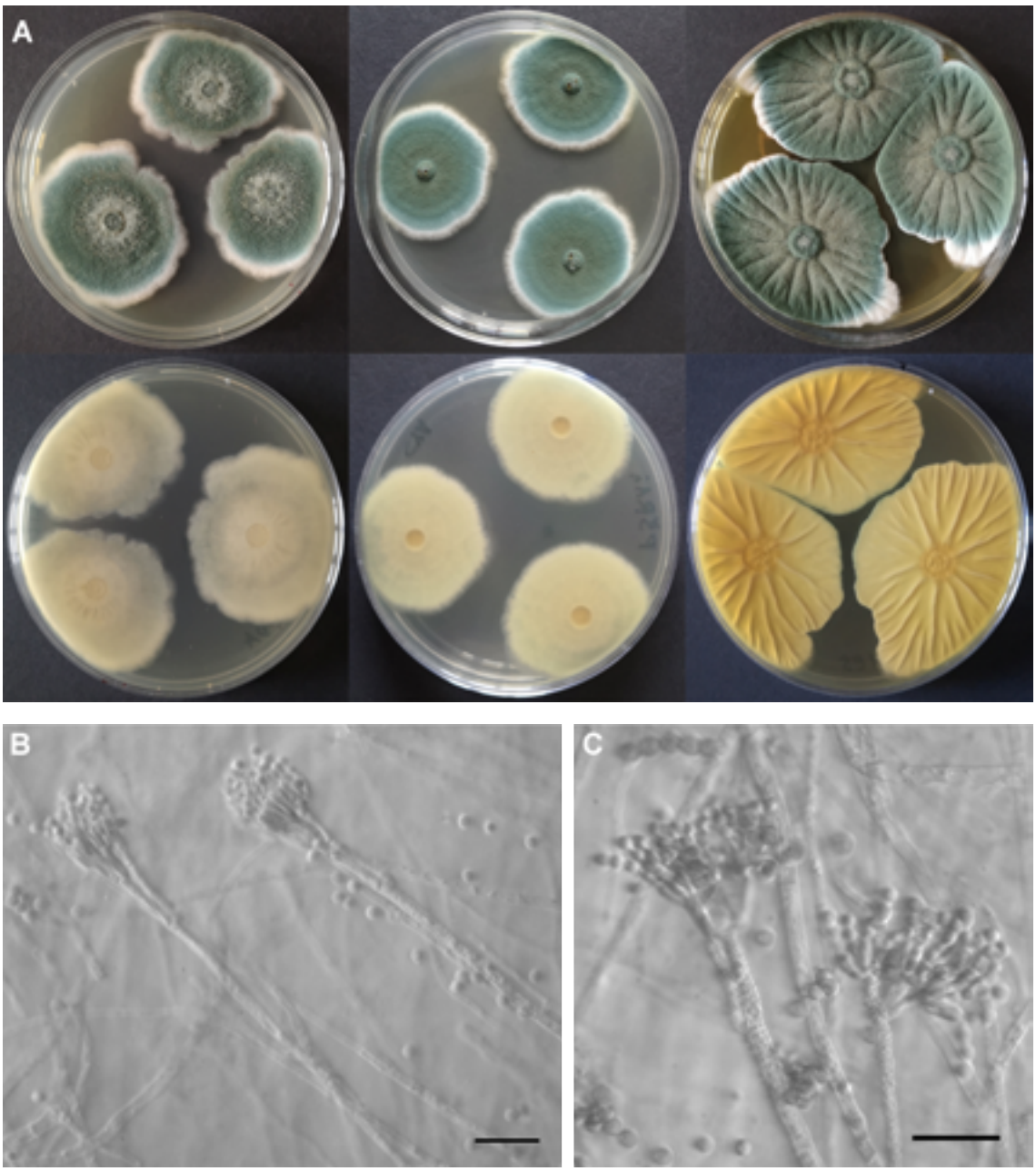

Figure 4. *Penicillium paneum*. A. Colonies on MEA, CYA and YES (left to right): obverse (top row) and reverse (bottom row) of isolate PSR403/3. B, C. Conidiophores and conidia (Scale bar = 20 μm).

observed in *P. paneum* isolates. The observed reactions indicated that *P. crustosum* and *P. expansum* isolates did not produce cyclopiazonic acid, but produced other alkaloids.

Pathogenicity and virulence of *Penicillium expansum*, *P. crustosum* and *P. paneum* on sugar beet

All tested isolates induced light brown rot on inoculated sugar beet slices, with sporulation mainly present at the inoculation site, confirming their pathogenicity. Control slices remained symptomless. Fungi reisolated from inoculated sugar beet slices exhibited identical morphological characteristics as the original isolates. The most virulent *Penicillium* species was *P. expansum* (average lesion size 20.8 mm), followed by moderately virulent *P. crustosum* (14.06 mm), and less virulent *P. paneum* (10 mm) ($p<0.05$). Considering isolates of the same species, differences in virulence were observed within *P. crustosum* (isolate PSR1748 was more virulent than PSR400/1z and PSR470), while no difference in virulence was noted within *P. paneum* and *P. expansum* (with the exception of PSR469) (Figure 5).

DISCUSSION

In this study, *P. expansum*, *P. crustosum*, and *P. paneum* were identified on sugar beet roots grown in Serbia. Two species, *P. crustosum* and *P. paneum*, were isolated at harvest, while *P. expansum* was dominant on overwintering sugar beet roots, followed by *P. crustosum* and *P. paneum*. The presence of these three species on sugar beet is a novel finding for Serbia, while *P. crustosum* had not been previously reported in scientific literature on sugar beet.

Penicillium expansum (section *Penicillium*) is the most prevalent and economically significant causal agent of blue mold in stored apples and pears globally (Jurick & Cox, 2017: Rosenberger, 2014). This species has also been isolated from a wide variety of other fruits, as well as vegetables, meat, cheese, grains, apple juice, dry fish, etc (Pitt & Hocking, 2009). *Penicillium expansum* has also been reported as a postharvest pathogen of sugar beet (Strausbaugh, 2018; Uchino, 2001). In this study, *P. expansum*, a well-documented pome fruit pathogen in Serbia (Žebeljan et al., 2021a), was isolated from overwintering sugar beet roots, revealing sugar beet as a novel host of this species in Serbia.

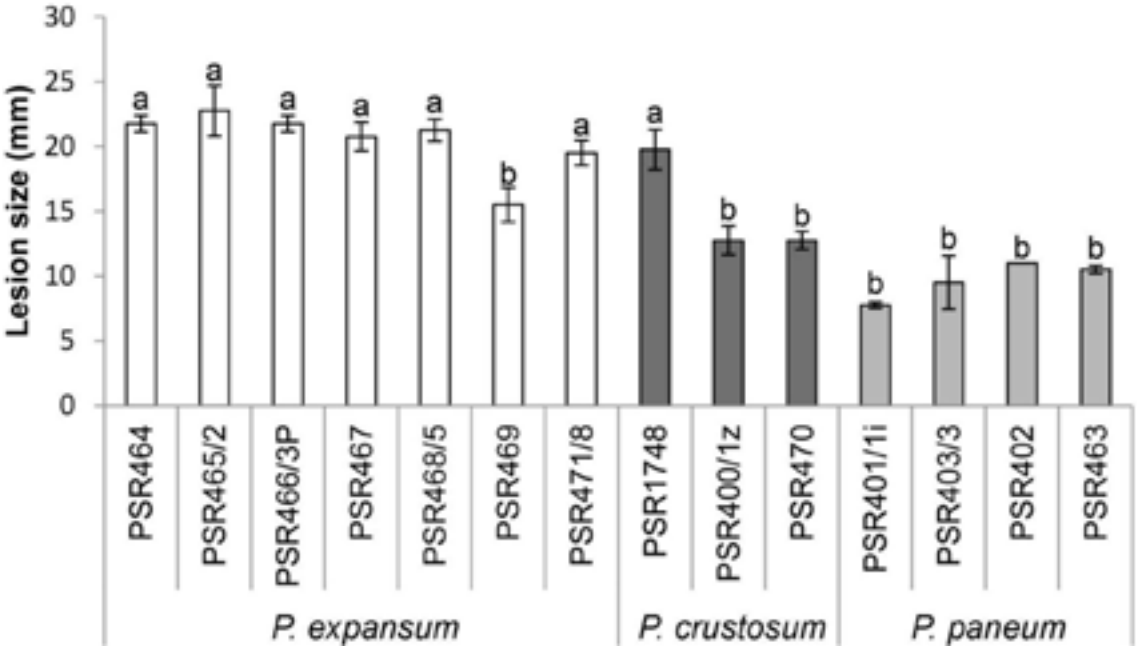

Figure 5. Virulence of *Penicillium expansum*, *P. crustosum*, and *P. paneum* on sugar beet slices. Numbers labelled with different letters represent means of lesion size that are significantly different among isolates according to Tukey's HSD test ($p<0.05$). Vertical bars represent standard errors of the mean.

The second identified species, *P. crustosum* (section *Fasciculata*), is a ubiquitous spoilage fungus, which has been isolated from cereal and animal feed samples such as maize, processed meats, cheese, biscuits, cakes and fruit juices (Pitt & Hocking, 2009). *Penicillium crustosum* has also been frequently isolated worldwide from pome fruits with blue mold (Andersen & Thrane, 2006; Louw & Korsten, 2014; Sholberg & Haag, 1996; Žebeljan et al., 2021a). In this study, *P. crustosum* was isolated from sugar beet roots collected at harvest, as well as from overwintering sugar beet roots, which represents the first report of this *Penicillium* species on sugar beet worldwide.

The morphological characteristics and growth rates of *P. expansum* and *P. crustosum* isolates from sugar beet were consistent with previously published descriptions for each species (Frisvad & Samson, 2004; Pitt & Hocking, 2009). Interestingly, when compared to previously characterized *P. expansum* and *P. crustosum* isolates from pome fruits from Serbia (Žebeljan et al., 2021a), similarities were observed in the presence of subgroups within *P. expansum* isolates from sugar beet based on their morphology (colony reverse on PDA, MEA and CYA), growth and phylogeny, as well as within *P. crustosum* based on *RPB2* locus and virulence. These results confirmed the morpho-genetic diversity within *P. expansum* and *P. crustosum*, opening opportunities for further studies of their populations across various hosts in Serbia.

Penicillium paneum (section *Roquefortorum*), known to occur on baled grass silage, has been reported as a significant contaminant of cereal grains, and has also been described as a pathogen of apple fruit (Boysen et al., 2000; Frisvad & Samson, 2004; O'Brien et al. 2008; Yin et al. 2017). Previously reported in Serbia on grape marc (Jovicic-Petrovic et al. 2016), *P. paneum* has now been identified as the causal agent of blue mold of sugar beet. In this study, we observed that *P. paneum* is clearly distinguishable from the other two *Penicillium* species by its larger conidia and more rapid growth, all consistent with the species description (An et al. 2009; Frisvad & Samson, 2004; O'Brien et al., 2008). Described as a postharvest pathogen of sugar beet, *P. paneum* dominates under storage conditions at 20°C (Liebe et al. 2016).

Penicillium is the most diverse genus of soil fungi (Visagie et al., 2014). Some species are opportunistic plant pathogens that colonize plants at the end of the growing season, affecting senescent plants or their parts, and are therefore often encountered postharvest. To assess the pathogenic potential of *Penicillium* isolates obtained at harvest or from overwintering sugar beet roots, we conducted artificial inoculation tests on sugar beet under conditions that maximally favor pathogenicity. Our results have shown that *P. expansum* isolates were the most virulent, which is consistent with previous findings (Strausbaugh, 2018). In comparison, *P. crustosum* displayed moderate virulence, while *P. paneum* showed lower virulence. Overall, the higher virulence of *P. expansum* compared to other *Penicillium* species, such as *P. crustosum,* was also observed on other plant species, such as apples (Louw & Korsten, 2014; Sanderson & Spotts, 1995; Žebeljan et al., 2021a). Research on the *Penicillium*-apple pathosystem has shown that *P. expansum* induces more intense and dynamic metabolic changes than *P. crustosum*, aiding the pathogen to overcome host defenses. This results in significantly reduced levels of phenolics and glutathione during *P. expansum*-mediated decay, compared to *P. crustosum*-mediated decay (Žebeljan et al. 2019, 2021b), leading to higher virulence of *P. expansum* and thus faster decay of the infected apple fruit.

Sugar beet roots stored in piles are vulnerable to *Penicillium* infection. Prolonged storage periods under higher temperatures can increase the likelihood of infection events. *Penicillium* species are characterized by abundant sporulation, which enhances their dispersal and survival, serving as an evolutionary strategy that allows their persistence in agricultural soils and on crop residues. In this study, the most virulent species, *P. expansum*, as well as *P. crustosum* and *P. paneum*, were isolated from overwintering sugar beet, indicating that inoculum persists in the roots throughout the winter. Although *Penicillium* species are generally less virulent than other postharvest pathogens of sugar beet, such as *Phoma betae* or *Botrytis cinerea*, their abundant sporulation can produce substantial airborne inoculum, potentially leading to destructive blue mold under prolonged or improper storage conditions for sugar beet (Fugate & Campbell, 2009). While *P. crustosum* is less virulent than *P. expansum*, it demonstrates a significant capacity to produce large quantities of conidia (Pitt & Hocking, 2009). The occurrence and persistence of less virulent *Penicillium* species, such as *P. crustosum*, may therefore be supported by the ability to sporulate abundantly (Žebeljan et al., 2021a).

Colonization by *Penicillium* species can negatively impact sugar beet by causing root decay, significantly reducing sucrose content, and introducing the risk of mycotoxin contamination (Fugate & Campbell, 2009). Though this study has identified three *Penicillium* species colonizing sugar beet, further detailed research is needed to determine their prevalence and impact

both as postharvest pathogens of sugar beet roots or as contaminants of sugar beet pulp silage in Serbia. However, based on their presence and pathogenic potential, these species are the likely contributors to postharvest losses in sugar beet. Additionally, the three species identified on sugar beet, *P. expansum*, *P. crustosum*, and *P. paneum*, are toxigenic. *P. expansum* is the primary producer of patulin, and also produces citrinin, roquefortine C and other mycotoxins; *P. crustosum* produces penitrem A, roquefortine C and other mycotoxins, while *P. paneum* produces roquefortine C and patulin (Perrone & Susca, 2017). The production of cyclopiazonic acid, a mycotoxin, is used for *Penicillium* spp. characterization (Frisvad & Samson, 2004; Visagie et al., 2014). Although the production of this extrolite is typically associated with *P. expansum* and *P. crustosum*, the Ehrlich test performed in this study showed that *P. expansum* and *P. crustosum* isolates from sugar beet did not produce cyclopiazonic acid, suggesting a variability in production across different substrates. However, other alkaloids were produced. Nevertheless, colonization by toxigenic species is particularly concerning if the infected roots are used as livestock feed. Although *P. paneum* has been detected in hard-pressed beet fibers, the impact on contamination remains unconfirmed (Boysen et al., 2000). Boudra et al. (2015) found low mycotoxin levels in sugar beet pulp, with roquefortine C detected in only a few samples and no detectable patulin. These findings suggest that further studies under diverse conditions and involving a broader range of mycotoxins are needed to verify the low-risk mycotoxin status of sugar beet pulp silage.

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REFERENCES

An, K.D., Kiyuna, T., Kigawa, R., Sano, C., Miura, S., & Sugiyama, J. (2009). The identity of *Penicillium* sp. 1, a major contaminant of the stone chambers in the Takamatsuzuka and Kitora Tumuli in Japan, is *Penicillium paneum*. *Antonie Van Leeuwenhoek*, *96*, 579-592. https://doi.org/10.1007/s10482-009-9373-0

Andersen, B., Thrane, U. (2006). Food-borne fungi in fruit and cereals and their production of mycotoxins. *Advances in Food Mycology, 571*, 137-152. https://doi.org/10.1007/0-387-28391-9_8

Boudra, H., Rouillé, B., Lyan, B., Morgavi, D.P. (2015). Presence of mycotoxins in sugar beet pulp silage collected in France. *Animal Feed Science and Technology, 205*, 131-135.

Boysen, M.E., Jacobsson, K.G., & Schnürer, J. (2000). Molecular identification of species from the *Penicillium roqueforti* group associated with spoiled animal feed. *Applied and Environmental Microbiology, 66*, 1523-1526.

Bugbee, W.M. (1975). *Penicillium claviforme* and *Penicillium variabile*: Pathogens of stored sugar beets. *Phytopathology 65,* 926-927. https://doi.org/10.1094/Phyto-65-926

Bugbee, W.M., & Nielsen, G.E. (1978). *Penicillium cyclopium* and *Penicillium funiculosum* as sugarbeet storage rot pathogens. *Plant Disease Reporter, 62*, 953.

Day, J.P., & Shattock, R.C. (1997). Aggressiveness and other factors relating to displacement of populations of *Phytophthora infestans* in England and Wales. *European Journal of Plant Pathology, 103*, 379-391.

Duduk, N., Bekčić, F., Žebeljan, A., Vučković, N., & Vico, I. (2021) First report of blue mold caused by *Penicillium crustosum* on nectarine fruit in Serbia. *Plant Disease, 105*(2), 489. https://doi.org/10.1094/PDIS-07-20-1632-PDN

Duduk, N., Lazarević, M., Žebeljan, A., Vasić, M., & Vico, I. (2017). Blue mould decay of stored onion bulbs caused by *Penicillium polonicum*, *P. glabrum* and *P. expansum*. *Journal of Phytopathology, 165*(10), 662-669. https://doi.org/10.1111/jph.12605

Dugan, F.M., & Strausbaugh, C.A. (2019). Catalog of *Penicillium* spp. causing blue mold of bulbs, roots, and tubers. *Mycotaxon 134*, 197-213.

Frisvad, J.C., & Samson, R.A. (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Studies in Mycology, 49*, 1-174.

Fugate, K., & Campbell, L. (2009). Postharvest deterioration of sugar beet. R. M. In: Harveson, R.M., Hanson, L.E., & Hein, G.L. (eds), *Compendium of Beet Diseases and Pests*, 2nd edn. (pp 92-94). St. Paul: APS Press.

Glass, N.L., & Donaldson, G.C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology, 61*(4), 1323-1330. https://doi.org/10.1128/aem.61.4.1323-1330.1995

Hong, S.B., Cho, H.S., Shin, H.D., Frisvad, J.C., & Samson, R.A. (2006). Novel *Neosartorya* species isolated from soil in Korea. *International Journal of Systematic and Evolutionary Microbiology, 56*(2),477-486. https://doi.org/10.1099/ijs.0.63980-0

Jovičić-Petrović, J., Stanković, I., Bulajić, A., Krstić, B., Kiković, D., & Raičević, V. (2016). Filamentous fungi isolated from grape marc as antagonists of *Botrytis cinerea*. *Genetika 48*, 37-48. https://doi.org/10.2298/GENSR1601037J

Jurick, II W.M., & Cox, K.D. (2017). Pre- and postharvest fungal apple diseases. In Evans (ed), *Achieving sustainable cultivation of apples*, 1st edn. (ch 14, pp 12). London: Burleigh Dodds Science Publishing.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution, 35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Kusstatscher, P., Cernava, T., Harms, K., Maier, J., Eigner, H., Berg, G., & Zachow, C. (2019). Disease incidence in sugar beet fields is correlated with microbial diversity and distinct biological markers. *Phytobiomes Journal, 3,* 22-30.

Liebe, S., & Varrelmann, M. (2016). Effect of environment and sugar beet genotype on root rot development and pathogen profile during storage. *Phytopathology, 106*, 65-75. doi: 10.1094/PHYTO-07-15-0172-R

Liebe, S., Wibberg, D., Winkler, A., Pühler, A., Schlüter, A., & Varrelmann, M. (2016). Taxonomic analysis of the microbial community in stored sugar beets using high-throughput sequencing of different marker genes. *FEMS Microbiology Ecology, 92*(2), fiw004.

Liu, Y.J., Whelen, S., & Hall, B.D. (1999). Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution, 16*(12),1799-1808. https://doi.org/10.1093/oxfordjournals.molbev.a026092.

Louw, J.P., & Korsten, L. (2014). Pathogenic *Penicillium* spp. on apple and pear. *Plant Disease, 98*(5), 590-598. https://doi.org/10.1094/PDIS-07-13-0710-RE

Lund, F. (1995). Differentiating *Penicillium* species by detection of indole metabolites using a filter paper method. *Letters in Applied Microbiology, 20*(4), 228-231. https://doi.org/10.1111/j.1472-765X.1995.tb00434.x

O'Brien, M., Egan, D., O'Kiely, P., Forristal, P.D., Doohan, F.M., & Fuller H.T. (2008). Morphological and molecular characterisation of *Penicillium roqueforti* and *P. paneum* isolated from baled grass silage. *Mycological Research, 112*(8), 921-932. https://doi.org/10.1016/j.mycres.2008.01.023

Perrone, G., & Susca, A. (2017). *Penicillium* species and their associated mycotoxins. In: Moretti, A., & Susca, A. (eds), *Mycotoxigenic Fungi. Methods in Molecular Biology, vol 1542*. (pp 107-119). New York: Humana Press. https://doi.org/10.1007/978-1-4939-6707-0_5

Pitt, J.I., & Hocking, A.D. (2009). *Penicillium* and related genera. In: Pitt, J.I., & Hocking, A.D. (eds), *Fungi and Food Spoilage* (pp 169-273). Boston: Springer. https://doi.org/10.1007/978-0-387-92207-2_7

Rosenberger, D.A. (2014). Blue mold. In: Sutton, T.B., Aldwinkle, H.S., Angello, A.M., & Walgenbach, J.F. (eds), *Compendium of apple and pear diseases and pests*, 2nd edn. (p. 76). St Paul: APS Press.

Sanderson, P., & Spotts, R. (1995). Postharvest decay of winter pear and apple fruit caused by species of *Penicillium*. *Phytopathology, 85,* 103-110. https://doi.org/10.1094/Phyto-85-103

Sholberg, P.L., & Haag, P.D. (1996). Incidence of postharvest pathogens of stored apples in British Columbia. *Canadian Journal of Plant Pathology, 18*, 81-85. https://doi.org/10.1080/07060669609500661

Staden, R., Beal, K.F., & Bonfield, J.K. (2000). The Staden package, 1998. In: *Bioinformatics Methods and Protocols* (Methods in Molecular Biology, 132; pp 115-130). https://doi.org/10.1385/1-59259-192-2:115

Stošić, S., Ristić, D., Savković, Ž., Ljaljević Grbić, M., Vukojević, J., & Živković, S. (2021a). *Penicillium* and *Talaromyces* species as postharvest pathogens of pear fruit (*Pyrus communis*) in Serbia. *Plant Disease, 105*(11), 3510-3521. https://doi.org/10.1094/PDIS-01-21-0037-RE

Stošić, S., Ristić, D., Trkulja, N., & Živković, S. (2025). *Penicillium* species associated with postharvest blue mold rots of garlic in Serbia. *Plant Disease, 109*, 149-161. doi: 10.1094/PDIS-04-24-0890-RE

Stošić, S., Ristić, D., & Živković, S. (2021b). Postharvest decay of mandarin fruit in Serbia caused by *Penicillium expansum*. *Zbornik Matice srpske za prirodne nauke, 140*, 29-44.

Strausbaugh, C.A. (2018). Incidence, distribution, and pathogenicity of fungi causing root rot in Idaho long-term sugar beet storage piles. *Plant Disease, 102*(11), 2296-2307. https://doi.org/10.1094/PDIS-03-18-0437-RE

Strausbaugh, C.A., & Dugan, F. (2017). A novel *Penicillium* sp. causes rot in stored sugar beet roots in Idaho. *Plant Disease, 101*(10), 178-1787. https://doi.org/10.1094/PDIS-03-17-0410-RE

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., & Higgins, D.G. (1997). The CLUSTAL_X Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*(24), 4876-4882. https://doi.org/10.1093/nar/25.24.4876

Uchino H (2001) Studies on storage rot in sugar beet [*Beta vulgaris*]. *Memoirs of the Graduate School of Agriculture - Hokkaido University*, *23*, 319-401.

Vico, I., Duduk, N., Vasić, M., & Nikolić, M. (2014). Identification of *Penicillium expansum* causing postharvest blue mold decay of apple fruit. *Pesticidi i fitomedicina, 29*(4), 257-266. https://doi.org/10.2298/PIF1404257V

Visagie, C.M., Houbraken, J., Frisvad, J.C., Hong, S.B., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Varga, J., Yaguchi, T., & Samson, R.A. (2014). Identification and nomenclature of the genus *Penicillium*. *Studies in Mycology, 78*, 343–371. https://doi.org/10.1016/j.simyco.2014.09.001

White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., & White, T.J. (eds), *PCR protocols: A guide to methods and applications* (pp 315-322). New York: Academic Press. http://dx.doi.org/10.1016/B978-0-12-372180-8.50042-1

Yin, G., Zhang, Y., Pennerman, K.K., Wu, G., Hua, S.S.T., Yu, J., Jurick, W.M., Guo, A., & Bennett, J.W. (2017). Characterization of blue mold *Penicillium* species isolated from stored fruits using multiple highly conserved loci. *Journal of Fungi, 3*, 12. https://doi.org/10.3390/jof3010012

Žebeljan, A., Duduk, N., Vučković, N., Jurick, W.M., & Vico, I. (2021a). Incidence, speciation, and morphogenetic diversity of *Penicillium* spp. causing blue mold of stored pome fruits in Serbia. *Journal of Fungi, 7*(12), 1019. https://doi.org/10.3390/jof7121019

Žebeljan, A., Vico, I., Duduk, N., Žiberna, B., & Krajnc, A.U. (2019). Dynamic changes in common metabolites and antioxidants during *Penicillium expansum*-apple fruit interactions. *Physiological and Molecular Plant Pathology, 106*, 166-174

Žebeljan, A., Vico, I., Duduk, N., Žiberna, B., & Krajnc, A.U. (2021b). Profiling changes in primary metabolites and antioxidants during apple fruit decay caused by *Penicillium crustosum*. *Physiological and Molecular Plant Pathology, 113*, 101586.

Živković, S., Ristić, D., & Stošić, S. (2021). First report of *Penicillium olsonii* causing postharvest fruit rot on tomato in Serbia. *Plant Disease, 105*(8), 2246. https://doi.org/10.1094/PDIS-02-21-0323-PDN

Penicillium expansum, *P. crustosum* i *P. paneum* prouzrokovači plave truleži šećerne repe u Srbiji

REZIME

U ovom istraživanju kao prouzrokovači plave truleži korena šećerne repe identifikovane su tri vrste iz roda *Penicillium: P. expansum*, *P. crustosum* i *P. paneum*, što predstavlja novi nalaz za Srbiju. Osim toga, naročito je značajno da je *P. crustosum* prvi put opisan kao prouzrokovač plave truleži korena šećerne repe u svetu. Izolati *Penicillium* spp. iz korenova šećerne repe prikupljenih u toku vađenja i iz prezimelih korenova molekularno i morfološki su okarakterisani i procenjen je njihov potencijal kao postžetvenih patogena. Test patogenosti na veštački inokulisanim korenovima potvrdili su da sve izolovane *Penicillium* spp. mogu izazvati trulež korena šećerne repe, pri čemu je najvirulentnija vrsta bila *P. expansum*, a zatim *P. crustosum* i *P. paneum*. Pošto su vrste roda *Penicillium* značajni postžetveni patogeni koji mogu dovesti do ekonomskih gubitaka i kontaminacije mikotoksinima, dalja istraživanja njihovog prisustva i uticaja nakon vađenja repe, u toku čuvanja, su od suštinskog značaja. Dobijeni rezultati doprinose znanju o vrstama roda *Penicillium* koje mogu kolonizovati koren šećerne repe i proširuju naše razumevanje raznovrsnosti vrsta ovog roda u Srbiji.

Ključne reči: šećerna repa, plava trulež, karakterizacija, filogenija, virulentnost

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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).

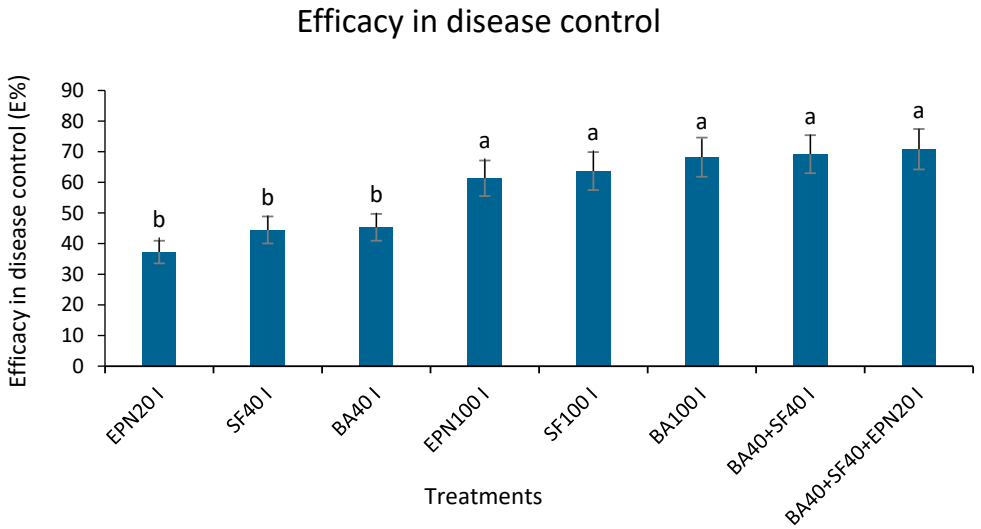

Figure 1. Efficacy of single or combined biological agents (BA – bacterium *Bacillus amyloliquefaciens* B-241, SF – actinobacterium *Streptomyces flavovirens* A06, EPN – entomopathogenic nematode *Steinernema feltiae*) in the control of green mould disease agent (I – artificial inoculation with *Trichoderma 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 ($p<0.05$).

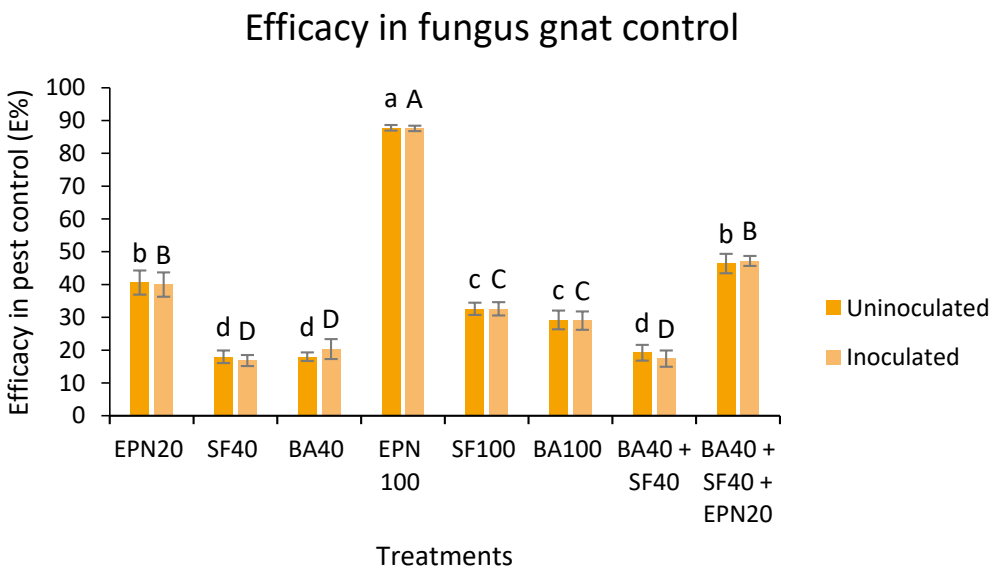

Figure 2. Efficacy of single or combined biological agents (BA – bacterium *Bacillus amyloliquefaciens* B-241, SF – actinobacterium *Streptomyces flavovirens* A06, EPN – entomopathogenic nematode *Steinernema feltiae*) in the control of fungus gnat *Lycoriella ingenua* fourth instar larvae on *Agaricus 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).

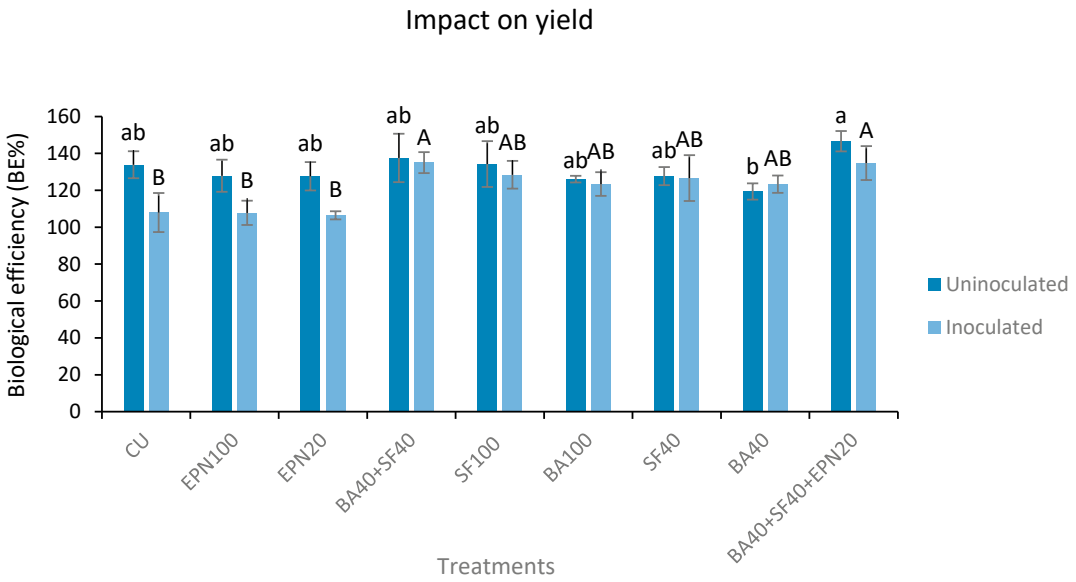

Figure 3. Impact of single or combined biological agents (BA – bacterium *Bacillus amyloliquefaciens* B-241, SF – actinobacterium *Streptomyces flavovirens* A06, EPN – entomopathogenic nematode *Steinernema feltiae*) on the yield (BE%) of *Agaricus bisporus* uninoculated or artificially inoculated with *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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REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology, 18*(2), 265-267. DOI: https://doi.org/10.1093/jee/18.2.265a

Burns, K.C. (1999). Compatibility of the entomopathogenic nematode, *Steinernema carpocapsae*, with *Bacillus* spp. used in the biological control of plant pathogens. (Master's Thesis). Knoxville, TN, USA: University of Tennessee. Retrieved from https://trace.tennessee.edu/utk_gradthes/6709

Caldas, C., Cherqui, A., Pereira, A., & Simões, N. (2002). Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression. *Applied and Environmental Microbiology*, *68*(3), 1297–1304. DOI: https://doi.org/10.1128/AEM.68.3.1297-1304.2002

Chrysayi-Tokousbalides, M., Kastanias, M.A., Philippoussis, A., & Diamantopoulou, P. (2007). Selective fungitoxicity of famaxadone, tebuconazole and trifloxystrobin between *Verticillium fungicola* and *Agaricus bisporus*. *Crop Protection*, *26*(4), 469-475. DOI: https://doi.org/10.1016/j.cropro.2006.02.016

Clarke, J., Grogan, H., Fitzpatrick, D., & Kavanagh, K. (2022). Characterising the proteomic response of mushroom pathogen *Lecanicillium fungicola* to *Bacillus velezensis* QST 713 and Kos biocontrol agents. *European Journal of Plant Pathology, 163,* 369-379. DOI: https://doi.org/10.1007/s10658-022-02482-1

Coles, P.S., & Berber, W. (2002). Cultural control. In *Mushroom integrated pest management handbook* (pp. 27-32). University Park, PA, USA: The Pennsylvania State University, Penn State College of Agricultural Sciences; AMI. Retrieved from http://www.cas.psu.edu

Drobnjaković, T., Marčić, D., Potočnik, I., Rekanović, E., Prijović, M., Milijašević-Marčić, S., & Stepanović, M. (2019). Control of mushroom sciarid fly *Lycoriella ingenua* (Dufour) with an azadirachtin-based insecticide. *Pesticides and Phytomedicine*, *34*(2), 111-121. DOI: https://doi.org/10.2298/PIF1902111D

Drobnjaković, T., Grujić, N., Luković, J., Anđelković, N., Potočnik, I., Milijašević-Marčić, S. Šantrić, Lj., Popović, A., & Marčić, D. (2025). Potential of *Steinernema feltiae* (Nematoda: Steinernematidae) native populations in the biocontrol of *Lycoriella ingenua* (Diptera: Sciaridae) and their impact on mushroom production. *Agriculture*, *15*(5), 537. DOI: https://doi.org/10.3390/agriculture15050537

El-Ashry, R.M., & El-Marzoky, A.M. (2018). Compatibility of entomopathogenic nematodes, *Heterorhabditis bacteriophora* Poinar and *Steinernema carpocapsae* Weiser with some chemical and biopesticides. *Zagazig Journal of Agricultural Research*, *45*(3), 905-916. DOI: https://doi.org/10.21608/ZJAR.2018.49129

Fatimah, N., Askary, T.H., & Abd-Elgawad, M.M.M. (2025). Factors influencing the performance of entomopathogenic nematodes: from laboratory to field conditions. *Egypt Journal of Biological Pest Control*, *35*, 29. DOI: https://doi.org/10.1186/s41938-025-00864-1

Furgani, G., Böszörményi, E., Fodor, A., Máthé-Fodor, A., Forst, S., Hogan, J. S., Katona, Z., Klein, M.G., Stackebrandt, E., Szentirmai, A., Sztaricskai, F., & Wolf, S.L. (2008). *Xenorhabdus* antibiotics: a comparative analysis and potential utility for controlling mastitis caused by bacteria. *Journal of Applied Microbiology*, *104*(3), 745–758. DOI: https://doi.org/10.1111/j.1365-2672.2007.03613.x

Gea, F.J., Navarro, M.J., & Tello, J.C. (2005). Reduced sensitivity of the mushroom pathogen *Verticillium fungicola* to prochloraz-manganese *in vitro*. *Mycological Research*, *109*(Pt 6), 741-745. DOI: https://doi.org/10.1017/s095375620500242x

Grewal, P., & Hand, P. (1992). Effects of bacteria isolated from a saprophagous rhabditid nematode *Caenorhabditis elegans* on the mycelial growth of *Agaricus bisporus*. *Journal of Applied Microbiology*, *72*(3), 173-179. DOI: https://doi.org/10.1111/j.1365-2672.1992.tb01820.x

Jess, S., Schweizer, H., & Kilpatrick, M. (2005). Mushroom applications. In Grewal, P.S., Ehlers, R.-U., & Shapiro-Ilan, D.I. (Eds.). *Nematodes as biocontrol agents* (pp. 191-213). Wallingford, UK: CABI Publishing. DOI: https://doi.org/10.1079/9780851990170.0191

Keil, C.B. (2002). Arthropod pests. Pest species biology and control. In *Mushroom integrated pest management handbook* (pp. 47–51). University Park, PA, USA: The Pennsylvania State University, Penn State College of Agricultural Sciences; AMI. Retrieved from www.americanmushroom.org/clientuploads/IPM/mushroomIPMhandbook.pdf

Kosanović, D., Potočnik, I., Duduk, B., Vukojević, J., Stajić, M., Rekanović, E., & Milijašević-Marčić, S. (2013). *Trichoderma* species on *Agaricus bisporus* farms in Serbia and their biocontrol. *Annals of Applied Biology*, *163*(2), 218-230. DOI: https://doi.org/10.1111/aab.12048

Marčić, D., Milijašević-Marčić, S., Drobnjaković, T., Luković, J., Šantrić, Lj., Grujić, N., & Potočnik, I. (2025). Bioprotection of the button mushroom from pests and diseases. *Agronomy*, *15*(6), 1323. DOI: https://doi.org/10.3390/agronomy15061323

McGee, C.F. (2017). Microbial ecology of the *Agaricus bisporus* mushroom cropping process. *Applied Microbiology and Biotechnology*, *102*(3), 1075-1083. DOI: https://doi.org/10.1007/s00253-017-8683-9

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. DOI: https://doi.org/10.1007/s10658-016-1107-3

Milijašević-Marčić, S., Šantrić, Lj., Luković, J., Potočnik, I., Grujić, N., Drobnjaković, T., & Marčić, D. (2024).

Altering microbial communities in substrate to stimulate the growth of healthy button mushrooms. *Agriculture*, *14*(7), 1152. DOI: https://doi.org/10.3390/agriculture14071152

Menzel, F., & Mohrig, W. (1999). Revision der paläarktischen Trauermücken (Diptera: Sciaridae) [A revision of the Palaearctic black fungus gnats (Diptera: Sciaridae)]. In Stark, A., & Menzel, F. (Eds.). *Studia Dipterologica – Suppl.6* (pp. 1-761). Halle an der Saale, Germany: Ampyx-Verlag. [In German]

Potočnik, I., Šantrić, Lj., Luković, J., Grujić, N., Anđelković, N., Majić, I., Drobnjaković, T., Marčić, D., & Milijašević-Marčić, S. (2025). Discovering ecological interactions between biocontrol bacterial strains and entomopathogenic nematodes in button mushroom production. *Microorganisms, 13*(3), 505. DOI: https://doi.org/10.3390/microorganisms13030505

Půža, V., & Tarasco, E. (2023). Interactions between entomopathogenic fungi and entomopathogenic nematodes. *Microorganisms*, *11*(1), 163. DOI: https://doi.org/10.3390/microorganisms11010163

Richer, D.L. (1987). Synergism – A patent view. *Pesticide Science*, *19*(4), 309-315. DOI: https://doi.org/10.1002/ps.2780190408

Rijal, R., Maity, P., & Kumar, A. (2021). Pests of mushroom and their ecological management strategies: A review. *Biological Forum – An International Journal*, *13*(1), 375-387.

Rinker, D.L., Olthof, Th.H.A., Dano, J., & Alm, G. (1995). Effects of entomopathogenic nematodes on control of a mushroom-infesting sciarid fly and on mushroom production. *Biocontrol Science and Technology*, *5*(1), 109–120. DOI: https://doi.org/10.1080/09583159550040051

Sharma, H.S.S., Lyons, G., & Chambers, J. (2000). Comparison of the changes in mushroom (*Agaricus bisporus*) compost during windrow and bunker stages of phase I and II. *Annals of Applied Biology*, *136*(1), 59-68. DOI: https://doi.org/10.1111/j.1744-7348.2000.tb00009.x

Shi, D., An, R., Zhang, W., Zhang, G., & Yu, Z. (2017). Stilbene derivatives from *Photorhabdus temperata* SN259 and their antifungal activities against phytopathogenic fungi. *Journal of Agricultural and Food Chemistry*, *65*(1), 60–65. DOI: https://doi.org/10.1021/acs.jafc.6b04303

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: https://doi.org/10.2298/ABS151104073S

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*, *126*(5), 104944. DOI: https://doi.org/10.1016/j.cropro.2019.104944

StatSoft Inc. (2004). STATISTICA (data analysis software system), version 7. Retrieved from https://www.statsoft.com

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology*, *56*(4), 845-857. DOI: https://doi.org/10.1111/j.1365-2958.2005.04587.x

Šantrić, Lj., Potočnik, I., Radivojević, Lj., Gajić Umiljendić, J., Rekanović, E., Duduk, B., & Milijašević-Marčić, S. (2018). Impact of a native *Streptomyces flavovirens* from mushroom compost on green mold control and yield of *Agaricus bisporus*. *Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes*, *53*(10), 677-684. DOI: https://doi.org/10.1080/03601234.2018.1474559

Xu, X.-M., Jeffries, P., Pautasso, M., & Jeger, M.J. (2011). Combined use of biocontrol agents to manage plant diseases in theory and practice. *Phytopathology*, *101*(9), 1024-1031. DOI: https://doi.org/10.1094/PHYTO-08-10-0216

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

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Efficacy of biofungicide *Bacillus subtilis* Ch-13 in the suppression of *Hypomyces odoratus* (cobweb disease) in a large-scale white button mushroom production

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SUMMARY

More frequent application of the biofungicide *Bacillus subtilis* Ch-13 enhanced its efficacy against natural infestation of *Hypomyces odoratus* (cobweb disease) in white button mushrooms (*Agaricus bisporus*), as well as its positive impact on mushroom production. In two different application procedures, strain *B. subtilis* Ch-13 was used at a final concentration of 60 ml per m^2 of casing layer (1×10^8 CFU ml^{-1}). In comparison to the low efficacy of the fungicide prochloraz (53%), optimal efficacy of *B. subtilis* Ch-13 in the suppression of *H. odoratus* was recorded. Substantially higher efficacy of this biofungicide in cobweb disease control was achieved when it was applied in three split doses (42%), rather than two (30%), in the large-scale production of white button mushrooms. The greatest improvement in white button mushroom production, in comparison to the untreated control, was achieved when *B. subtilis* Ch-13 was applied in three split doses (biological efficiency, BE=15%), rather than two (BE=7%). For suppression of the mycopathogen *H. odoratus* on white button mushrooms, this study supports the application of the biofungicide *B. subtilis* Ch-13 in three split doses of 30 + 15 + 15 ml m^{-2} on the second day after casing, two weeks after casing, and after the first fruiting flush, respectively.

Keywords: edible mushrooms; biocontrol agent; disease control

INTRODUCTION

The causal agent of cobweb disease, *Hypomyces odoratus* (G.R.W. Arnold) [formerly *Cladobotryum mycophilum* (Oudemans) W. Gams & Hooz.], is one of the major fungal pathogens of the white button mushroom [*Agaricus bisporus* (Lange) Imbach] in mainland Europe (Gea et al., 2012; Tamm & Põldmaa, 2013; Carrasco et al., 2016; 2017a; Luković et al., 2021), North America (McKay et al., 1999), Asia (Back et al., 2010; Muhammad et al., 2019), Australia (McKay et al., 1999; Tamm & Põldmaa, 2013), and Africa (Chakwiya et al., 2015; 2019), while *Hypomyces rosellus* (Alb. & Schwein.) Tul. [formerly *Cladobotryum dendroides* (Bull.: Fr.) W. Gams & Hooz.] is detected in the

British Isles (McKay et al., 1999; Fletcher, 2002; Grogan, 2006) and North America (McKay et al., 1999; Tamm & Põldmaa, 2013). These pathogens are soil-borne fungi that are spread by black peat soil, the main component of casing soil in the mushroom substrate. Cobweb disease agents induce crop losses of 28-40%, which recently reduced the number of white button mushroom producers (Grogan, 2006; Carrasco et al., 2016). Cobweb disease agents may also appear mixed with other substantial mycopathogens, such as *Zarea fungicola* (Preuss) Khons., Thanakitp. and Luangsa-ard. [formerly *Lecanicillium fungicola* (Preuss) Zare & W. Gams], *Hypomyces perniciosus* Magnus [formerly *Mycogone perniciosus* (Magnus) Delacroix], and *Trichoderma aggressivum* Samuels & W. Gams. Symptoms of cobweb disease manifest as white fluffy colonies that quickly cover the casing soil and mushroom fruiting bodies, changing colour with sporulation into yellow or pink. Mild symptoms include cap spotting and sunken lesions in mushrooms. Conidia of *Hypomyces odoratus* are dry and easily spread by air flow throughout the growing chamber (Carrasco et al., 2016).

Cobweb disease agents are difficult to suppress, as the genus *Hypomyces* is characterized by a lack of recombinations, resulting in the quick spread of fungicide resistance after single point mutations occur (Tamm & Põldmaa, 2013). For example, development of resistance in *H. odoratus* (Grogan, 2006) and *Z. fungicola* (Grogan et al., 2000; Gea et al., 2005) to the fungicide prochloraz resulted in its withdrawal from the EU market in June 2023 (Clarke et al., 2024), whereas it is still used in China (Shi et al., 2020) and Australia (Australian Pesticides and Veterinary Medicines Authority [APVMA], 2025). Strains of *Hypomyces* tolerant to the fungicide metrafenone were detected shortly after its introduction (Clarke et al., 2024) as the only chemical fungicide registered in the EU and North America for the control of *Hypomyces* spp. and *Z. fungicola* in cultivated mushrooms (Carrasco et al., 2017b). Chlorothalonil is authorized in some EU countries (France, Poland, Spain) and North America for use in mushrooms (Carrasco et al., 2017b; United States Environmental Protection Agency [US EPA], 2020). However, tolerance development to chlorothalonil in *H. odoratus* (Beyer & Kremser, 2004), as well as fungicide toxicity in mushroom mycelia were recorded (Challen & Elliot, 1985; Fletcher, 2002).

Thus, the development of pathogen resistance, in addition to the negative impacts of fungicides on humans, non-target organisms, and the environment, has shifted disease control towards the broader use of biological agents. Mushroom substrate is composed of various beneficial and harmful microorganisms, and its supplementation with suitable microbials helps mushrooms to resist pathogen infestation (Marčić et al., 2025). Different *Bacillus* species produce a multitude of biomolecules with remarkable pesticidal properties (Stanojević et al., 2019). Furthermore, *B. subtilis* (Ehrenberg) Cohn is considered a safe (Generally Recognized as Safe [GRAS]) microorganism for humans and the environment (Food and Drug Administration [FDA], 1999), and is also the growth promoter of cultivated plants and mushrooms (Liu et al., 2015). Though many studies concerning the suppression of various mycoparasites with biofungicides, such as *Bacillus* spp., are available, few studies and industrial-scale experiments have focused on the control of *H. odoratus*. For example, a large-scale study conducted by Regnier and Combrinck (2010) evaluated the application rate (40 µl l^{-1}) of essential oils of lemon verbena, thyme, and lemongrass, as well as their main components nerol and thymol, against natural infestation of *H. perniciosus*.

The aim of this study was to use two different procedures to (*1*) compare the efficacy of strain *B. subtilis* Ch-13 to that of the fungicide prochloraz in cobweb disease control in natural infestation conditions, and (*2*) compare the effects of *B. subtilis* Ch-13 and prochloraz on white button mushroom production. In these two procedures, the same final concentration of *B. subtilis* Ch-13 was applied either in two or three split doses.

MATERIAL AND METHODS

Antifungal agents

Efficacy of the biofungicide *Bacillus subtilis* Ch-13 (Ekstrasol F SC, BioGenesis d.o.o., Belgrade, Serbia; content of active ingredient [a.i.] 1×10^8 CFU ml^{-1}) was evaluated in the suppression of *H. odoratus* (cobweb disease agent) on naturally infected white button mushrooms (*A. bisporus*). The experiment was organized in a mushroom growing room (A2) of the mushroom factory Delta Danube d.o.o., Kovin, Serbia. Efficacy in pathogen control and biological efficiency (impact on mushroom yield) of *B. subtilis* Ch-13 were compared to those of the chemical fungicide prochloraz (Mirage® EC, ADAMA Agricultural Solutions UK Ltd., UK; content of a.i. 450 ml l^{-1}).

Large-scale experiment in mushroom growing room

The mushroom substrate used in this study consisted of mushroom compost and casing soil. The mushroom compost (Phase III) was produced from fermented and

pasteurized straw and chicken manure (Champicomp d.o.o., Pločica, Kovin, Serbia). Plastic briquettes sized 0.6 × 0.4 × 0.25 m (*l* x *w* x *h*) contained 18 kg of mushroom compost inoculated with 0.7% of grain spawn of *A. bisporus* F56 (Italspawn, Onigo di Pederobba, Italy). The surface area of five briquettes was estimated at 1 m^2. Casing soil made of black peat soil (Pešter peat soil, Dallas Company d.o.o., Tutin, Serbia) was layered onto the briquettes at a thickness of 40-50 mm (7 kg per briquette), and disinfected using 90 ml of 0.02% peracetic acid (Peral-S 15%, Vetprom, Belgrade, Serbia) per m^2 of casing layer. Mushroom mycelia were incubated at 25°C in the substrate for 16 days. After casing time (day one), the air temperature was gradually lowered to 17°C for 7 days for the case run. The experiment was conducted according to the standard PP 1/270(1) methodology (European and Mediterranean Plant Protection Organization [EPPO], 2010). Bio/fungicides were applied on the casing soil as a drench application. The fungicide prochloraz was used at the standard product application rate of 3 ml per m^2 of casing layer, in two split applications (2 × 1.5 ml in 1 l of water per m^2, four days after casing and after the first fruiting flush). The biofungicide *B. subtilis* Ch-13 was used at a rate of 60 ml m^{-2} in two protocols: (*1*) three split applications (30 + 15 + 15 ml in 1 l of water per m^2, on the second and 15th day after casing, and after the first flush, respectively); (*2*) two split applications (2 × 30 ml in 1 l of water per m^2, on the second day after casing and after the first flush). Bio/fungicides were applied with an automatic "fir" sprayer with 10 full cone nozzles, in the amount of 1 l per m^2 of casing layer. The untreated mushroom control was sprayed with tap water.

Each treatment was repeated twice (repetitions I and II) in a randomized block design, on 43 m^2 of casing surface per block. Each block consisted of 214 briquettes. Mushrooms were picked from the 14th to the 21st day of the first fruiting flush, and from the 24th to the 34th day of the second flush. The harvested mushrooms were classified as healthy and diseased, and then weighed. The effect of bio/fungicides on mushroom yield was calculated via biological efficiency (BE) (Chrysayi-Tokousbalides et al., 2007). Bio/fungicide efficacy (E) was evaluated using Abbott's formula (Abbott, 1925) based on disease incidence (DI) (proportion of diseased mushrooms and total yield) in the untreated control and each treated sample (Gea et al., 2010).

Statistical analyses

Data concerning the efficacy of bio/fungicides (E) in the suppression of *H. odoratus* and their effect on yield (biological efficiency, BE) of white button mushrooms were analyzed using the one-way analysis of variance (ANOVA). Average values for E and BE, obtained in two repetitions of bio/fungicide treatments against *H. odoratus*, were compared and any statistically significant differences were detected using the *F*-test. The level of significance was determined at $p<0.05$ (Sokal & Rohlf, 1995). Statistical data analysis was conducted using the software Statistica for Windows 7.0 (StatSoft Inc., 2004).

RESULTS

Cobweb disease symptoms in white button mushrooms, including white fluffy colonies on casing soil and mushroom fruiting bodies, were detected from the 20th to the 34th day of casing time.

Table 1. Efficacy of bio/fungicides in relation to the untreated control or standard fungicide treatment against *Hypomyces odoratus* on naturally infected white button mushrooms

Treatments	Fungicide application rate	$^1E_{st}$ (%)	^2E_c (%)	SE
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1} (II)	2 × 30 ml m^{-2}	56.78 b[3]	30.13 c	0.75
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1} (III)	30 ml m^{-2} + 2 × 15 ml m^{-2}	79.12 a	41.98 b	0.58
Prochloraz 450 ml l^{-1}	2 × 1.5 ml m^{-2}	100.00	53.06 a	0.61
Untreated Control	–	1.88 c	–	1.25

The data represent means (of two repetitions, each including 214 experimental units) ± SE, standard error of the mean; efficacy in relation to the fungicide prochloraz (standard) set to 100% $^1(E_{st})$, or untreated control $^2(E_c)$; SED, standard error of difference = 32.64; df, degrees of freedom = 3; $F = 69.54$; p-value = 0.001. [3]Values marked with same letter within each repetition series are not significantly different according to the *F*-test ($p<0.05$).

The incidence of cobweb disease in mushrooms varied depending on different treatments (Figure 1). Mushrooms in the untreated control had the highest disease incidence (12.62-12.8%). Reduction of disease symptoms was recorded in briquettes treated with *B. subtilis* Ch-13 in two (DI=8.84-8.92%) or three (DI=7.27-7.48%) split doses. The lowest disease incidence was observed after treatment of mushrooms with the fungicide prochloraz (DI=5.81-6.12%). Efficacy of each bio/fungicide in disease control was estimated in two ways: in relation to standard prochloraz treatment set to 100% efficacy (E_{st}), or in relation to the untreated control (E_c) (Table 1). A statistically significant difference in the efficacy of bio/fungicides in cobweb disease control was detected between prochloraz and the biofungicide *B. subtilis* Ch-13 used in both application protocols (two or three split doses). In relation to the untreated control (E_c), prochloraz showed the highest efficacy against *H. odoratus* (53.1%), followed by the biofungicide *B. subtilis* Ch-13 applied three times (42%) (Table 1). The biofungicide *B. subtilis* Ch-13 used twice exhibited the lowest efficacy against *H. odoratus* (30.1%). In relation to the fungicide prochloraz when its efficacy was set to 100% (E_{st}), the biofungicide *B. subtilis* Ch-13 used three times showed 28% higher efficacy in pathogen suppression (79.1%), than when applied twice (56.8%) (Table 1). Furthermore, when the biofungicide *B. subtilis* Ch-13 was used three times, it considerably diminished the incidence of cobweb disease of white button mushrooms, by up to 58% compared to the untreated control.

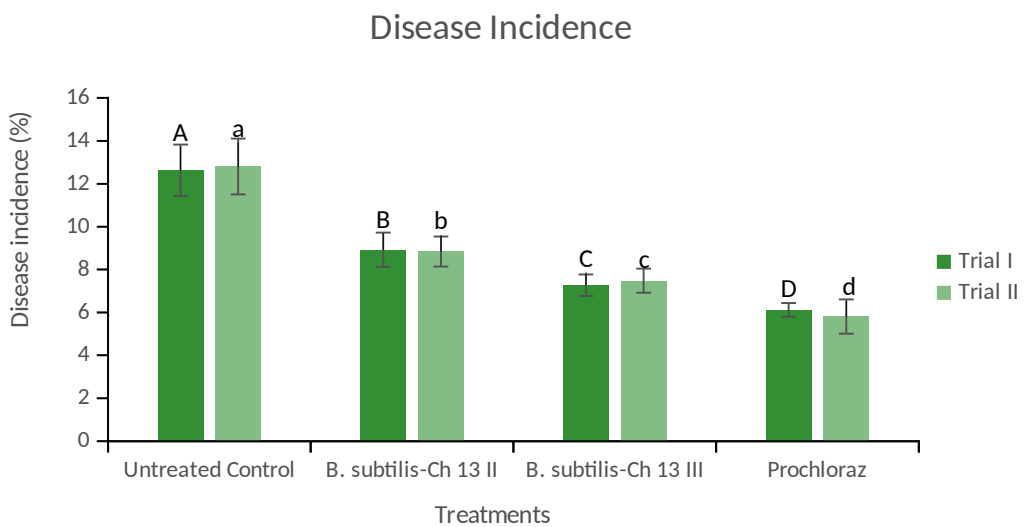

Figure 1. Incidence of cobweb disease (*Hypomyces odoratus*) after using bio/fungicides on white button mushrooms. The biofungicide *Bacillus subtilis* Ch-13 was used in two (II) or three (III) split doses. Data represent means of repetition trials I and II (each performed on a set of 214 experimental briquettes) ± SE, standard error of the mean; SED, standard error of difference = 0.8 (I); 0.9 (II); df, degrees of freedom = 3; $F = 62.52$ (I); 73.64 (II); p-value = 0.001. Values marked with the same letter within each repetition series (I – capital letters; II – lowercase letters) are not significantly different according to the F-test ($p<0.05$).

Table 2. Biological efficiency (BE%) of bio/fungicides in relation to the untreated control or standard fungicide treatment of white button mushrooms naturally infected with *Hypomyces odoratus*

Treatments	Fungicide application rate	$^1BE_{st}$ (%)	^2BE_c (%)	SE
Bacillus subtilis Ch-13 1×10^8 CFU ml^{-1} (II)	2×30 ml m^{-2}	104.82 b[3]	107.41 b	2.05
Bacillus subtilis Ch-13 1×10^8 CFU ml^{-1} (III)	30 ml m^{-2} + 2×15 ml m^{-2}	112.66 a	115.44 a	2.22
Prochloraz 450 ml l^{-1}	2×1.5 ml m^{-2}	100.00	102.47 b	2.02
Untreated Control	–	97.57 c	100.00	2.42

The data represent means (of two repetitions, each including 214 experimental units) ± SE, standard error of the mean; fungicide prochloraz (standard) impact [1](BE_{st}) or untreated control impact [2](BE_c) value is set to 100%; SED, standard error of difference = 32; df, degrees of freedom = 3; $F = 28.32$; p-value = 0.001. [3]Values marked with same letter within each repetition series are not significantly different according to the F-test ($p<0.05$).

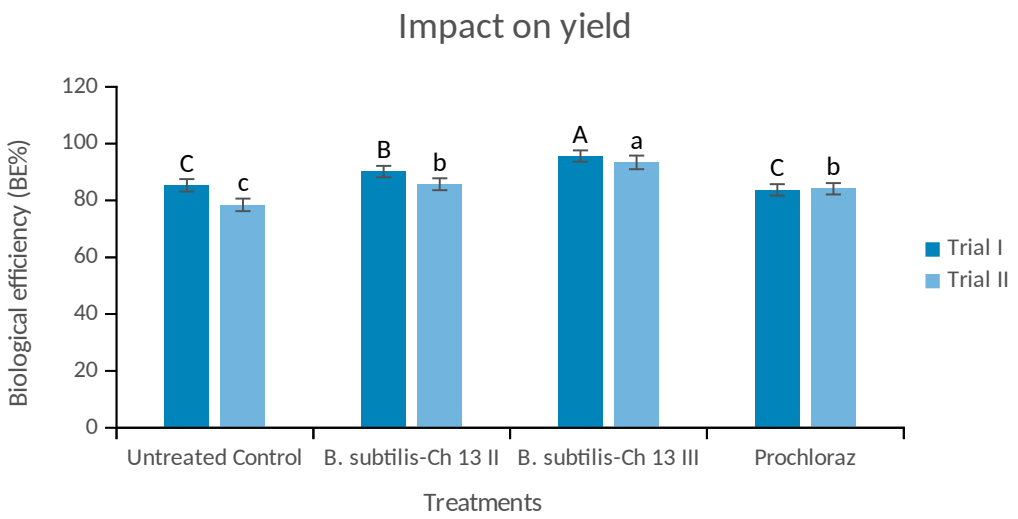

Figure 2. The impact of bio/fungicides on yield (biological efficiency – BE%) of white button mushrooms naturally infected with *Hypomyces odoratus*. The biofungicide *Bacillus subtilis* Ch-13 was used in two (II) or three (III) split doses. Data represent means of repetition trials I and II (each including 214 experimental briquettes) ± SE, standard error of the mean; SED, standard error of difference = 2.1 (I); 2.2 (II); df, degrees of freedom = 3; $F = 30.2$ (I); 28.8 (II); p-value = 0.001. Values marked with the same letter within each repetition series (I – capital letters; II – lowercase letters) are not significantly different according to the F-test ($p<0.05$).

The effect of bio/fungicides on white button mushroom production was estimated with biological efficiency (BE) (Table 2; Figure 2). In comparison to the untreated control (BE=78.4-85.3%), application of *B. subtilis* Ch-13 significantly improved mushroom yield, whether used in three (BE=93.3-95.6%) or two split doses (BE=85.7-90.1%). No significant difference in biological efficiency was recorded between the untreated control (BE=78.4-85.3%) and prochloraz treatment (BE=75.4-78.4%) (Figure 2). The effect of bio/fungicides on mushroom production (biological efficiency) was evaluated in two ways: in relation to the untreated control (BE_c) or standard prochloraz treatment (BE_{st}), when the value of each was set to 100% biological efficiency (Figure 2). Compared to the untreated control set at 100% (BE_c), *B. subtilis* Ch-13 applied in three split doses significantly increased mushroom production (BE=115.4%), whereas *B. subtilis* Ch-13 applied in two split doses and prochloraz treatment did not significantly affect mushroom production (BE=107.4; 102.5%, respectively) (Table 2). When prochloraz treatment was set to 100% biological efficiency (BE_{st}), three split doses of the biofungicide *B. subtilis* Ch-13 promoted a mushroom yield (BE=112.7%) that was 7% higher than the yield obtained with two split doses (BE=104.8%) (Table 2). Additionally, in relation to the fungicide prochloraz (BE_{st}), mushroom yield obtained with the biofungicide *B. subtilis* Ch-13 applied in two split doses significantly differed from that of the untreated control (BE=97.6%) (Table 2). The use of the biofungicide in three split doses significantly increased mushroom production, in contrast to its application in two split doses, the fungicide prochloraz treatment, or the untreated control. In comparison to the untreated control and prochloraz treatment, the application of strain *B. subtilis* Ch-13 in three split doses significantly increased white button mushroom production: up to 15% and 13%, respectively.

DISCUSSION

Symptoms found in all experimental briquettes with white button mushrooms resembled those of cobweb disease caused by *H. odoratus* (Luković et al., 2021). Tested for the suppression of natural infestation of cobweb disease agent *H. odoratus*, the biofungicide *B. subtilis* Ch-13 was applied at a concentration of 60 ml (1×10^8 CFU ml^{-1}) per m^2 of casing layer in two procedures – using two (2×30 ml m^{-2}) or three (30 ml $m^{-2} + 2 \times 15$ ml m^{-2}) split doses – whereas the fungicide prochloraz was applied at the standard application rate used in industrial-scale conditions of white button mushroom cultivation. The effects of *B. subtilis* Ch-13 and prochloraz on mushroom production were also estimated.

Strain *B. subtilis* Ch-13 has been registered as a microbiological fertilizer, fungicide, and wheat seed disinfectant in several countries (Chebotar et al., 2009; Kayin et al., 2015; Potočnik et al., 2019). Concerning the protection of naturally infected white button mushrooms, *B. subtilis* Ch-13 was previously tested only against the mycopathogen *T. aggressivum* in industrial-scale cultivation (Potočnik et al., 2021). The biofungicide *B. subtilis* Ch-13 displayed optimal efficacy in the suppression of *H. odoratus* when used in three split doses (≈42%) and to a certain extent when applied twice (≈30%), compared to the fungicide prochloraz (≈53%). The efficacy of *B. subtilis* Ch-13 against *H. odoratus* was substantially higher when applied three times, rather than twice, despite the same total concentration of *B. subtilis* Ch-13 used in both applications. The efficacy of three split doses of *B. subtilis* Ch-13 (≈42%) against *H. odoratus* was comparable to the very low efficacy of the fungicide prochloraz (≈53%).

When the strain *B. subtilis* Ch-13 was previously tested against *T. aggressivum* in an industrial-scale experiment, Potočnik et al. (2021) found that it displayed higher efficacy when applied in three (53.6%) or two split doses (46.4%), than when used in the same manner to control *H. odoratus* in the present study. Additionally, the fungicide prochloraz showed much lower efficacy against *H. odoratus* (53%) in the current study than found by Potočnik et al. (2021) in the suppression of *T. aggressivum* (71%). The decreased efficacy of prochloraz in cobweb disease control implies the importance of replacing it with effective beneficial microorganisms to suppress *H. odoratus*.

Strains *B. velezensis* QST 713 (formerly *B. subtilis*), QST 713 (Ehrenberg) Cohn, *B. amyloliquefaciens* subsp. *plantarum* D747, and *B. amyloliquefaciens* MBI 600 were authorized for use against *Trichoderma harzianum* Rifai and *T. aggressivum* on white button mushrooms worldwide (Marčić et al., 2025). Strain *B. velezensis* QST 713 (5.13×10^{10} CFU g^{-1}) used at the standard application rate showed distinct efficacies in the suppression of various mushroom disease agents: 45-62% against *Z. fungicola* (Stanojević et al., 2019), 48-58% against *T. aggressivum* (Milijašević-Marčić et al., 2017; Potočnik et al., 2018; 2019; Stanojević et al., 2019), 44% against *T. harzianum* (Kosanović et al., 2013; Milijašević-Marčić et al., 2017), and 17-21% against *H. perniciosus* (Navarro et al., 2023). Strain *B. amyloliquefaciens* subsp. *plantarum* D747 (5×10^{13} CFU g^{-1}) used at the standard application rate exhibited an efficacy of 15-19% against *H. perniciosus* (Navarro et al., 2023). On the other hand, *B. subtilis* QST 713 failed to control cobweb disease (*H. rosellus*) in the experiment conducted by Ślusarski et al. (2012).

Various native *Bacillus* spp. strains also showed different efficacies against white button mushroom pathogens: *B. velezensis* Kos, 30-40% against *H. odoratus* (Clarke et al., 2024), similar to *B. subtilis* Ch-13 in the current study (30-42%); *B. subtilis* B-38, 50% against *T. harzianum* (Milijašević-Marčić et al., 2017); *B. subtilis* B-38, 36% (Milijašević-Marčić et al., 2017) and *B. amyloliquefaciens* B-241, 53-68% against *T. aggressivum* (Stanojević et al., 2019); and *B. amyloliquefaciens* B-241, 46-58% against *Z. fungicola* (Stanojević et al., 2019).

Furthermore, various *Bacillus* spp. strains distinctly improved mushroom yield: *B. velezensis* QST 713, 15% (Milijašević-Marčić et al., 2017; Navarro et al., 2023); *B. velezensis,* 18-26% (Büchner et al., 2021); *B. amyloliquefaciens* subsp. *plantarum* D747, 4-8% (Navarro et al., 2023); and *B. subtilis* Ch-13, 8-12% (Potočnik et al., 2019; Potočnik et al., 2021), consistent with data reported in the current study (7-15%).

More specifically, findings from the current study support the application of strain *B. subtilis* Ch-13 at a concentration of 60 ml m^{-2} (1×10^8 CFU ml^{-1}) in three split doses to control cobweb disease and improve the yield of white button mushrooms.

CONCLUSION

More frequent application of the biofungicide *B. subtilis* Ch-13 enhanced its efficacy against natural infestation of *H. odoratus* in white button mushrooms. Used at a final concentration of 60 ml m^{-2} (1×10^8 CFU ml^{-1}), strain *B. subtilis* Ch-13 was substantially more effective in the large-scale production of white button mushrooms when applied in three split doses (42% efficacy), rather than two (30% efficacy). Compared to the low efficacy of the fungicide prochloraz (53%), optimal efficacy of *B. subtilis* Ch-13 was recorded in the suppression of *H. odoratus*. The greatest improvement in mushroom production, in comparison to the untreated control, was found when *B. subtilis* Ch-13 was applied in three split doses (15%), rather than two (7%). Therefore, current data support the application of the biofungicide *B. subtilis* Ch-13 in three split doses (30 + 15 + 15 ml m^{-2} on the second day after casing, two weeks after casing, and after the first fruiting flush, respectively) against the mycopathogen *H. odoratus* in white button mushrooms.

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REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology, 18*(2), 265-267. DOI: https://doi.org/10.1093/jee/18.2.265a

Australian Pesticides and Veterinary Medicines Authority (APVMA). (2025). Retrieved from https://portal.apvma.gov.au/es/permits

Back, C.-G., Kim, Y.-H., Jo, W.-S., Chung, H., & Jung, H.-Y. (2010). Cobweb disease on *Agaricus bisporus* caused by *Cladobotryum mycophilum* in Korea. *Journal of Plant Pathology*, *76*(3), 232-235. DOI: https://doi.org/10.1007/s10327-010-0236-3

Beyer, D.M., & Kremser, I.J. (2004). Evaluation of fungicide tolerance and control for three fungal diseases of mushrooms. In Romaine, C.P., Keil, C.B., Rinker, D.L., & Royse, D.J. (Eds.). *Mushroom Science XVI*, *Proceedings of the XVI International Congress on the Science and Cultivation of Edible and Medicinal Fungi*, *Miami, USA*, *March 14-17, 2004* (pp 521-529). Philadelphia, PA, USA: Pennsylvania State University Press.

Büchner, R., Vörös, M., Allaga, H., Varga, A., Bartal, A., Szekeres, A., Varga, S., Bajzát, J., Bakos-Barczi, N., Misz, A., Csutorás, C., Hatvani, L., Vágvölgyi, C., & Kredics, L. (2022). Selection and characterization of a *Bacillus* strain for potential application in industrial production of white button mushroom (*Agaricus bisporus*). *Agronomy*, *12*(2), 467. DOI: https://doi.org/10.3390/agronomy12020467

Carrasco, J., Navarro, M.J., Santos, M., Diánez, F., & Gea, F.J. (2016). Incidence, identification and pathogenicity of *Cladobotryum mycophilum*, causal agent of cobweb disease on *Agaricus bisporus* mushroom crops in Spain. *Annals of Applied Biology*, *168*(2), 214-224. DOI: https://doi: 10.1111/aab.12257

Carrasco, J., Navarro, M.J., & Gea, F.J. (2017a). Cobweb, a serious pathology in mushroom crops: A review. *Spanish Journal of Agricultural Research*, *15*(2), e10R01. DOI: https://doi.org/10.5424/sjar/2017152-10143

Carrasco, J., Navarro, M.J., Santos, M., & Gea, F.J. (2017b). Effect of five fungicides with different modes of action on mushroom cobweb disease (*Cladobotryum mycophilum*) and mushroom yield. *Annals of Applied Biology*, *171*(1), 62-69. DOI: https://doi.org/10.1111/aab.12352

Chakwiya, A., Van der Linde, E.J., & Korsten, L. (2015). *In vitro* sensitivity testing of *Cladobotryum mycophilum* to carbendazim and prochloraz manganese. *South African Journal of Science*, *111*(11/12), 1-7. DOI: https://doi.org/10.17159/sajs.2015/20140408

Chakwiya, A., Van der Linde, E.J., Chidamba, L., & Korsten, L. (2019). Diversity of *Cladobotryum mycophilum* isolates associated with cobweb disease of *Agaricus bisporus* in the South African mushroom industry. *European Journal of Plant Pathology*, *154*, 767-776. DOI: https://doi.org/10.1007/s10658-019-01700-7

Challen, M.P., & Elliott, T.J. (1985). The *in vitro* responses to a range of fungicides of two strains of the mushroom *Agaricus bisporus* and the pathogen *Verticillium fungicola*. *Mycopathologia*, *90*, 161-164. DOI: https://doi.org/10.1007/BF00436732

Chebotar, V.K., Makarova, N.M., Shaposhnikov, A.I., & Kravchenko, L.V. (2009). Antifungal and phytostimulating characteristics of *Bacillus subtilis* Ch-13 rhizospheric strain, producer of biopreparations. *Applied Biochemistry and Microbiology*, *45*(4), 465-469. DOI: https://doi.org/10.1016/j.micres.2006.04.001

Chrysayi-Tokousbalides, M., Kastanias, M.A., Philippoussis, A., & Diamantopoulou, P. (2007). Selective fungitoxicity of famoxadone, tebuconazole and trifloxystrobin between *Verticillium fungicola* and *Agaricus bisporus*. *Crop Protection*, *26*(4), 469-475. DOI: https://doi.org/10.1016/j.cropro.2006.02.016

Clarke, J., McGuinness, B., Fitzpatrick, D., Kavanagh, K., & Grogan, H. (2024). Response of the mushroom pathogen *Cladobotryum mycophilum* to the fungicides prochloraz and metrafenone and two *Bacillus*-based biological control agents in mushroom crop trials. *Crop Protection*, *177*, 106530. DOI: https://doi.org/10.1016/j.cropro.2023.106530

European and Mediterranean Plant Protection Organization (EPPO). (2010). Efficacy evaluation of fungicides: Fungal diseases on cultivated mushrooms of *Agaricus* spp. PP 1/270(1) in EPPO Standards. *OEPP/EPPO Bulletin*, *40*(3), 270-273. DOI: https://doi.org/10.1111/j.1365-2338.2010.02384.x

Fletcher, J.T. (2002). Cobweb disease, a new challenge. *Pest Control (Mushroom News)*, *50*, 20-23.

Food and Drug Administration (FDA). (1999). Code of Federal Regulations, Title 21: Food and drugs. In: *Chapter 1: Food and Drug Administration Department of Health and Human Services, Part 184: Direct food substances affirmed as Generally Recognized as Safe* (pp. 892-896). Washington, D.C., USA: US Government Printing Office.

Gea, F.J., Navarro, M.J., & Tello, J.C. (2005). Reduced sensitivity of the mushroom pathogen *Verticillium fungicola* to prochloraz-manganese *in vitro*. *Mycological Research*, *109*(Pt 6), 741-745. DOI: https://doi.org/10.1017/s095375620500242x

Gea, F.J., Tello, J.C., & Navarro, M.J. (2010). Efficacy and effect on yield of different fungicides for control of wet bubble disease of mushroom caused by the mycoparasite *Mycogone perniciosa*. *Crop Protection*, *29*(9), 1021-1025. DOI: https://doi.org/10.1016/j.cropro.2010.06.006

Gea, F.J., Navarro, M.J., Carrasco, J., González, A.J., & Suz, L.M. (2012). First report of cobweb on white button mushroom (*Agaricus bisporus*) in Spain caused by *Cladobotryum mycophilum*. *Plant Disease*, *96*(7), 1067. DOI: https://doi.org/10.1094/PDIS-02-12-0120-PDN

Grogan, H.M., Keeling, C., & Jukes, A.A. (2000). *In vitro* response of the mushroom pathogen *Verticillium fungicola* (dry bubble) to prochloraz-manganese. In *The BCPC 2000, Proceedings of the British Crop Protection Conference – Pests & Diseases, Brighton, UK, November 13-16, 2000* (pp 273-278). Farnham, UK: British Crop Protection Council.

Grogan, H.M. (2006). Fungicide control of mushroom cobweb disease caused by *Cladobotryum* strains with different benzimidazole resistance profiles. *Pest Management Science*, *62*(2), 153-161. DOI: https://doi.org/10.1002/ps.1133

Kayin, G.B., Öztüfekçi, S., Akin, H.F., Karaata, E.U., Katkat, A.V., & Turan, M.A. (2015). Effect of *Bacillus subtilis* Ch-13, nitrogen and phosphorus on yield, protein and gluten content of wheat (*Triticum aestivum* L.). *Journal of Agricultural Faculty of Uludag University*, *29*(1), 19-28. DOI: https://dergipark.org.tr/tr/download/article-file/154225

Kosanović, D., Potočnik, I., Duduk, B., Vukojević, J., Stajić, M., Rekanović, E., & Milijašević-Marčić, S. (2013). *Trichoderma* species on *Agaricus bisporus* farms in Serbia and their biocontrol. *Annals of Applied Biology*, *163*(2), 218-230. DOI: https://doi.org/10.1111/aab.12048

Liu, C., Sheng, J., Chen, L., Zhen, Y., Lee, D.Y.W., Yang, Y., Xu, M., & Shen, L. (2015). Biocontrol activity of *Bacillus subtilis* isolated from *Agaricus bisporus* mushroom compost against pathogenic fungi. *Journal of Agriculture and Food Chemistry*, *63*(26), 6009-6018. DOI: https://doi.org/10.1021/acs.jafc.5b02218

Luković, J., Milijašević-Marčić, S., Hatvani, L., Kredics, L., Szűcs, A., Vágvölgyi, C., Duduk, N., Vico, I., & Potočnik, I. (2021). Sensitivity of *Trichoderma* strains from edible mushrooms to the fungicides prochloraz and metrafenone. *Journal of Environmental Science and Health, Part B*, *56(*1), 54-63. DOI: https://doi.org/10.1080/03601234.2020.1838821

Marčić, D., Milijašević-Marčić, S., Drobnjaković, T., Luković, J., Šantrić, Lj., Grujić, N., & Potočnik, I. (2025). Bioprotection of the button mushroom from pests and diseases. *Agronomy*, *15*(6), 1323. DOI: https://doi.org/10.3390/agronomy15061323

McKay, G.J., Egan, D., Morris, E., Scott, C., & Brown, A.E. (1999). Genetic and morphological characterization of *Cladobotryum* species causing cobweb disease of mushrooms. *Applied and Environmental Microbiology, 65*(2), 606-610. DOI: https://doi.org/10.1128/aem.65.2.606-610.1999

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. DOI: https://doi.org/10.1007/s10658-016-1107-3

Muhammad, I., Sossah, F.L., Yang, Y., Li, D., Li, S., Fu, Y., & Li, Y. (2019). Identification of resistance to cobweb disease caused by *Cladobotryum mycophilum* in wild and cultivated strains of *Agaricus bisporus* and screening for bioactive botanicals. *RSC Advances*, *9*(26), 14758-14765. DOI: https://doi.org/10.1039/c9ra00632j

Navarro, M.J., Santos, M., Dianez, F., & Gea, F.J. (2023). Chemical and biological control of wet bubble disease (*Hypomyces perniciosus*) in mushroom crops. *Agronomy*, *13*(7), 1672. DOI: https://doi.org/10.3390/agronomy13071672

Potočnik, I., Todorović, B., Rekanović, E., Luković, J., Paunović, D., & Milijašević-Marčić, S. (2018). Impact of *Bacillus subtilis* QST 713 mushroom grain spawn treatment on yield and green mould control. *Pesticides and Phytomedicine*, *33*(3-4), 205-211. DOI: https://doi.org./10.2298/PIF1804205P

Potočnik, I., Rekanović, E., Todorović, B., Luković, J., Paunović, D., Stanojević, O., & Milijašević-Marčić, S. (2019). The effects of casing soil treatment with *Bacillus subtilis* Ch-13 biofungicide on green mould control and mushroom yield. *Pesticides and Phytomedicine*, *34*(1), 53-60. DOI: https://doi.org/10.2298/PIF1901053P

Potočnik, I., Todorović, B., Milijašević-Marčić, S., Luković, J., Kanižai Šarić, G., Majić, I., & Rekanović, E. (2021). A large-scale study on the effectiveness of a *Bacillus subtilis* Ch-13-based biofungicide against green mould

disease and mushroom yield improvement. *Pesticides and Phytomedicine*, *36*(2), 83-90. DOI: https://doi.org/10.2298/PIF2102083P

Regnier, T., & Combrinck, S. (2010). *In vitro* and *in vivo* screening of essential oils for the control of wet bubble disease of *Agaricus bisporus*. *South African Journal of Botany*, *76*(4), 681-685. DOI: https://doi.org/10.1016/j.sajb.2010.07.018

Shi, N., Ruan, H., Jie, Y., Chen, F., & Du, Y. (2020). Sensitivity and efficacy of fungicides against wet bubble disease of *Agaricus bisporus* caused by *Mycogone perniciosa*. *European Journal of Plant Pathology, 157*(4), 873-885. DOI: https://doi.org/10.1007/s10658-020-02047-0

Ślusarski, C., Uliński, Z., Szumigaj-Tarnowska, J., & Miszczak, A. (2012). Preliminary appraisal of the new preparations for protection of the white button mushroom against fungal diseases. *Progress in Plant Protection*, *52*(4), 1058-1063.

Sokal, R.R., & Rohlf, F.J. (1995). *Biometry: The principles and practice of statistics in biological research* (3rd edition). New York, NY, USA: W.H. Freeman and Company.

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*, *126*(5), 104944. DOI: https://doi.org/10.1016/j.cropro.2019.104944

StatSoft Inc. (2004). STATISTICA (data analysis software system), version 7. Retrieved from https://www.statsoft.com

Tamm, H., & Põldmaa, K. (2013). Diversity, host associations, and phylogeography of temperate aurofusarin-producing *Hypomyces/Cladobotryum* including causal agents of cobweb disease of cultivated mushrooms. *Fungal Biology*, *117*(5), 348-367. DOI: https://doi.org/10.1016/j.funbio.2013.03.005

United States Environmental Protection Agency (US EPA). Grower resources. *Integrated Pest Management. Pesticides. Fungicides*. Retrieved from https://www.americanmushroom.org/integrated-pest-management/fungicides/

Efikasnost biofungicida *Bacillus subtilis* Ch-13 u suzbijanju prouzrokovača paučinaste plesni (*Hypomyces odoratus*) u uslovima industrijske proizvodnje šampinjona

REZIME

Učestalija primena biofungicida *Bacillus subtilis* Ch-13 povećala je njegovu efikasnost u suzbijanju prirodne zaraze *Hypomyces odoratus* (paučinate plesni) i pozitivan uticaj na prinos šampinjona (*Agaricus bisporus*). Soj *B. subtilis* Ch-13 je bio primenjen u ukupnoj koncentraciji od 60 ml m^{-2} (1×10^8 CFU ml^{-1}). Značajno veća efikasnost biofungicida u suzbijanju prouzrokovača paučinaste plesni je postignuta kada je primenjen u tri podeljene doze (42%), umesto u dve (30%), u uslovima industrijske proizvodnje šampinjona. Zadovoljavajuća efikasnost *B. subtilis* Ch-13 u suzbijanju *H. odoratus* je uočena u poređenju sa vrlo smanjenom efikasnošću fungicida prohloraza (53%). Najveće povećanje prinosa je zabeleženo kod primene *B. subtilis* Ch-13 u tri podeljene doze (15%), umesto u dve (7%), u poređenju sa netretiranom kontrolom. Može se preporučiti primena *B. subtilis* Ch-13 u tri podeljene doze: 30 + 15 +15 ml po m^2 (drugog dana, dve nedelje nakon pokrivanja i posle prve berbe) u suzbijanju *H. odoratus*.

Ključne reči: jestive gljive; agens biološke zaštite; zaštita od bolesti

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Biofungicide *Bacillus subtilis* Ch-13 in the control of *Hypomyces perniciosus* (wet bubble disease) in industrial-scale mushroom cultivation

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SUMMARY

The current study aimed to evaluate the efficacy of the biofungicide *Bacillus subtilis* Ch-13 in the suppression of natural infection of white button mushrooms (*Agaricus bisporus*) with *Hypomyces perniciosus* (causal agent of wet bubble disease), as well as its impact on mushroom yield in industrial-scale cultivation. The biofungicide *B. subtilis* Ch-13 was applied at a total concentration of 60 ml per m^2 of casing layer in two different procedures – using either three (30 + 2 × 15 ml m^{-2}) or two split doses (2 × 30 ml m^{-2}) – and then its effects were compared to those of the fungicide prochloraz applied at the standard application rate. The efficacy of the biofungicide was significantly higher when applied in three split doses (29.7%), than in two (15.7%). Though the efficacy of *B. subtilis* Ch-13 (≈30%) against *H. perniciosus* was low in comparison to that of prochloraz (≈68%), *B. subtilis* Ch-13 slightly reduced wet bubble symptoms. Furthermore, the highest increase in mushroom yield was achieved when *B. subtilis* Ch-13 was applied in three split doses (14%), rather than two (2%), compared to the untreated control. In comparison to prochloraz, three and two split applications of *B. subtilis* Ch-13 enhanced mushroom yield by up to 17% and 4%, respectively. Regarding its efficacy in wet bubble disease control and augmentation of mushroom yield, *B. subtilis* Ch-13 was much more effective when applied in three split doses, than in two. Therefore, this study supports the application *B. subtilis* Ch-13 in three split doses (30 + 2 × 15 ml m^{-2}, on the second day and two weeks after casing, and after the first fruiting flush, respectively) to suppress *H. perniciosus* and increase mushroom yield.

Keywords: edible mushrooms; biocontrol agent; disease control

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INTRODUCTION

The causal agent of wet bubble disease, *Hypomyces perniciosus* Magnus [formerly *Mycogone perniciosa* (Magnus) Delacroix], is a significant fungal pathogen of white button mushrooms [*Agaricus bisporus* (Lange) Imbach] worldwide (Umar & Van Griensven, 1999; Meyer & Korsten, 2008; Glamočlija et al., 2008; Siwulski et al., 2011; Shi et al., 2020). As a soil-borne fungus, *H. perniciosus* spreads mainly by black peat used for casing soil, which serves as a cover layer for the mushroom compost. Mostly appearing in mixed infestations with other significant mycopathogens – such as *Zarea fungicola* (Preuss) Khons., Thanakitp. and Luangsa-ard. [formerly *Lecanicillium fungicola* (Preuss) Zare & W. Gams], *Cladobotryum mycophilum* (Oudem.) W. Gams & Hoozem., and *Trichoderma aggressivum* Samuels & W. Gams – *H. perniciosus* has occasionally caused yield losses of 15-30% (Gea et al., 2010; Potočnik et al., 2010; Shi et al., 2020). Symptoms of wet bubble disease manifest as large undifferentiated tumorous fruiting bodies with amber-coloured droplets on the surface (Umar & Van Griensven, 1999). After *Z. fungicola* (Gea et al., 2005) and *C. mycophilum* (Grogan, 2006) developed resistance to prochloraz, the fungicide was withdrawn from the EU market in June 2023 (Clarke et al., 2024). However, prochloraz is still used in China (Shi et al., 2020) and Australia (Australian Pesticides and Veterinary Medicines Authority [APVMA], 2025). Furthermore, strains of *Cladobotryum* tolerant to metrafenone, the only chemical fungicide approved in the cultivation of mushrooms in the EU (Carrasco et al., 2017), have already been detected (Clarke et al., 2024). However, no evidence of *H. perniciosus* resistance to fungicides has been recorded (Gea et al., 2010; Shi et al., 2020). Resistance of pathogens to fungicides, in addition to the harmful effects of fungicides on human health and the environment, has redirected mushroom disease control to the application of biocontrol agents, predominantly antagonistic microorganisms. Mushrooms are highly connected with the microbiota in their substrate, which may be beneficial or harmful for their growth. Supplementing the substrate with favourable microbial species helps the mushrooms to cope with pathogen infection (Marčić et al., 2025). Biofungicides based on various *Bacillus* species compete with pathogens for space and nutrients by producing antibiotics, enzymes, iron chelators, and various volatile compounds (Pandin et al., 2018). Moreover, *B. subtilis* (Ehrenberg) Cohn is regarded as harmless to the environment and humans, and has been classified as a safe organism (i.e. generally recognized as safe [GRAS]) (Food and Drug Administration [FDA], 1999). The biocontrol agent *B. subtilis* also promotes the growth of cultivated plants and mushrooms (Liu et al., 2015). A commercial biofungicide based on the strain *Bacillus velezensis* (formerly *B. subtilis*) QST 713 (Ehrenberg) Cohn has been registered for use against many fungal pathogens of cultivated plants and mushrooms (Védie & Rousseau, 2008; Pandin et al., 2018; Marčić et al., 2025), and was recently approved for use in Serbia. However, the efficacy of *B. velezensis* QST 713 against different mushroom pathogens varies, especially in the suppression of *H. perniciosus* (Navarro et al., 2023) and *C. mycophilum* (Clarke et al., 2024). As a commercial strain with antifungal and phytostimulating properties, *B. subtilis* Ch-13 was approved for use as a microbiological fertilizer, fungicide, and wheat seed disinfectant in Serbia and several other countries (Chebotar et al., 2009; Kayin et al., 2015; Potočnik et al., 2019). Strain *B. subtilis* Ch-13 was tested against *T. aggressivum* f. *europaeum* and compared to strain *B. velezensis* QST 713 and the fungicide prochloraz (Potočnik et al., 2019; Potočnik et al., 2021). Few industrial-scale studies of disease control in cultivated mushrooms are available, especially those concerning the biological control of *H. perniciosus*. Only one such study was performed by Regnier and Combrinck (2010), who determined the application rate (40 µl l^{-1}) of non-formulated plant essential oils (lemon verbena, thyme, lemongrass, and their main components nerol and thymol) in the suppression of *H. perniciosus* on naturally infected mushrooms.

The goal of the current study was to compare the biofungicide *B. subtilis* Ch-13 to the fungicide prochloraz in relation to their efficacy in wet bubble disease control in natural infection conditions, as well as their impact on mushroom yield. For this purpose, two different application protocols for *B. subtilis* Ch-13 (two *vs.* three split doses) on naturally infected mushrooms were evaluated.

MATERIAL AND METHODS

Antifungal agents

The efficacy of *B. subtilis* Ch-13 (Ekstrasol F SC, BioGenesis d.o.o., Belgrade, Serbia; content of active ingredient [a.i.] 1×10^8 CFU ml^{-1}) against the

mycopathogen *H. perniciosus* (wet bubble disease agent) was tested on naturally infested white button mushrooms (*A. bisporus*). The trial was conducted in a growing room (A2) of mushroom producer Delta Danube d.o.o., Kovin, Serbia. Efficacy in disease control and impact on yield (biological efficiency) of the biofungicide were evaluated and compared to those of the commercial fungicide prochloraz (Mirage® EC, ADAMA Agricultural Solutions UK Ltd., UK; content of a.i. 450 ml l^{-1}).

Tests in the mushroom growing room

Mushroom compost (fermented and pasteurized straw, supplemented with chicken manure) for phase III was produced by the compost factory Champicomp d.o.o., Pločica, Kovin, Serbia. The compost was packed in plastic briquettes sized 0.6 × 0.4 × 0.25 m (*l* x *w* x *h*). Each briquette contained 18 kg of compost inoculated with 0.7% of grain spawn of *A. bisporus* F56 (Italspawn, Onigo di Pederobba, Italy). Compost in each briquette was covered with a 50 mm layer of casing soil (7 kg) made of black peat soil (Pešter peat soil, Dallas Company d.o.o., Tutin, Serbia), and disinfected with 90 ml of 0.02% peracetic acid (Peral-S 15%, Vetprom, Belgrade, Serbia) per m^2 of casing layer. To calculate the doses for fungicide application, the surface area of the casing layer of five briquettes was estimated at 1 m^2. After the substrate was incubated at 25°C for 16 days, the air temperature was gradually reduced for 8 days to 17°C for the case-run. The day of casing time was regarded as day one. The trial was prepared according to standard PP 1/270 (1) methodology (European and Mediterranean Plant Protection Organization [EPPO], 2010). The fungicide prochloraz and biofungicide *B. subtilis* Ch-13 were applied onto the casing soil as a drench application. Prochloraz was applied at the standard product application rate – divided into two split applications (2 × 1.5 ml in 1 l of water per 1 m^2 of casing surface, four days after casing time and after the first fruiting flush). The biofungicide *B. subtilis* Ch-13 was applied in the total amount of 60 ml per m^2 of casing surface using two different application procedures: (*1*) three split applications (30 + 2 × 15 ml in 1 l of water per m^2 of casing surface, two days after casing, two weeks after casing, and after the first fruiting flush, respectively); and (*2*) two split applications (2 × 30 ml in 1 l of water per m^2 of casing surface, two days after casing, and after the first fruiting flush). The fungicide and biofungicide were applied using an automatic "fir" sprayer with 10 full cone nozzles, in the amount of 1 l per m^2 of casing surface. Control (untreated) briquettes were sprayed with tap water.

Each treatment was repeated in a randomized block design, on 43 m^2 of casing area per block. Each block consisted of 214 briquettes (replicates) with mushroom substrate. The fruiting bodies were hand-picked during the first production flush (14 to 21 days after casing) and the second flush (24 to 34 days after casing). The harvested mushrooms were classified into two groups, healthy and diseased, and then weighed. The impact of the biofungicide and fungicide on mushroom yield was evaluated via biological efficiency (BE) according to Chrysayi-Tokousbalides et al. (2007). Biofungicide and fungicide efficacy (E) were estimated using Abbott's formula (Abbott, 1925), i.e. the calculation was based on disease incidence (proportion of healthy and diseased mushrooms) in treated mushrooms (Gea et al., 2010).

Statistical analyses

Data obtained for efficacy in disease control and biological efficiency (impact on mushroom yield) were analyzed using the one-way analysis of variance (ANOVA); means were compared using the *F*-test. The *F*-test was used to compare mean values for efficacy obtained from repeated treatments of mushrooms with the fungicide, biofungicide, and water against the *H. perniciosus* mycopathogen *in vivo*, and to detect any significant difference among the treatments. The level of significance was determined at $p<0.05$ (Sokal & Rohlf, 1995). Statistical data analysis was performed using the software Statistica for Windows 7.0 (StatSoft Inc., 2004).

RESULTS

The efficacy of the biofungicide *B. subtilis* Ch-13, applied at the total concentration of 60 ml m^{-2} using two different application protocols – two (2 × 30 ml m^{-2}) or three (30 + 2 × 15 ml m^{-2}) split doses – was compared to that of the fungicide prochloraz in industrial-scale conditions of white button mushroom cultivation and their natural infestation with *H. perniciosus*. The impact of prochloraz and *B. subtilis* Ch-13 on mushroom yield was also evaluated. Wet bubble disease symptoms developed and were detected 18 to 34 days after casing time.

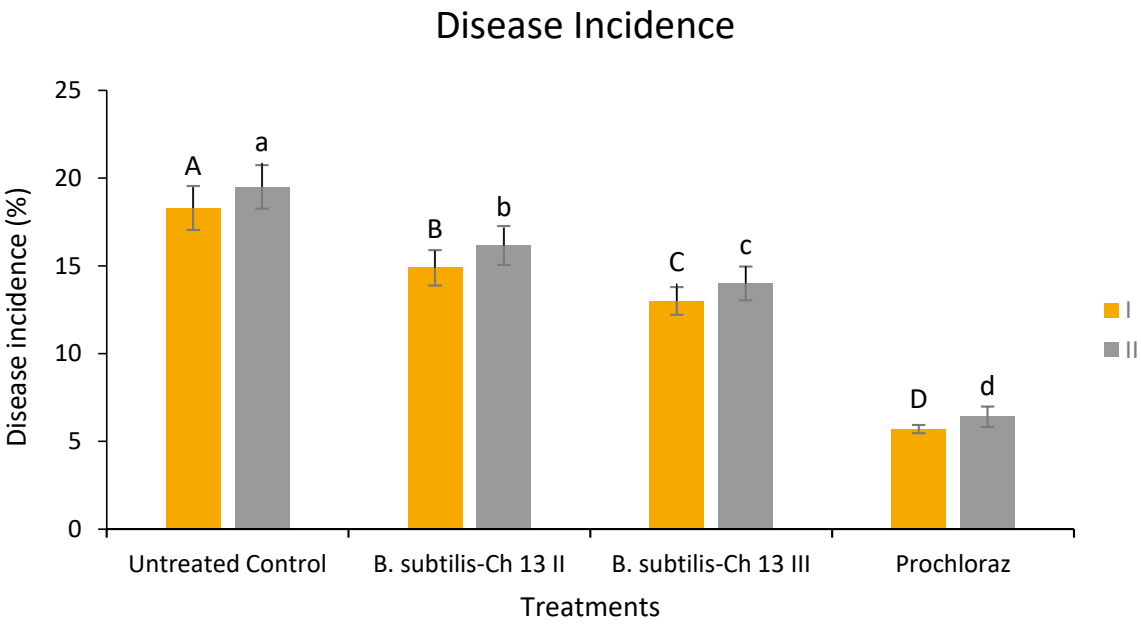

Figure 1. Incidence of wet bubble disease caused by *Hypomyces perniciosus* after treatment of *Agaricus bisporus* with bio/fungicides (*Bacillus subtilis* Ch-13 II/III – used in two/three split doses); data represent means of repetitions I and II (214 experimental briquettes per repetition) ± SE, standard error of the mean; SEDs, standard error of difference = 8.5 (I); 9.7 (II); df, degrees of freedom = 3; $F = 65.22$ (I); 78.32 (II); p-value = 0.001. Values marked with the same letter within each repetition series (I – capital letters; II – lowercase letters) are not significantly different according to the F-test ($p<0.05$).

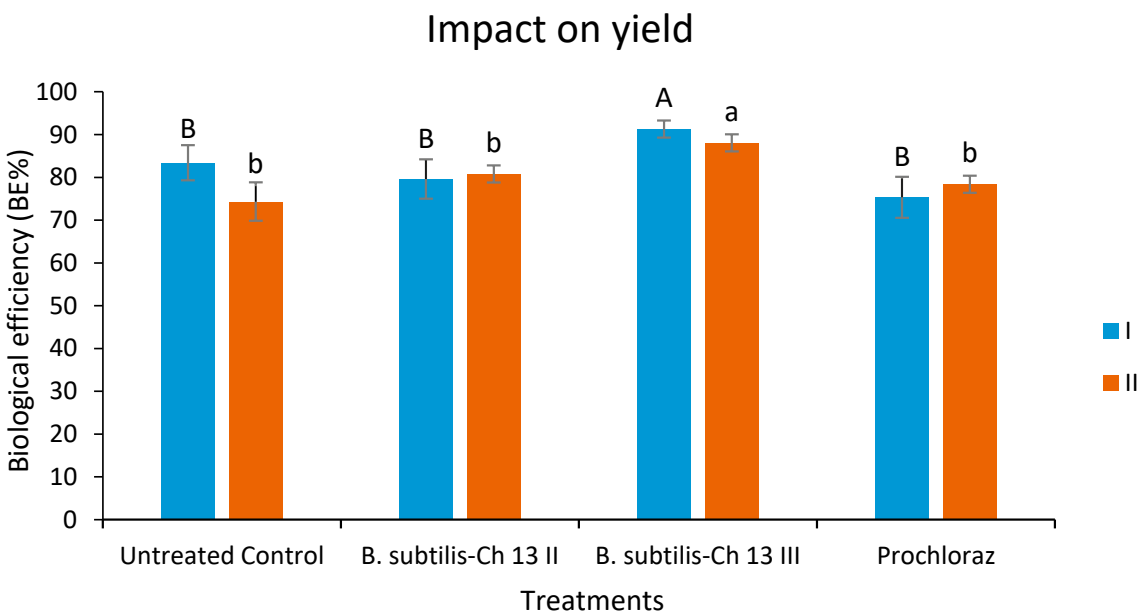

Figure 2. Impact of bio/fungicides on yield (biological efficiency – BE%) of *Agaricus bisporus*, naturally infested with *Hypomyces perniciosus* (*Bacillus subtilis* Ch-13 II/III – used in two/three split doses). Data represent means of repetitions I and II (214 experimental briquettes per repetition) ± SE, standard error of the mean; SEDs, standard error of difference = 37.7 (I); 35.8 (II); df, degrees of freedom = 3; $F = 28.4$ (I); 32.6 (II); p-value = 0.001. Values marked with the same letter within each repetition series (I – capital letters; II – lowercase letters) are not significantly different according to the F-test ($p<0.05$).

Effects of bio/fungicides on wet bubble disease incidence in white button mushrooms are shown in Figure 1. The highest disease incidence was found in the untreated mushroom control (18.3-19.5%). Disease incidence significantly decreased by applying biofungicide *B. subtilis* Ch-13 in two (14.9-16.2%) or three (13-14%) split doses. The lowest disease incidence was found in plots treated with prochloraz (5.7-6.4%). Efficacy in disease control was calculated in two ways: in relation to the fungicide prochloraz as a standard (E_{st}), which was set to 100% efficacy, or to the untreated control (E_c) (Table 1). Statistically significant differences in efficacy against the fungal pathogen were recorded for all treatments, with respect to either the fungicide standard or the untreated control. In relation to the untreated control (E_c), prochloraz exhibited the highest efficacy in the suppression of wet bubble disease (67.9%), followed by *B. subtilis* Ch-13 applied in three split doses (28.7%) (Table 1). The biofungicide *B. subtilis* Ch-13 used in two split doses showed the lowest efficacy in the suppression of *H. perniciosus* (15.7%), even though the same final concentration of *B. subtilis* Ch-13 was used in both protocols (with two or three split applications). Relative to efficacy of prochloraz set to 100% (E_{st}), *B. subtilis* Ch-13 used in three split doses demonstrated 55% higher efficacy in disease control (42.3%), than when used in two split doses (23.1%) (Table 1). Therefore, *B. subtilis* Ch-13 used three times significantly decreased the incidence of wet bubble disease of white button mushrooms, by up to 29% compared to the untreated control. In addition, efficacy of *B. subtilis* Ch-13 against *H. perniciosus* was significantly higher when the biofungicide was applied in three split doses, instead of two, regardless of the same total concentration of *B. subtilis* Ch-13 used in both protocols. Although efficacy of *B. subtilis* Ch-13 (≈30%) against *H. perniciosus* was low in the current study, treatment of mushrooms with *B. subtilis* Ch-13 slightly diminished wet bubble symptoms.

The impact of prochloraz and *B. subtilis* Ch-13 on mushroom yield (Table 2; Figure 2) was calculated

Table 1. Efficacy of bio/fungicides in the suppression of *Hypomyces perniciosus* on naturally infested *Agaricus bisporus,* in relation to the fungicide prochloraz (standard) or untreated control

Treatments	Fungicide application rate	[2]E_{st} (%)	[2]E_c (%)	SE
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1} II[1]	2 × 30 ml m^{-2}	23.10 c[4]	15.70 c	0.36
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1} III	30 + 2 × 15 ml m^{-2}	42.30 b	28.74 b	0.48
Prochloraz 450 ml l^{-1}	2 × 1.5 ml m^{-2}	100.00	67.94 a	0.22
Untreated Control	–	8.60 a	–	0.57

[1]*Bacillus subtilis* Ch-13 II/III – used in two/three split doses. The data represent means of two repetitions (214 experimental units per repetition) ± SE, standard error of the mean; efficacy in relation to the fungicide prochloraz (standard) set to 100% [2](E_{st}), or the untreated control [3](E_c); SEDs, standard error of difference = 36.75; df, degrees of freedom = 3; $F = 71.77$; p-value = 0.001. [4]Values marked with the same letter within each repetition series are not significantly different according to the F-test ($p<0.05$).

Table 2. Biological efficiency (BE%) of bio/fungicides in relation to the untreated control or fungicide prochloraz (standard) treatment of *Agaricus bisporus* naturally infested with *Hypomyces perniciosus*

Treatments	Fungicide application rate	[2]BE_{st} (%)	[3]BE_c (%)	SE
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1} II[1]	2 × 30 ml m^{-2}	104.35 b[4]	101.67 b	0.86
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1} III	30 + 2 × 15 ml m^{-2}	116.71 a	113.72 a	2.28
Prochloraz 450 ml l^{-1}	2 × 1.5 ml m^{-2}	100.00	97.44 b	2.14
Untreated Control	–	102.63 b	100.00	6.43

[1]*Bacillus subtilis* Ch-13 II/III – used in two/three split doses. The data represent means of two repetitions (214 briquettes per repetition) ± SE, standard error of the mean; fungicide prochloraz (standard) impact [2](BE_{st}) or untreated control impact [3](BE_c) is set to 100 %; SEDs, standard error of difference = 36; df, degrees of freedom = 3; $F = 30.5$; p-value = 0.001. [4]Values marked with the same letter within each repetition series are not significantly different according to the F-test ($p<0.05$).

by using the formula for biological efficiency (BE) according to Chrysayi-Tokousbalides et al. (2007). A statistically significant increase in mushroom yield relative to the untreated control was found when *B. subtilis* Ch-13 was applied in three split doses (88.1-91.3%), while no significant difference in yield was recorded between the untreated control (74.3-83.4%) and treatments with either prochloraz (75.4-78.4%) or *B. subtilis* Ch-13 applied in two split doses (80-81%) (Figure 2). Biological efficiency was calculated in two ways: relative to prochloraz (standard) (BE_{st}) or to the untreated control (BE_c), each set to 100% biological efficiency (Table 2). Relative to the untreated control, *B. subtilis* Ch-13 applied in three split doses had the highest and statistically most significant impact on mushroom yield (113.7%) (Table 2). In contrast, treatment of mushrooms with *B. subtilis* Ch-13 applied in two split doses, as well as with prochloraz, resulted in respective yield values of 101.7% and 97.4%, which did not significantly differ from that of the untreated control (Table 2). Relative to prochloraz set to 100% biological efficiency (BE_{st}), *B. subtilis* Ch-13 used in three split doses supported a mushroom yield (116.7%) that was 12.4% higher than the yield it supported when applied in two split doses (104.3%) (Table 2). Concerning its impact on yield, *B. subtilis* Ch-13 used in two split doses did not significantly differ from the untreated control in relation to prochloraz (BE_{st}) set to 100%, and did not significantly differ from prochloraz in relation to the untreated control (BE_c) set to 100%. The application of *B. subtilis* Ch-13 in three split doses significantly increased mushroom yield in contrast to its application in two split doses, the untreated control, or prochloraz. The biofungicide *B. subtilis* Ch-13, when applied in three split doses, considerably improved mushroom yield: by up to 14% and 17% relative to the untreated control and the fungicide prochloraz, respectively.

DISCUSSION

Large tumorous fruiting bodies with extracellular fluid on the surface of diseased mushrooms, detected in all experimental briquettes, were consistent with symptoms of wet bubble disease caused by *H. perniciosus* and described by Umar and Van Griensven (1999).

Rather low efficacy of *B. subtilis* Ch-13 applied in three (≈29%) or two split doses (≈16%) was recorded against *H. perniciosus,* in comparison to that of the fungicide prochloraz (≈68%). However, wet bubble symptoms were slightly reduced after *B. subtilis* Ch-13 treatment relative to the untreated control. Having found that two commercial strains, *B. velezensis* QST 713 (5.13×10^{10} CFU g^{-1}) and *B. amyloliquefaciens* subsp. *plantarum* D747 (5×10^{13} CFU g^{-1}) – both applied in two doses, at a concentration of 3 g m^{-2} on the first and fourth day after casing time – were not effective against *H. perniciosus*, Navarro et al. (2023) stated that *Bacillus* strains were not applicable against *H. perniciosus* on white button mushrooms. Nevertheless, when Navarro et al. (2023) evaluated strains *B. velezensis* QST 713 and *B. amyloliquefaciens* subsp. *plantarum* D747 at a concentration of 1×10^5 CFU m^{-2} against *H. perniciosus* in artificially infested mushrooms, they recorded efficacy ranges of 14.8-19% and 17-20.7%, respectively, which correspond to disease incidence values found after natural infestation of mushrooms with the pathogen in the present study. Efficacy of strain *B. subtilis* Ch-13 applied in the current study in two split doses (≈16%) was consistent with the previously reported findings of Navarro et al. (2023). However, strain *B. subtilis* Ch-13 applied in three split doses in the present study achieved higher efficacy (28.7%) than strains *B. velezensis* QST 713 and *B. amyloliquefaciens* subsp. *plantarum* D747 applied at much higher concentrations by Navarro et al. (2023). Strain *B. subtilis* Ch-13 exhibited higher efficacy against compost pathogen *T. aggressivum* f. *europaeum* and also increased mushroom yield more than *B. velezensis* QST 713, when both were applied at a concentration (10^7 CFU m^{-2}) lower than their standard application rates (Potočnik et al., 2019). This finding suggests that the timing of *Bacillus* spp. application is of great importance for its effect on mushroom production.

The biofungicide *B. subtilis* Ch-13 showed higher efficacy against *Trichoderma aggressivum* when applied either in three (53.6%) or two split doses (46.4%) in a large-scale experiment (Potočnik et al., 2021), than when used at the same concentration against *H. perniciosus* in the current study. The fungicide prochloraz exhibited efficacy in the control of *T. aggressivum* (71%) (Potočnik et al., 2021) similar to that against *H. perniciosus* in the present study (68%).

In several studies, the biofungicide widely used for plant and mushroom protection, *B. velezensis* QST 713 (Védie & Rousseau, 2008), displayed different efficacies against green mould disease agents *Trichoderma* spp. on *A. bisporus*: against *T. harzianum,* 44% (Kosanović et al., 2013; Milijašević-Marčić

et al., 2017), and against *T. aggressivum,* 48% (Milijašević-Marčić et al., 2017), 53% (spawn treatment) (Potočnik et al., 2018), 55% (Potočnik et al., 2019), and 54-58% (Stanojević et al., 2019). Stanojević et al. (2019) recorded an efficacy of 45-62% of strain *B. velezensis* QST 713 in the suppression of *Z. fungicola* (dry bubble disease). Clarke et al. (2024) noted that *B. velezensis* QST 713 prevents disease symptoms caused by *C. mycophilum* with an efficacy of 30-40%. Variation in biofungicide efficacy may be based either on differences in compost quality or among pathogen strains. Recently, strain *B. amyloliquefaciens* subsp. *plantarum* D747 was approved for use against *T. aggressivum,* while *B. amyloliquefaciens* MBI 600 was approved for suppression of *T. harzianum* and *T. aggressivum* on white button mushrooms (Marčić et al., 2025).

Milijašević-Marčić et al. (2017) recorded efficacies of 50% and 36% of the indigenous strain *B. subtilis* B-38 in the control of green mould agents *T. harzianum* and *T. aggressivum*, respectively. Stanojević et al. (2019) found that the native strain *B. amyloliquefaciens* B-241 suppressed *Z. fungicola* and *T. aggressivum* f. *europaeum* with efficacies of 46-58% and 53-68%, respectively. Clarke et al. (2024) found that the native strain *B. velezensis* Kos reduced *C. mycophilum* symptoms by up to 30-40%. Carrasco and Preston (2020) noted that native *Bacillus* spp. may diminish wet bubble disease symptoms only if the concentration of disease inoculum is low, whilst having a significant role in increasing mushroom yield.

Liu et al. (2015) confirmed that the indigenous strain *B. subtilis* B154 significantly increased the yield of *A. bisporus* infected with red bread mould *Neurospora sitophila* in comparison to the untreated control. Büchner et al. (2022) recorded that native strains of *B. velezensis,* used against *Trichoderma* spp. and *Z. fungicola,* increased mushroom yield by up to 18-26%. Milijašević-Marčić et al. (2017) found that *B. velezensis* QST 713 improved mushroom production by up to 15% in plots inoculated with *T. aggressivum* f. *europaeum,* while in uninoculated treatments, no increase in yield was found. Navarro et al. (2023) found that strains *B. velezensis* QST 713 and *B. amyloliquefaciens* subsp. *plantarum* D747 increased mushroom yield significantly in comparison to the untreated control in one out of six trials. In a small-scale trial, Potočnik et al. (2019) recorded that strain *B. subtilis* Ch-13 applied in two split doses (two days after casing and after the first flush) at a final concentration of 60 ml per m^2 (1×10^8 CFU ml^{-1}) enhanced mushroom yield by up to 12% in pathogen-free briquettes. Though applied at the same total concentration (60 ml per m^2, 1×10^8 CFU ml^{-1}) in the current study, *B. subtilis* Ch-13 increased mushroom yield much more when applied more frequently, in three split doses (14% and 17%, relative to the untreated control and prochloraz, respectively), than when applied in two split doses (2% and 4%, relative to the untreated control and prochloraz, respectively). Therefore, the results of this study support the application of strain *B. subtilis* Ch-13 at a concentration of 60 ml per m^2 (1×10^8 CFU ml^{-1}) in three split doses to suppress *H. perniciosus* and improve *A. bisporus* yield.

CONCLUSION

In the industrial-scale cultivation of *A. bisporus,* the efficacy of *B. subtilis* Ch-13, used at a total concentration of 60 ml per m^2 (1×10^8 CFU ml^{-1}) against *H. perniciosus* on naturally infected white button mushrooms, was significantly higher when applied in three split doses (28.7%), than in two (15.7%). Although the efficacy of *B. subtilis* Ch-13 (≈29%) against *H. perniciosus* was low in comparison to that of the fungicide prochloraz (≈68%), wet bubble symptoms in mushrooms were slightly reduced. Additionally, the greatest increase in mushroom yield was achieved when *B. subtilis* Ch-13 was applied in three split doses (14%), rather than in two (2%), compared to the untreated control. Therefore, the results of this study support that *B. subtilis* Ch-13 should be applied in three split doses ($30 + 2 \times 15$ ml m^{-2} on the second day after casing, two weeks after casing, and after the first fruiting flush, respectively) to suppress *H. perniciosus*.

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REFERENCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology, 18*(2), 265-267. DOI: https://doi.org/10.1093/jee/18.2.265a

Australian Pesticides and Veterinary Medicines Authority (APVMA) (2025). Retrieved from https://portal.apvma.gov.au/es/permits

Büchner, R., Vörös, M., Allaga, H., Varga, A., Bartal, A., Szekeres, A., Varga, S., Bajzát, J., Bakos-Barczi, N., Misz, A., Csutorás, C., Hatvani, L., Vágvölgyi, C., & Kredics, L. (2022). Selection and characterization of a *Bacillus* strain for potential application in industrial production of white button mushroom (*Agaricus bisporus*). *Agronomy*, *12*(2), 467. DOI: https://doi.org/10.3390/agronomy12020467

Carrasco, J., Navarro, M.J., & Gea, F.J. (2017). Cobweb, a serious pathology in mushroom crops: A review. *Spanish Journal of Agricultural Research*, *15*(2), e10R01. DOI: https://doi.org/10.5424/sjar/2017152-10143

Carrasco, J., & Preston, G.M. (2020). Growing edible mushrooms: A conversation between bacteria and fungi. *Environmental Microbiology*, *22*(3), 858-872. DOI: https://doi.org/10.1111/1462-2920.14765

Chebotar, V.K., Makarova, N.M., Shaposhnikov, A.I., & Kravchenko, L.V. (2009). Antifungal and phytostimulating characteristics of *Bacillus subtilis* Ch-13 rhizospheric strain, producer of biopreparations. *Applied Bichomistry and Microbiology*, *45*(4), 465-469. DOI: https://doi.org/10.1016/j.micres.2006.04.001

Chrysayi-Tokousbalides, M., Kastanias, M.A., Philippoussis, A., & Diamantopoulou, P. (2007). Selective fungitoxicity of famoxadone, tebuconazole and trifloxystrobin between *Verticillium fungicola* and *Agaricus bisporus*. *Crop Protection*, *26*(4), 469-475. DOI: https://doi.org/10.1016/j.cropro.2006.02.016

Clarke, J., McGuinness, B., Fitzpatrick, D., Kavanagh, K., & Grogan, H. (2024). Response of the mushroom pathogen *Cladobotryum mycophilum* to the fungicides prochloraz and metrafenone and two *Bacillus*-based biological control agents in mushroom crop trials. *Crop Protection*, *177*, 106530. DOI: https://doi.org/10.1016/j.cropro.2023.106530

European and Mediterranean Plant Protection Organization (EPPO) (2010). Efficacy evaluation of fungicides: Fungal diseases on cultivated mushrooms of *Agaricus* spp. PP 1/270(1) in EPPO Standards. *OEPP/EPPO Bulletin*, *40*(3), 270-273. DOI: https://doi.org/10.1111/j.1365-2338.2010.02384.x

Food and Drug Administration (FDA) (1999). Code of Federal Regulations, Title 21: Food and drugs. In: *Chapter 1: Food and Drug Administration Department of Health and Human Services, Part 184: Direct food substances affirmed as Generally Recognized as Safe* (pp. 892-896). Washington, D.C., USA: US Government Printing Office.

Gea, F.J., Navarro, M.J., & Tello, J.C. (2005). Reduced sensitivity of the mushroom pathogen *Verticillium fungicola* to prochloraz-manganese *in vitro*. *Mycological Research*, *109*(Pt 6), 741-745. DOI: https://doi.org/10.1017/s095375620500242x

Gea, F.J., Tello, J.C., & Navarro, M.J. (2010). Efficacy and effect on yield of different fungicides for control of wet bubble disease of mushroom caused by the mycoparasite *Mycogone perniciosa*. *Crop Protection*, *29*(9), 1021-1025. DOI: https://doi.org/10.1016/j.cropro.2010.06.006

Glamočlija, J., Soković, M., Ljaljević-Grbić, M., Vukojević, J., Milenković, I., & Van Griensven, L. (2008). Morphological characteristics and mycelial compatibility of different *Mycogone perniciosa* isolates. *Journal of Microscopy*, *232*(3), 489-492. DOI: https://doi.org/10.1111/j.1365-2818.2008.02145.x

Grogan, H.M. (2006). Fungicide control of mushroom cobweb disease caused by *Cladobotryum* strains with different benzimidazole resistance profiles. *Pest Management Science*, *62*(2), 153-161. DOI: https://doi.org/10.1002/ps.1133

Kayin, G.B., Öztüfekçi, S., Akin, H.F., Karaata, E.U., Katkat, A.V., & Turan, M.A. (2015). Effect of *Bacillus subtilis* Ch-13, nitrogen and phosphorus on yield, protein and gluten content of wheat (*Triticum aestivum* L.). *Journal of Agricultural Faculty of Uludag University*, *29*(1), 19-28. https://dergipark.org.tr/tr/download/article-file/154225

Kosanović, D., Potočnik, I., Duduk, B., Vukojević, J., Stajić, M., Rekanović, E., & Milijašević-Marčić, S. (2013). *Trichoderma* species on *Agaricus bisporus* farms in Serbia and their biocontrol. *Annals of Applied Biology*, *163*(2), 218-230. DOI: https://doi.org/10.1111/aab.12048

Liu, C., Sheng, J., Chen, L., Zhen, Y., Lee, D.Y.W., Yang, Y., Xu, M., & Shen, L. (2015). Biocontrol activity of *Bacillus subtilis* isolated from *Agaricus bisporus* mushroom compost against pathogenic fungi. *Journal of Agriculture and Food Chemistry*, *63*(26), 6009-6018. DOI: https://doi.org/10.1021/acs.jafc.5b02218

Marčić, D., Milijašević-Marčić, S., Drobnjaković, T., Luković, J., Šantrić, Lj., Grujić, N., & Potočnik; I. (2025). Bioprotection of the button mushroom from pests

and diseases. *Agronomy*, *15*(6), 1323. DOI: https://doi.org/10.3390/agronomy15061323

Meyer, L., & Korsten, L. (2008). A nested PCR for the detection of *Mycogone perniciosa* causing wet bubble disease of white button mushrooms. *Mushroom Science, 17*(1), 554-564.

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. DOI: https://doi.org/10.1007/s10658-016-1107-3

Navarro, M.J., Santos, M., Dianez, F., & Gea, F.J. (2023). Chemical and biological control of wet bubble disease (*Hypomyces perniciosus*) in mushroom crops. *Agronomy*, *13*(7), 1672. DOI: https://doi.org/10.3390/agronomy13071672

Pandin, C., Védie, R., Rousseau, T., Le Coq, D., Aymerich, S., & Briandet, R. (2018). Dynamics of compost microbiota during the cultivation of *Agaricus bisporus* in the presence of *Bacillus velezensis* QST 713 as biocontrol agent against *Trichoderma aggressivum*. *Biological Control, 127,* 39-54. DOI: https://doi.org./10.1016/j.biocontrol.2018.08.022

Potočnik, I., Vukojević, J., Stajić, M., Tanović, B., & Rekanović, E. (2010). Sensitivity of *Mycogone perniciosa*, pathogen of culinary-medicinal button mushroom *Agaricus bisporus* (J. Lge) Imbach (Agaricomycetideae), to selected fungicides and essential oils. *International Journal of Medicinal Mushrooms*, *12*(1), 91-98. DOI: https://doi.org/10.1615/IntJMedMushr.v12.i1.90

Potočnik, I., Todorović, B., Rekanović, E., Luković, J., Paunović, D., & Milijašević-Marčić, S. (2018). Impact of *Bacillus subtilis* QST 713 mushroom grain spawn treatment on yield and green mould control. *Pesticides and Phytomedicine*, *33*(3-4), 205-211. DOI: https.//doi.org./10.2298/PIF1804205P

Potočnik, I., Rekanović, E., Todorović, B., Luković, J., Paunović, D., Stanojević, O., & Milijašević-Marčić, S. (2019). The effects of casing soil treatment with *Bacillus subtilis* Ch-13 biofungicide on green mould control and mushroom yield. *Pesticides and Phytomedicine*, *34*(1), 53-60. DOI: https://doi.org/10.2298/PIF1901053P

Potočnik, I., Todorović, B., Milijašević-Marčić, S., Luković, J., Kanižai Šarić, G., Majić, I., & Rekanović, E. (2021). A large-scale study on the effectiveness of a *Bacillus subtilis* Ch-13-based biofungicide against green mould disease and mushroom yield improvement. *Pesticides and Phytomedicine*, *36*(2), 83-90. DOI: https://doi.org/10.2298/PIF2102083P

Regnier, T., & Combrinck, S. (2010). *In vitro* and *in vivo* screening of essential oils for the control of wet bubble disease of *Agaricus bisporus*. *South African Journal of Botany*, *76*(4), 681-685. DOI: https://doi.org/10.1016/j.sajb.2010.07.018

Siwulski, M., Sobieralski, K., Górski, R., Lisiecka, J.M., & Sas-Golak, I. (2011). Temperature and pH impact on the mycelium growth of *Mycogone perniciosa* and *Verticillium fungicola* isolates derived from Polish and foreign mushroom growing houses. *Journal of Plant Protection Research*, *51*(3), 268-272. DOI: https://10.2478/v10045-011-0044-6

Shi, N., Ruan, H., Jie, Y., Chen, F., & Du, Y. (2020). Sensitivity and efficacy of fungicides against wet bubble disease of *Agaricus bisporus* caused by *Mycogone perniciosa*. *European Journal of Plant Pathology, 157*(4)*,* 873-885. DOI: https://doi.org/10.1007/s10658-020-02047-0

Sokal, R.R., & Rohlf, F.J. (1995). *Biometry: The principles and practice of statistics in biological research* (3rd edition). New York, NY, USA: W.H. Freeman and Company.

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*, *126*(5), 104944. DOI: https://doi.org/10.1016/j.cropro.2019.104944

StatSoft Inc. (2004). STATISTICA (data analysis software system), version 7. Retrieved from https://www.statsoft.com

Umar, M.H., & Van Griensven, L.J.L.D. (1999). Studies on the morphogenesis of *Agaricus bisporus*: Dilema of normal versus abnormal fruit body development. *Mycological Research*, *103*(10), 1235-1244. DOI: https://doi.org/10.1017/S0953756299008473

Védie, R., & Rousseau, T. (2008). Serenade biofungicide: une innovation mjeure dans les champignonnières françaises pour lutter contre *Trichoderma aggressivum*, agent de la moisissure verte du compost. *La Lettre du CTC*, *21*, 1-2.

Biofungicid *Bacillus subtilis* Ch-13 u suzbijanju *Hypomyces perniciosus* (mokra trulež) u uslovima industrijske proizvodnje šampinjona

REZIME

Cilj rada je bio da se ispita efikasnost biofungicida *Bacillus subtilis* Ch-13 u suzbijanju prirodne zaraze *Hypomyces perniciosus* (prouzrokovača bolesti mokre truleži) i njegovog uticaja na prinos u uslovima industrijske proizvodnje šampinjona (*Agaricus bisporus*). Biofungicid *B. subtilis* Ch-13 je bio primenjen u ukupnoj koncentraciji od 60 ml m^{-2} na dva različita načina: u tri ($30 + 2 \times 15$ ml m^{-2}) ili dve podeljene doze (2×30 ml m^{-2}) u poređenju sa standardnom dozom primene fungicida prohloraza. Efikasnost biofungicida je bila značajno veća kada je primenjen u tri podeljene doze (28.7%), nego u dve (15.7%). Iako je uočena niska efikasnost *B. subtilis* Ch-13 (≈29%) u suzbijanju *H. perniciosus* u poređenju sa fungicidom prohlorazom (≈68%), simptomi mokre truleži su smanjeni u određenoj meri. Takođe, najveće povećanje prinosa šampinjona je postignuto kada je *B. subtilis* Ch-13 primenjen u tri podeljene doze (14%), umesto u dve (2%), u poređenju sa netretiranom kontrolom. Tri podeljene doze biofungicida *B. subtilis* Ch-13 su povećale prinos šampinjona 17%, a dve podeljene doze 4%, u poređenju sa fungicidom prohlorazom. Efikasnost biofungicida *B. subtilis* Ch-13 u suzbijanju prouzrokovača mokre truleži, kao i u povećanju prinosa šampinjona, bila je veća kada je primenjen u tri podeljene doze, umesto u dve. Dakle, preporučuje se primena biofungicida u tri podeljene doze ($30 + 2 \times 15$ ml m^{-2}, drugog dana i dve nedelje nakon pokrivanja i posle prve berbe) radi efikasnog suzbijanja *H. perniciosus* i povećanja proizvodnje šampinjona.

Ključne reči: jestive gljive; agens biološke zaštite; zaštita od bolesti

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Dedić, B. (2012). Testing sunflower inbred lines for tolerance to phoma black stem. *Pesticides* & *Phytomedicine*, *27*(4), 299-303. doi:10.2298/PIF1204299D

Abbaspoor, M. & Streibig, J.C. (2005). Clodinafop changes the chlorophyll fluorescence induction curve. *Weed Science, 53*(1), 1-9. doi:10.1614/WS-04-131R

Abbaspoor, M., Teicher, H.B. & Streibig, J.C. (2006). The effect of root-absorbed PSII inhibitors on Kautsky curve parameters in sugar beet. *Weed Research, 46*(3), 226-235. doi:10.1111/j.1365-3180.2006.00498.x

Books: name(s) of author(s) or editor(s), year of publication, title, place of publication and name of publisher.

Timbrell, J. (2000). *Principles of biochemical toxicology* (3rd ed). London, UK: Taylor and Francis Ltd.

Frank, R. H. & Bernanke, B. (2007). *Principles of macroeconomics* (3rd ed.). Boston, MA: McGraw-Hill/Irwin.

Saari L.L. & Thill, D.C. (Eds.). (1994). *Resistance to acetolactate synthase inhibiting herbicides: Herbicide resistance in plants*. Boca Raton, FL, USA: CRC Press.

Dissertations: author's name, year of presentation, title, full name of the institution at which dissertation was defended.

Stepanović, M. (2012). *Osetljivost izolata Alternaria solani (Sorauer) iz različitih krajeva Srbije na fungicide i rizik rezistentnosti*. (Doktorska disertacija). Univerzitet u Beogradu, Poljoprivredni fakultet, Beograd.

Book chapters and articles in conference proceedings: author(s), year of publication, title of chapter/article/abstract, source title (with editors names), pages, place of publication and publisher.

Hammond, K. R. & Adelman, L. (1986). Science, values, and human judgment. In H. R. Arkes & K. R. Hammond (Eds.), *Judgement and decision making: An interdisciplinary reader* (pp. 127-143). Cambridge, England: Cambridge

University Press.

Edwards, J.P., Fitches, E.C., Audsley, N. & Gatehouse, J.A. (2002). Insect neuropeptide fusion proteins – A new generation of orally active insect control agents. In T. Margini (Ed.), *Proceedings of the BCPC – Pests and diseases* (pp. 237-242). Brighton, UK: University of Brighton Press.

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Graora, D., & Spasić, R. (2008). Prirodni neprijatelji *Pseudaulacaspis pentagona* Targioni-Tozzetti u Srbiji. *Pesticidi i fitomedicina*, *23*(1) 11-16. Retrieved from http://www.pesting.org.rs/media/casopis/2008/no.1/23_1_11-16.pdf

Radunović, D., Gavrilović, V., Gašić, K., Krstić, M. (2015). Monitoring of *Erwinia amylovora* in Montenegro. *Pesticides and Phytomedicine*, 30(3), 179-185. doi 10.2298/PIF1503179R or http://www.pesting.org.rs/media/casopis/2015/no.3/30-3_179-185.pdf

Kerruish, R.M. & Unger, P.W. (2010). *Plant protection 1 – Pests, diseases and weeds*. Retrieved from APPS at http://www.appsnet.org/Publications/Kerruish/PP1.pdf

Tables need to be numbered in Arabic numerals consecutively as they appear in text. Tables should be made exclusively in Word for Windows using the toolbar menu Table-Insert-Table, Times New Roman font, 12 pt, and single line spacing. Footnotes immediately below the table body should be given priority over other explanation in table header or in table cells, and text should be in Times New Roman font, 10 pt. Each table must have a header. Tables should be submitted as supplementary (separate) files, and their approximate location in the text marked.

Graphs should be processed in Microsoft Excel and all data in Times New Roman black font. Explanations should be provided in captions, consecutively and marked with Arabic numerals. Graphs should be submitted as supplementary files, and their approximate location in the text marked.

Figures and diagrams should be processed in Adobe Illustrator (version 9 or later) and all data written in Times New Roman black font. Diagrams should be submitted as supplementary files and their approximate locations in the text marked.

Photos need to be submitted in JPG or TIFF formate and the resolution should be at least 300 dpi. Photos need to be marked with Arabic numerals in consecutive order. Provide each photo with a caption, mark its approximate location in the text and submit it as a supplementary file.

Any use of artificial inteligence tools (e.g. ChatGpT) should be explained (e.g. for improving English text, which is acceptable).

Authors are expected to use the accepted International System of Units (SI). Abbreviations should be defined in brackets at their first in-text mention. Provide full Latin names along with common names of organisms, and italicize only Latin names of genera and species, e.g. Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). After first mention, the Latin name can be abbreviated (e.g. *L. decemlineata*).

Review articles need to contain an introduction, appropriate subtitles and a reference list.

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Uputstvo autorima

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Časopis *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* objavljuje naučne radove iz oblasti: toksikologije i ekotoksikologije pesticida; fitopatologije; primenjene entomologije i zoologije; herbologije; zaštite bilja i prehrambenih proizvoda; primene pesticida u poljoprivredi, komunalnoj higijeni i javnom zdravstvu.

Časopis *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* predstavlja nastavak publikacije *Pesticidi*, koja je pod tim imenom izlazila u periodu 1986-2003.

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Radovi koji se prilažu moraju biti napisani na engleskom jeziku, sa rezimeom na engleskom i srpskom jeziku.

Od 2020. godine, časopis izlazi četvoromesečno (tri broja godišnje).

Od 2021. godine, časopis Pesticidi i fitomedicina (Pesticides and Phytomedicine) objavljuje sveske samo u **elektronskom obliku**, bez štampane verzije. Osnovne platforme na kojima se postavljaju sadržaji časopisa su: Scindeks (http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949) i zvanična veb stranica izdavača (http://www.pesting.org.rs/).

Časopis se indeksira u sledećim bazama: Chemical Abstracts, CAB International; DOAJ, EBSCO, AGRIS, Scindeks.

Tokom 2011. godine, časopis je prešao na sistem onlajn uređivanja (Elektronsko uređivanje – e-Ur) na portalu SCIndeks Asistent **http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949**. Ovaj sistem uređivanja omogućava funkcionalniju prijavu radova i komunikaciju uredništva sa recenzentima i autorima, a obuhvata i servise za kontrolu kvaliteta radova: *CrossRef* za dodelu DOI i iThenticate za prevenciju plagijarizma. Elektronsko uređivanje je usaglašeno sa Aktom o uređivanju časopisa Ministarstva prosvete, nauke i tehnološkog razvoja RS i obezbeđuje vođenje evidencije koje ovaj akt nalaže.

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Autori u radu NE NAVODE ključne reči. Njih će predložiti urednik, a autori imaju pravo da dodeljene ključne reči prihvate ili da neke od njih zamene.

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Rad treba pripremiti u programu za obradu teksta Word (format A4, margine 25 mm, font Times New Roman 12 pt). Radovi treba da budu isključivo na engleskom jeziku sa naslovom i rezimeom na oba jezika (engleskom i srpskom).

Naslov treba da bude kratak i da upućuje na temu. Puna imena i prezimena svih autora, puni nazivi i adrese institucija svih autora i njihove email adrese treba navesti ispod naslova rada. U slučaju neslaganja ovih podataka u samom tekstu rada i u prijavi na platformi za uređivanje, prioritet će se dati podacima u samom tekstu rada. U toku faze lekture, tražiće se da svi autori u pisanom obliku potvrde svoje autorstvo.

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Uvod treba da sadrži najnužniji pregled istraživanja u datoj oblasti i ciljeve istraživanja.

Materijal i metode treba opisati dovoljno detaljno da omogućе ponavljanje ispitivanja. Poznate metode i tehnike označiti samo odrednicom iz literature.

Rezultate predstaviti logičnim redosledom, jasno i precizno, koristeći prigodne tabele i grafičke prikaze. Izbegavati ponavljanje rezultata u tabelama i grafikonima, ali i u tekstu rada.

Diskusija treba da istakne značaj dobijenih rezultata, kao i njihovo mesto u kontekstu prethodnih istraživanja. Kad god je to moguće, diskusiju treba odvojiti od rezultata.

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Dedić, B. (2012). Testing sunflower inbred lines for tolerance to phoma black stem. *Pesticides* & *Phytomedicine*, *27*(4), 299-303. doi:10.2298/PIF1204299D

Abbaspoor, M., & Streibig, J.C. (2005). Clodinafop changes the chlorophyll fluorescence induction curve. *Weed Science, 53*(1), 1-9. doi:10.1614/WS-04-131R

Abbaspoor, M., Teicher, H.B., & Streibig, J.C. (2006). The effect of root-absorbed PSII inhibitors on Kautsky curve parameters in sugar beet. *Weed Research, 46*(3), 226-235. doi:10.1111/j.1365-3180.2006.00498.x

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Timbrell, J. (2000). *Principles of biochemical toxicology* (3rd ed). London, UK: Taylor and Francis Ltd.

Frank, R. H., & Bernanke, B. (2007). *Principles of macroeconomics* (3rd ed.). Boston, MA: McGraw-Hill/Irwin.

Saari L.L., & Thill, D.C. (Eds.). (1994). *Resistance to acetolactate synthase inhibiting herbicides: Herbicide resistance in plants*. Boca Raton, FL, USA: CRC Press.

Disertacije: autor, godina odbrane, naslov, i puni naziv institucije u kojoj je disertacija odbranjena.

Stepanović, M. (2012). *Osetljivost izolata Alternaria solani (Sorauer) iz različitih krajeva Srbije na fungicide i rizik rezistentnosti*. (Doktorska disertacija). Univerzitet u Beogradu, Poljoprivredni fakultet, Beograd.

Poglavlja u knjigama i radovi u zbornicima: autor(i), godina publikovanja, naslov poglavlja/rada/apstrakta, naslov izvornika sa imenom (imenima) urednika, strane priloga, mesto publikovanja i naziv izdavača.

Hammond, K. R., & Adelman, L. (1986). Science, values, and human judgment. In H. R. Arkes & K. R. Hammond (Eds.), *Judgement and decision making: An interdisciplinary reader* (pp 127-143). Cambridge, UK: Cambridge University Press.

Edwards, J.P., Fitches, E.C., Audsley, N. & Gatehouse, J.A. (2002). Insect neuropeptide fusion proteins – A new generation of orally active insect control agents. In T. Margini (Ed.), *Proceedings of the BCPC – Pests and diseases* (pp 237-242). Brighton, UK: University of Brighton Press.

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Radunović, D., Gavrilović, V., Gašić, K., Krstić, M. (2015). Monitoring of *Erwinia amylovora* in Montenegro. *Pesticides and Phytomedicine*, 30(3), 179-185. doi 10.2298/PIF1503179R or http://www.pesting.org.rs/media/casopis/2015/no.3/30-3_179-185.pdf

Kerruish, R.M. & Unger, P.W. (2010). *Plant protection 1 – Pests, diseases and weeds*. Retrieved from APPS at http://www.appsnet.org/Publications/Kerruish/PP1.pdf

Tabele se obeležavaju arapskim brojevima prema predviđenom redosledu. Tabele se izrađuju isključivo u programu Word for Windows, kroz meni Table-Insert-Table, koristeći font Times New Roman, 12 pt i osnovni prored. Fusnotama neposredno ispod tabela treba dati prednost nad drugim objašnjenima u zaglavlju tabela ili u samim tabelama, a tekst se daje u fontu Times New Roman, 10 pt. Svaka tabela mora imati zaglavlje. Tabele se prilažu kao dopunske (zasebne) datoteke, a u samom tekstu se obeležava njihovo približno mesto.

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Pregledni radovi treba da sadrže uvod, odgovarajuće podnaslove i spisak literature.

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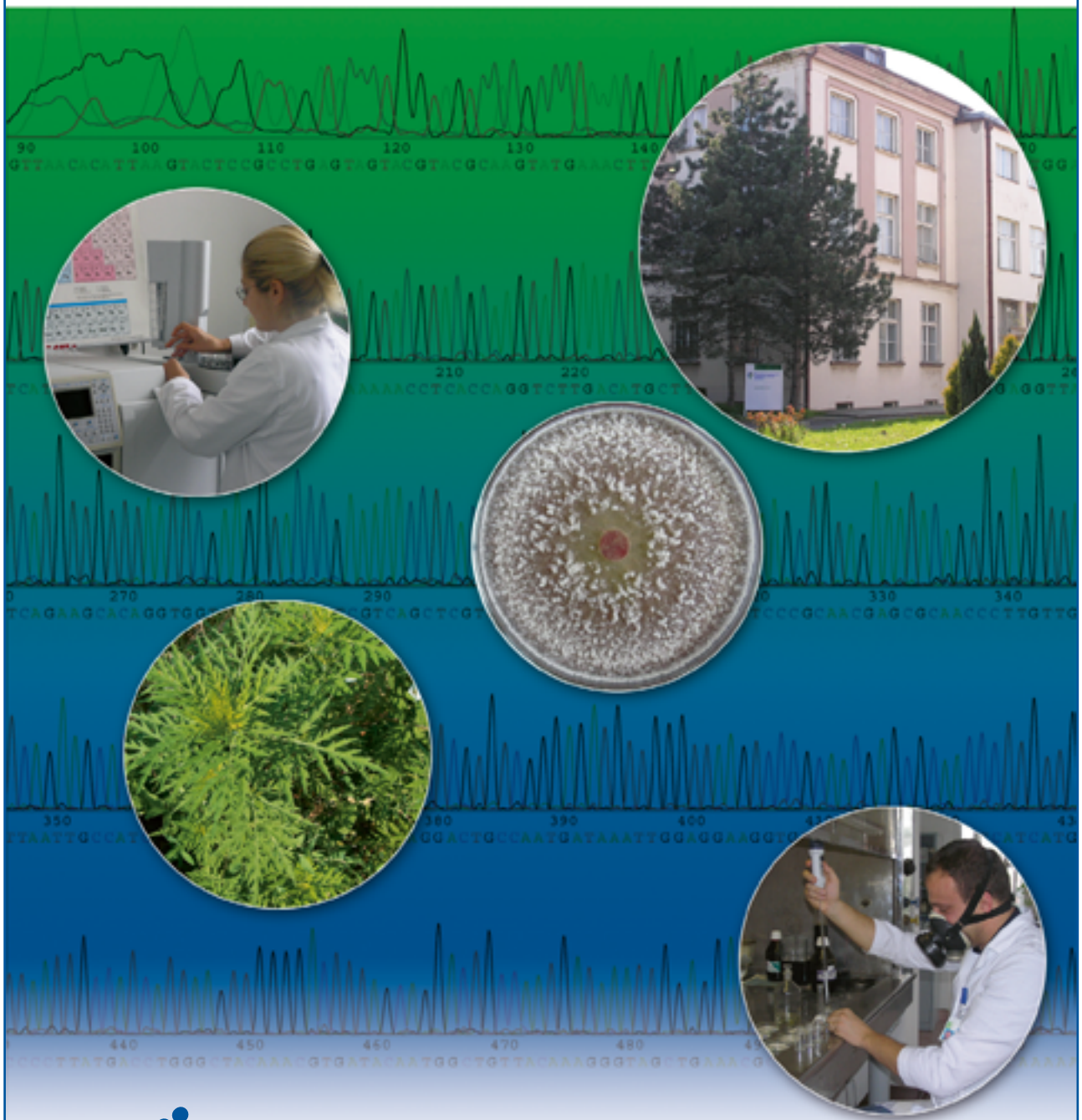

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