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Original scientific paper

A large-scale study on the effectiveness of a *Bacillus subtilis* Ch-13-based biofungicide against green mould disease and mushroom yield improvement

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SUMMARY

The aim of this study was to test a biofungicide based on *Bacillus subtilis* Ch-13 and its effectiveness in the control of green mould disease of cultivated mushroom in comparison with the fungicide prochloraz. Biofungicide effectiveness in disease control and impact on yield were evaluated on *Agaricus bisporus* after its natural infection with *Trichoderma aggressivum* in a commercial mushroom growing facility. An assay for testing the microbial efficacy of the biofungicide was conducted in two different procedures involving either three or two split doses. The highest statistically significant effectiveness in green mould control was shown by the fungicide prochloraz (71.43%), followed by the biofungicide applied in tree split doses (53.57%), and finally its two doses (45.46%). The biofungicide significantly improved yield in comparison with an untreated control and the fungicide prochloraz. Three split applications of *B. subtilis* strain Ch-13 enhanced mushroom yield to a larger extent than its two split doses, although the same final amount was used in both procedures. Biofungicide application in three split doses increased the total mass of harvested mushrooms 8.41% compared to the untreated control, and 10.53% compared to the fungicide prochloraz. These results implied that the biofungicide should be applied in three split applications: 30 ml (second day after casing) + 15 ml (two weeks after casing) + 15 ml (after first flush, 20-25 days after casing). The biofungicide *B. subtilis* Ch-13 should be further investigated regarding its joint usage with chemical fungicides in different application procedures, as it showed remarkable characteristics both in terms of promoting mushroom yield and inhibiting the spread of mycopathogenic *T. aggressivum*.

Keywords: *Bacillus subtilis*, biofungicides, edible mushroom, *Trichoderma aggressivum*, mushroom disease control

INTRODUCTION

The most devastating pathogen of cultivated mushroom (*Agaricus bisporus* L.) is *Trichoderma aggressivum* Samuels & W. Gams (Samuels et al., 2002) and, unlike other casing mycopathogens, it colonizes the substrate of *A. bisporus* and causes crop losses of between 60 and 100% (O'Brien et al., 2017). Kosanović et al. (2020) revealed that the concentration of *T. aggressivum* conidial suspension of 10^{-4} conidia per ml decreased mushroom yield 29-56%, while an inoculum of 10^{-3} conidia per ml caused 68-100% yield decrease. The fungicides prochloraz and metrafenone are allowed to be used in edible mushroom cultivation in the EU (Carrasco et al., 2017). These two fungicides have been registered in Serbia for other crops but not yet approved for use in mushrooms cultivation (Team of editors, 2020). Furthermore, prochloraz decomposes due to microbial degradation in casing soil, and its effectiveness in disease control is so rapidly lost after application (Grogan et al., 2000).

A good alternative to chemical control of mushroom diseases is the application of antagonistic microorganisms, primarily *Bacillus* species (Savoie et al. 2001). A biofungicide based on the most frequently used Canadian strain of *Bacillus velezensis* (Ehrenberg) Cohn, QST713, registered against many plant pathogens and mycopathogens (Védie & Rousseau, 2008; Pandin et al., 2018; Potočnik et al., 2018), is not available on the Serbian market at present. The Russian strain *Bacillus subtilis* (Ehrenberg) Cohn Ch-13 (Chebotar et al., 2009; Kayin et al., 2015), which has recently become available in Serbia, was compared with the chemical fungicide prochloraz and *B. velezensis* QST713 in a recent small-scale study in the experimental mushroom growing room (Potočnik et al., 2019). The biofungicide *B. subtilis* Ch-13 showed higher effectiveness against the compost pathogen *T. aggressivum,* and also increased mushroom yield more with its lower concentration than *B. velezensis* QST713 (Potočnik et al., 2019). Large-scale experiments with edible mushroom disease control are rather scarce. One of the few was conducted by Regnier and Combrinck (2010), establishing a suitable application regime (40 µl l^{-1}) for non-formulated essential oils of lemon, verbena, thyme and lemongrass, as well as two of their main components (nerol and thymol), against *M. perniciosa* in commercial growing facility under conditions of natural infection.

Based on the promising results of the previous small-scale experiment (Potočnik et al., 2019), the aim of this study was to compare the biofungicide based on *B. subtilis* Ch-13 and the fungicide prochloraz regarding green mould disease control under conditions of natural infection. The impact on mushroom yield was also estimated during this large-scale experiment in a commercial mushroom growing facility.

MATERIAL AND METHODS

Antifungal agents

The biofungicide Ekstrasol F SC (BioGenesis d.o.o., Belgrade, Serbia), based on *Bacillus subtilis* Ch-13 (1×10^8 CFU ml^{-1}), was tested as a potential antifungal agent for the control of *T. aggressivum* in natural infections of casing soil. The experiment was conducted in B8 growing chamber of the mushroom production facility of Delta Danube d.o.o., Kovin, Serbia. The biological efficacy and effectiveness of the biofungicide was evaluated by comparing it with the commercial fungicide prochloraz (Mirage® EC, ADAMA Agricultural Solutions UK Ltd., UK; content of a.i. 450 ml l^{-1}).

Tests in mushroom growing room

Treatments of casing soil in the mushroom growing chamber were carried out according to standard PP 1/270 (1) methodology (EPPO, 2010), using the biofungicide based on *B. subtilis* Ch-13 and the commercial prochloraz-based chemical fungicide.

Mushroom substrate packed in plastic bags sized 0.4 × 0.6 × 0.25 m ($l \times w \times h$), filled with 18 kg of compost and spawned with 0.7% of grain spawn of *A. bisporus* (Italspwan, Onigo di Pederobba, Italy), was provided by the compost producer Champicomp d.o.o., Pločica, Kovin, Serbia. Five plastic bags provided a casing surface of 1 m^2 which was used for treatment calculation. Compost was cased with 7 kg of black peat casing soil (Pešter peat soil, Dallas Company, Tutin, Serbia), and disinfected with peracetic acid 0.02% (Peral-S 15%, Vetprom, Belgrade, Serbia), 90 ml per m^2 of casing. Casing soil was cased in a 50 mm layer and incubated at 25°C for 8 days (case-run). The day of casing was regarded as day one. Over the next seven days air temperature was reduced in stages to 17°C. The fungicide prochloraz and the biofungicide were

repeatedly applied using an automatic "fir" sprayer with 10 full cone nozzles. Prochloraz was applied at the standard product application rate registered in the EU in two split applications, each treatment consisting of 1.5 ml in 1.8 l H_2O per 1 m^2 of casing surface on the fourth day after casing and after the first flush (approximately 20-25 days after casing). The biofungicide *B. subtilis* Ch-13 was used in two different application procedures in the same total amount of 60 ml per m^2 of casing surface: (1) three times: 30 ml (second day after casing) + 15 ml (two weeks after casing) + 15 ml (after first flush, approximately 20-25 days after casing); and (2) twice: 30 ml (second day after casing) + 30 ml (after first flush, approximately 20-25 days after casing). Each volume was diluted in 1 l of water and applied per m^2 of casing surface. Untreated control plots within groups were sprayed with tap water.

Each treatment and untreated control was repeated twice in a randomized block design experiment with casing area of 56 m^2 per block consisting of 224 bags of mushroom substrate (repetitions). The average values from both trials are presented. The fruiting bodies were hand-picked in two successive production flushes: the first from day 14 to 22 after casing, the second from day 23 to 35. The harvested mushrooms were weighed and divided into two groups based on visual observation, i.e. either with or without symptoms of green mould disease. Fungicide effectiveness was calculated by Abbott's formula (Abbott, 1925):

$$\% \text{ effectiveness} = (Ic - It)/Ic \times 100$$

where Ic - disease incidence in inoculated control; It - disease incidence in treated samples. Disease incidence was recorded as a percentage of fruiting bodies with symptoms compared with those without symptoms.

The effect of fungicides on mushroom productivity was evaluated as biological efficiency (BE), calculated as the ratio of fresh weight of total fruiting body yield and weight of dry spawned substrate, and expressed as percentages (Chrysayi-Tokousbalides et al., 2007) according to formula:

BE = (fresh total fruiting body yield/dry spawned substrate mass) × 100.

Statistical analyses

Data were examined using the one-way analysis of variance (ANOVA), including the comparison of means by the *F*-test. The test was used to compare the significance of differences among data for the average biological efficacy and effectiveness of different bio/fungicide treatments against *T. aggressivum* in the mushroom growing chamber. In all analyses, the level of significance was at least $P<0.05$ (Sokal & Rohlf, 1995). Statistical data analysis was performed using the software Statistica for Windows 6.0 (Stat Soft Italia, 1997).

RESULTS AND DISCUSSION

Dark green colonies were observed on the sides of compost surface eight days after casing, corresponding to first symptoms of green mould disease caused by *T. aggressivum* (Milijašević-Marčić et al., 2017).

Suppression of green mould disease incidence by using bio/fungicides is shown in Figure 1. The biofungicide *B. subtilis* Ch-13 significantly decreased disease incidence after natural *T. aggressivum* infection of cultivated mushrooms, compared to the chemical fungicide prochloraz and untreated control. The effectiveness of disease control was presented in two ways: in comparison with the standard fungicide prochloraz (E_{st}) set to 100%, and in relation to untreated control (E_k) (Table 1). The highest effectiveness in green mould control was shown by the fungicide prochloraz (71.43%), followed by the biofungicide *B. subtilis* Ch-13 applied in three split doses (53.57%). *B. subtilis* Ch-13 used in two split applications was the least effective against the pathogen (46.45%). Despite the same final concentration, the effectiveness of *B. subtilis* Ch-13 in green mould disease control was significantly higher when it was applied three times than in two applications. *B. subtilis* Ch-13 used in three split applications demonstrated effectiveness which was 17.86% lower than that of the standard chemical fungicide but still exceeded 50% in comparison with untreated control.

In the previous small-scale experiment, Potočnik et al. (2019) reported that *B. subtilis* Ch-13, applied at the concertation of 10^8 CFU per m^2, achieved 23% effectiveness when used in the amount of 10 ml m^{-2}; 27% in 20 ml m^{-2}, and 35% in 30 ml m^{-2} against *T. aggressivum*. The strain Ch-13 applied at the dose of 2×10^8 CFU per m^2 showed better efficacy (27.4%) than *B. velezensis* QST713 (23%) used at its higher concentration (5×10^9CFU per m^2). Prochloraz showed the highest effectiveness in disease control in both our experiments, 71% in the current large-scale study after natural infection, and 77% in the earlier small-scale assay after artificial infection with *T. aggressivum* 10^6 conidia per m^2 of casing soil (Potočnik et al., 2019).

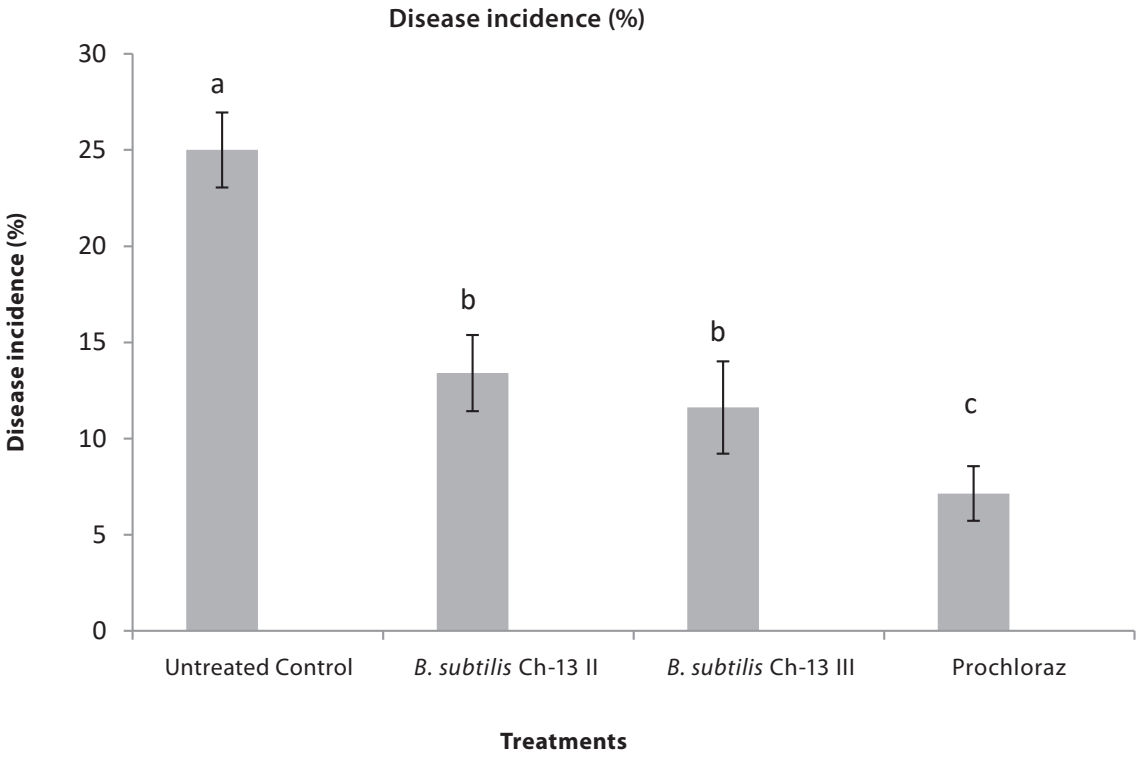

Figure 1. Suppression of disease incidence using bio/fungicides against naturally infected *Trichoderma aggressivum* on *Agaricus bisporus* in a large-scale assay; data are means of two trials, each including 224 replicate experimental bags ± SE, standard error of means; standard error of differences = 9.41; df, degrees of freedom = 3; $F = 70.22$; P-value = 0.001. Values within series marked with the same letters are not significantly different according to F-test ($P<0.05$).

Table 1. The effectiveness of biofungicide treatments in disease control on *Agaricus bisporus* naturally infected with *Trichoderma aggressivum* in a large-scale assay, as related to a standard fungicide (E_{st}) and untreated control (E_k)

Treatments	Bio/fungicide application rate (ml m^{-2})	E_{st}[1] (%)	E_k[2] (%)	SE
Bacillus subtilis Ch-13 1×10^8 CFU ml^{-1}	$1 \times 30 + 2 \times 15$	75.00 b[3]	53.57 b	4.79
Bacillus subtilis Ch-13 1×10^8 CFU ml^{-1}	2×30	65.03 c	46.45 c	3.95
Prochloraz 450 ml a.i. l^{-1}	2×1.5	100.00 a	71.43 a	2.85

Data are means of two trials, each including 224 replicates of experimental bags ± SE, standard error of means; Effectiveness (E)% in disease symptoms control, when standard fungicide effectiveness [1](E_{st}) is set to 100% or when effectiveness is related to untreated control [2](E_k); SEDs, standard error of differences=9.41; df, degrees of freedom=3; F=70.22; P-value=0.001. [3]Values within series marked with the same letters are not significantly different according to F-test ($P<0.05$).

A statistically significant increase in mushroom yield was noted when the biofungicide *B. subtilis* Ch-13 was used in two and three split doses, in comparison with the untreated control and prochloraz fungicide (Figure 2). The chemical fungicide (standard) did not significantly improve mushroom yield compared to the untreated control. Furthermore, impact on mushroom yield was shown as a biological efficiency coefficient (BE) when either the impact of untreated control (BE_{st}) or the standard fungicide prochloraz (BE_k) were set to 100 % (Table 2). The biofungicide increased mushroom yield more when it was used frequently, i.e. in three split applications, than only twice, although the same final amount was used in both treatments.

The strain *B. subtilis* Ch-13 used in three split applications improved the total mass of harvested mushrooms compared both with the untreated control (8.41%) and prochloraz fungicide (10.53%).

The previous small-scale experiment showed that treatments with *B. subtilis* Ch-13, used at concentrations 1-3 × 10^8 CFU ml^{-1}, resulted in considerably enhanced mushroom yield (72-76%), compared to all uninoculated treatments: control plots (66%), fungicide prochloraz plots (68%), and biofungicide *B. velezensis* QST713 plots (58-68%) applied at higher concentrations of 5 × 10^9 CFU ml^{-1} and 1 × 10^{10} CFU ml^{-1} (Potočnik et al., 2019).

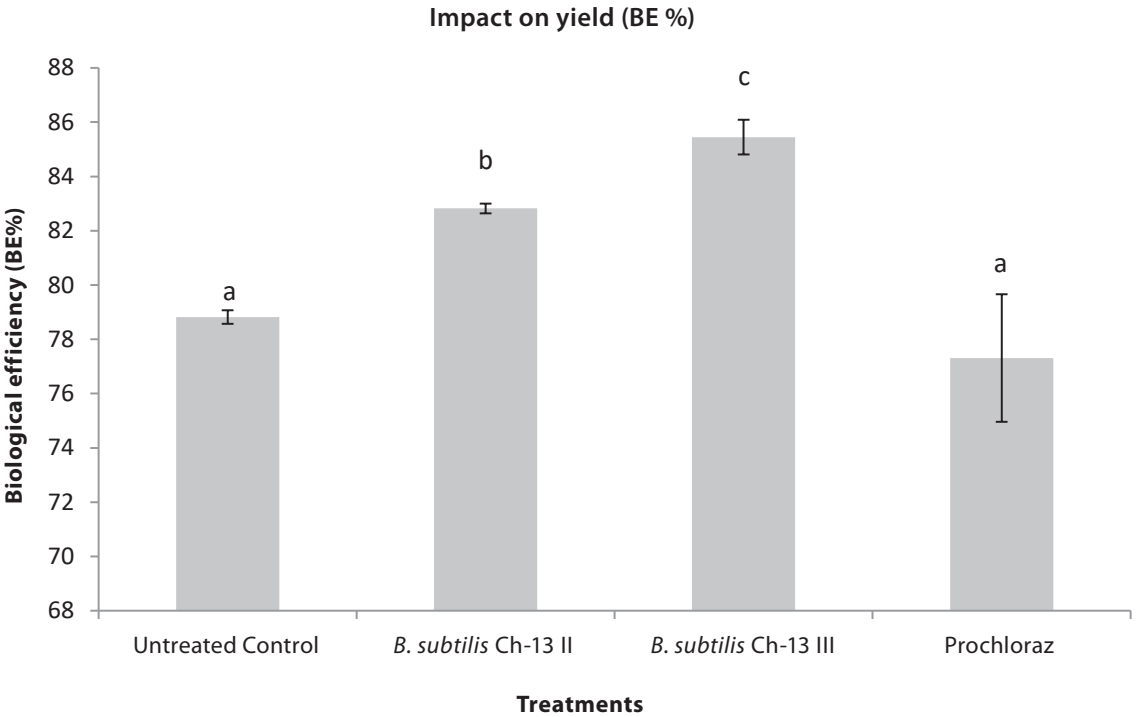

Figure 2. Impact of different bio/fungicides on the yield of cultivated mushroom (*Agaricus bisporus*) naturally infected with *Trichoderma aggressivum* in large-scale assays. Data are means of two replicates and each included 224 replicate experimental bags ± SE, standard error of means; BE% - Biological efficiency = ratio of the fresh weight of total mushroom yield and weight of dry spawned substrate; SEDs, standard error of differences=48; df, degrees of freedom=3; *F*=25; *P*-value=0.001. Values within series marked with the same letters are not significantly different according to *F*-test (P<0.05).

Table 2. Impact of bio/fungicides on the yield of cultivated mushroom (*Agaricus bisporus*) naturally infected with *Trichoderma aggressivum* in a large-scale assay

Treatments	Bio/fungicide application rate (ml m^{-2})	BE_{st}[1] (%)	BE_k[2] (%)	SE
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1}	1 × 30 + 2 × 15	110.53 a[3]	108.41 a	1.29
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1}	2 × 30	107.13 b	105.07 b	0.36
Prochloraz 450 ml a.i. l^{-1}	2 × 1.5	100.00 c	98.08 c	4.70
Untreated control	–	101.95 c	100.00 c	0.51

Data are means of two trials, each including 224 replicate experimental bags ± SE, standard error of means; Biological efficiency (BE) % = ratio of the fresh weight of total mushroom yield and weight of dry spawned substrate, when standard fungicide impact [1](BE_{st}) or untreated control impact [2](BE_k) is set to 100 %; SEDs, standard error of differences=48; df, degrees of freedom=3; *F*=25; *P*-value=0.001. [3]Values within series marked with the same letters are not significantly different according to *F*-test (P<0.05).

As for the inoculated treatments in the same experiment, the biofungicide *B. subtilis* Ch-13 used at the concentration of 1×10^8 CFU ml^{-1} increased yield (68%) more than *B. velezensis* QST713 (63%) used at its higher concentration of 5×10^9 CFU ml^{-1} (Potočnik et al., 2019). In the current large-scale experiment, the biofungicide *B. subtilis* Ch-13 concentration of 1×10^8 CFU ml^{-1}, and its dose of 60 ml m^{-2} of casing soil, improved yield 83-85%, while the biofungicide was applied in the small-scale experiment at lower doses and achieved proportionately lower yield: at the concentration of 10 ml m^{-2} - 68%, 20 ml m^{-2} - 72% and at 30 ml m^{-2} - 74% (Potočnik et al., 2019). Similar yields (79%) were obtained in untreated control plots in both experiments, i.e. in the previous small-scale trial (artificial infection) (Potočnik et al., 2019) and the current large-scale trial under conditions of natural infection.

The mode of action of *Bacillus* spp. biofungicides is based on competition for nutrients, substrate colonization (Chen et al., 2013), synthesis of antibiotics, iron chelators, antifungal volatile organic compounds and cell wall degrading enzymes (Manjula & Podile, 2005). Competition could also be responsible for the inhibition of *T. aggressivum* growth. Furthermore, *B. subtilis* strains are considered safe for the environment and harmless to human health and are generally recognized as safe (GRAS) organisms (FDA, 2020). Additionally, *Bacillus* spp. strains form endospores which ensure their survival and persistence in the environment (Cawoy et al., 2011). The current investigation of different procedures for the application of *B. subtilis* Ch-13 revealed benefits from applying three split doses to suppress the growth of *T. aggressivum,* an aggressive compost pathogen and causal agent of green mould disease, and to promote *A. bisporus* production.

CONCLUSION

The biofungicide based on *B. subtilis* Ch-13 showed better efficacy in green mould disease control and the highest positive impact on mushroom production when it was used in three split applications, rather than two. It suggests that the biofungicide should be applied three times: 30 ml (on the second day after casing) + 15 ml (two weeks after casing,) + 15 ml (after the first flush, approximately 20-25 days after casing). The microbial biofungicide *B. subtilis* Ch-13, which is harmless to the environment and non-target organisms, should be further investigated regarding its combinations with chemical fungicides in order to achieve better efficacy in disease control as it showed remarkable characteristics both in inhibiting the spread of the mycopathogen *T. aggressivum,* the causal agent of the most serious mushroom disease, and in promoting mushrooms production.

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Ispitivanje primene biofugicida na bazi *Bacillus subtilis* Ch-13 u suzbijanju prouzrokovača zelene plesni i pospešivanju prinosa šampinjona u industrijskim razmerama

REZIME

Cilj rada je ispitivanje mogućnosti primene biofungicida na bazi *Bacillus subtilis* Ch-13 u suzbijanju prouzrokovača zelene plesni i povećanju prinosa šampinjona. Biofungicid je testiran nakon prirodne zaraze *Trichoderma aggressivum* u komercijalnom gajilištu šampinjona i poređenjem sa fungicidom prohlorazom. Testirana je efikasnost mikrobiološkog biofungicida kroz dva postupka višestruke primene, u tri i u dve ponovljene doze. Najveću statistički značajnu efikasnost u suzbijanju prouzrokovača zelene plesni je ispoljio fungicid prohloraz 71,43%, zatim biofungicid primenjen u tri doze 53,57% i najmanju primenjen u dve doze 46,45%. Efikasnost *B. subtilis* Ch-13 u suzbijanju prouzrokovača zelene plesni je bila veća od 50% kada je primenjen u tri doze, za razliku od niže efikasnosti u dvokratnoj primeni. Testirani *B. subtilis* Ch-13 je značajnije povećao prinos šampinjona primenjen u tri podeljene doze nego u dve, iako sa istom ukupnom primenjenom količinom preparata. Biofungicid je znatno poboljšao prinos u poređenju sa netretiranom kontrolom i fungicidom prohlorazom. Soj *B. subtilis* Ch-13 je pokazao izuzetno pozitivan uticaj na prinos šampinjona primenjen u tri doze, sa povećanjem ukupne količine ubranih šampinjona 8,41% u odnosu na netretiranu kontrolu i 10,53% u odnosu na fungicid prohloraz. Ovi rezultati pokazuju da bi biofungicid na bazi *B. subtilis* Ch-13 trebalo primeniti u tri podeljene doze: 30 ml (drugi dan nakon stavljanja pokrivke) + 15 ml (dve nedelje nakon stavljanja pokrivke) + 15 ml (nakon prvog talasa plodonošenja, 20-25 dana nakon pokrivanja). Biofungicid *B. subtilis* Ch-13, neškodljiv za životnu sredinu i neciljne organizme, bi trebalo dalje ispitati u zajedničkoj primeni sa hemijskim fungicidima u različitim načinima primene da bi se obezbedila bolja efikasnost u suzbijanju prouzrokovača bolesti, jer je pokazao zadovoljavajuće osobine i u sprečavanju širenja mikopatogena *T. aggressivum* i povećanju prinosa.

Ključne reči: *Bacillus subtilis*, biofungicidi, šampinjon, *Trichoderma aggressivum*, suzbijanje bolesti pečuraka