Pestic. Phytomed. (Belgrade), 34(1), 2019, 53–60
DOI: https://doi.org/10.2298/PIF1901053P

UDC 632.937.1:632.4:635.82
Original scientific paper

# The effects of casing soil treatment with *Bacillus subtilis* Ch-13 biofungicide on green mould control and mushroom yield

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*Received: 11 April 2019*
*Accepted: 16 May 2019*

**SUMMARY**

The impact of a biofungicide based on *Bacillus subtilis* Ch-13 on mushroom yield and efficacy in suppression of *Trichoderma aggressivum* f. *europaeum* T77 from Serbia was estimated in comparision with a similar microbial fungicide, *Bacillus velezensis* QST713, and the chemical fungicide prochloraz manganese. The biofungicide *B. velezensis* QST713 is registered for treatments of mushrooms and other crops in many countries but it is not currently available on the Serbian market. The tested *B. subtilis* Ch-13 fungicide enhanced mushroom yield 12%, compared with an uninoculated control, and notably more than *B. velezensis* QST713 applied at its higher test concentrations. Regarding the efficacy of the biofungicides in control of the compost pathogen *T. aggressivum* f. *europaeum*, *B. subtilis* Ch-13 applied in concentration of $3 \times 10^8$ CFU per $m^2$ showed higher efficacy than the higher concentrations ($5 \times 10^9$ and $1 \times 10^{10}$ CFU per $m^2$) of *B. velezensis* QST713. The biofungicide based on *B. subtilis* Ch-13 should be further investigated regarding its different modes of application to ensure better efficacy in disease control as it showed beneficial features in both promoting *A. bisporus* production and suppressing the growth of the aggressive compost pathogen *T. aggressivum*, the causal agent of devastating green mould disease.

**Keywords**: cultivated mushroom; *Trichoderma aggressivum*; *Bacillus subtilis*; biofungicides

## INTRODUCTION

Green mould, caused by compost-inhabiting *Trichoderma aggressivum* Samuels & W. Gams (Seaby, 1996; Samuels et al., 2002), is the most serious fungal disease of cultivated mushroom (*Agaricus bisporus* L.). Serious outbreaks of disease result in great yield losses. In the 1990s, the aggressive species appeared

simultaneously in the British Isles and North America (Doyle, 1991; Romaine et al., 1996) and rapidly spread to other European countries, including Serbia, and to other continents (Kosanović et al., 2013). Its two forms, *Trichoderma aggressivum* f. *aggresivum* Samuels & W. Gams in North America and *T. aggressivum* f. *europaeum* Samuels & W. Gams in Europe, are phylogenetically closely related to *T. harzianum* Rifai. They emerged by population adaptation to their respective environmental conditions in mushroom-growing facilities and have never been found in the wild (Kredics et al., 2010).

Only a few fungicides have been registered and officially recommended for mushroom cultivation worldwide, i.e. prochloraz and metraphenone, while chlorothalonil, and the benzimidazoles thiabendazole and thiophanate-methyl are still in use in North America (Romaine et al., 1996; Grogan & Gaze, 2000). Over time, *Trichoderma* species have developed resistance to benzimidazoles in cultivated mushroom farms (Grogan & Fletcher, 1993; Grogan et al., 1996; Romaine et al., 2005; Grogan, 2008). The fungicide prochloraz has been shown to be very susceptible to degradation in soils. Grogan et al. (2000) reported a decrease in its concentration to less than 25% in casing soil, following two split applications. Also, the authors noted the rate of disappearance of the fungicide to be faster after the second spray (one week later) compared to the first application (after 18 days), which suggests microbial degradation.

Fungicides usually reduce the mycelial growth of *A. bisporus* to some extent, and their application must always be a balance between the benefit from disease control and reduced vigor of mushroom crop. Many chemicals have been withdrawn from the market over the past two decades. Moreover, there is an increased demand for biorational measures to control outbreaks rather than resorting to chemicals (Grogan, 2008). An alternative to chemical control of *Trichoderma* green mould is the application of beneficial microorganisms, mainly *Bacillus* species as biocontrol agents (Savoie et al. 2001; Védie & Rousseau, 2008). The most studied *Bacillus subtilis* (Ehrenberg) Cohn strain used as the biofungicide QST713 has been recently renamed to *Bacillus velezensis* (Pandin et al., 2018a,b). However, it is not registered in Serbia. The biocontrol strain *B. velezensis* QST713 was chosen for a correlation test with *B. subtilis* Ch-13, the newly introduced strain with antifungal and phytostimulating characteristics, which has been registered as a microbiological fertilizer, fungicide and wheat seed disinfectant in the Russian Federation, Kazakhstan and Moldova (Chebotar et al., 2009; Kayin et al., 2015). Both *Bacillus* strains were examined for their impact on mushroom yield and efficacy against *T. aggressivum* f. *europaeum* from Serbia, when applied to mushroom casing soil and in comparison with the fungicide prochloraz manganese in a mushroom growing room.

## MATERIAL AND METHODS

### Fungal species and culture conditions

The pathogenic fungus *T. aggressivum* f. *europaeum* T77, originally isolated from mushroom compost in a Serbian mushroom farm at Barajevo-Lisovići in 2010, was taken from the culture collection of the Institute of Pesticides and Environmental Protection (Belgrade, Serbia). The strain was then identified based on morpho-physiological characteristics and ITS1/ITS4 sequence analyses (Kosanović et al., 2013). The fungus was maintained on potato dextrose agar (PDA) medium (fresh-peeled potatoes; dextrose, Torlak, Serbia; agar, Torlak, Serbia) at 4°C. For inoculum preparation, agar discs with the fungal isolates were inoculated onto PDA medium and the plates were incubated for three days at 22°C. For *in vivo* trials, conidia from three-day old cultures were flooded with 10 ml of sterile distilled water and Tween 20 (v/v 0.01%), and filtered through double layers of cheesecloth.

### Antifungal agents

The biofungicide Ekstrasol F SC (BioGenesis d.o.o., Serbia), based on *Bacillus subtilis* Ch-13 $1 \times 10^7$ CFU $ml^{-1}$, was tested as a potential antifungal agent in treatment of casing soil on substrate spawned with *A. bisporus* Sylvan A15, against the isolate of *T. aggressivum* f. *eurpaeum* T77 in a mushroom growing room. The biological efficacy and effectiveness of the biofungicide was evaluated by comparison with the commercial biofungicide Serenade® WP (AgraQuest, Canada) based on *Bacillus velezensis* [*B. velezensis* QST 713 ($5.13 \times 10^{10}$ CFU $g^{-1}$) 15.7%; other ingredients 84.2%] and the chemical fungicide prochloraz manganese (Octave® WP, Bayer Crop Science, Germany, content of prochloraz manganese complex 50%; kaolin 35%; and other ingredients 15%) (Table 1).

### Tests in mushroom growing room

Mushroom substrate was provided by the compost producer »Uča & Co.« Vranovo, Smederevo, Serbia. Plastic boxes sized 0.340 × 0.215 × 0.130 m ($l \times w \times h$)

were filled with 1.5 kg of compost mixed with 15 g of grain spawn of *A. bisporus* A15 (Sylvan, Hungária zRt) to prepare 1% spawned substrate. Twelve plastic boxes were used in calculations as 1 $m^2$ of casing surface for treatment. Inoculation of *T. aggressivum* f. *europaeum* T77 was performed with the culture grown on PDA at 25°C for three days. Mycelia of the pathogen was scraped from the surface of PDA plates, mixed with water and Tween 20 (v/v 0.01%) (REANAL Finomvegyszergyár Rt., Hungary, No.: 805383) and filtered through sterile gauze. Spore concentration was determined by counting on a hemocytometer and the suspension was diluted to achieve the final concentration of $10^6$ conidia $ml^{-1}$. Inoculation of *T. aggressivum* f. *europaeum* T77 was performed two days after spawned compost was placed into boxes, by pipetting spore suspension ($10^6$ conidia per $m^2$) down the inner walls of each box. The boxes were incubated at 25°C (spawn-run) for 18 days. Compost was cased with 1.3 kg of black peat casing soil Terahum (Treset d.o.o., Veliko Gradište, Serbia), amended with limestone (1.4%, Tara, Dobanovci, Serbia) and disinfected with peracetic acid 0.02% (Peral-S 15%, Vetprom, Belgrade, Serbia), 90 ml per $m^2$ of casing. Soil was cased in a 50 mm layer and incubated at 22°C for 8 days (case-run). The day of casing was regarded as day one. The next seven days air temeperature was reduced in stages to 17°C. The fungicide prochloraz manganese was applied at the standard product application rate of 0.6 g of active ingredient (a.i.) in 1.8 l $H_2O$ per 1 $m^2$ of casing surface on the fourth day after casing. The biofungicide *B. subtilis* Ch-13 was used in three different doses: 10 ml ($1 \times 10^8$ CFU), 20 ml ($2 \times 10^8$ CFU) and 30 ml ($3 \times 10^8$ CFU), each volume diluted in 1 l of water and applied per $m^2$ of casing surface. The biofungicide *B. velezenzis* QST713 was also used in three different doses: 0.1 g ($5.13 \times 10^9$ CFU), 0.2 g ($1.03 \times 10^{10}$ CFU), and 3 g ($1.5 \times 10^{11}$ CFU), each volume diluted in 1 l of water and applied per $m^2$ of casing surface. Application doses of the two biofungicides were chosen based on their recomended doses. Also, it was not possible to apply the same dose of each biofungicide due to their different product formulation. Both biofungicides based on *Bacillus* spp. were applied on the second day after casing. Treatments with both biofungicides and the fungicide prochloraz manganese were repeated after the first flush, approximately 22 days after casing. All treatments were applied by spraying corresponding water suspensions on mushroom bed areas prepared for six plots, i.e. a total area of 0.5 $m^2$. The trial consisted of two groups, uninoculated plots and those inoculated with *T. aggressivum* f. *europaeum* T77. Control plots within both groups were sprayed with tap water.

The plots were arranged in a completely random design with six replicates per treatment. The experiment was repeated twice and average values from both repetitions were computed. 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. with and without symptoms of green mould disease. The effect of fungicides on mushroom productivity was evaluated by calculating biological efficacy (BE) as the ratio of fresh weight of total fruiting body yield and weight of dry spawned substrate, according to Chrysayi-Tokousbalides et al. (2007), and expressed as %:

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

Fungicide effectiveness was calculated by Abbott's formula (Abbott, 1925):

% effectiveness = [(Ic -It)/Ic] × 100

where Ic - disease incidence in inoculated control; It - disease incidence in treated samples (Gea et al., 2010). Disease incidence was recorded as a percentage of fruiting bodies with symptoms compared with those without symptoms.

## Statistical analysis

Data were examined using the one-way analysis of variance (ANOVA), including comparison of means by F-test. The test was used to compare the significance

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

| Trade name | Active ingredient | Concentration of active ingredient | Manufacturer |
|---|---|---|---|
| Octave® WP | Prochloraz manganese | 500 mg $l^{-1}$ | Bayer, Germany |
| Serenade® WP | *Bacillus velezensis* QST 713 | 15.7% ($5.13 \cdot 10^{10}$ CFU $g^{-1}$) | AgraQuest, Canada |
| Ekstrasol F SC | *Bacillus subtilis* Ch-13 | $1 \times 10^7$ CFU $ml^{-1}$ | BioGenesis d.o.o., Serbia |

of differences among data on the average biological efficacy and effectiveness of different bio/fungicide treatments against *T. aggressivum* f. *europaeum* T77 in the mushroom growing room. 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

Brown spots of a few millimeters were found on *A. bisporus* fruiting bodies in plots inoculated with *T. aggressivum* f. *europaeum* T77 16 days after casing. Larger spots and necrotic lesions of a few centimeters were found three days later. Small emerald green colonies, a few centimeters in diameter, were noted on the casing surface 28 days after casing. A few days later, colonies became larger as reported before (Milijašević-Marčić et al., 2017).

Comparison of the two microbial biofungicides was very difficult because the commercially available products had different formulations and concentrations of active ingredients, i.e. *B. subtilis* Ch-13 was formulated as a suspension concentrate $1 \times 10^7$ CFU $ml^{-1}$ and *B. velezensis* QST713 was formulated as a wettable powder $5.13 \times 10^{10}$ CFU $g^{-1}$ (Table 1). Also, it was not possible to apply these two biofungicides at the same dose because it would differ considerably from their recomended doses. Therefore, the tested doses of *B. subtilis* Ch-13 suspension were 10, 20 and 30 ml per $m^2$ of casing soil, to obtain concentrations of 1, 2, and $3 \times 10^8$ CFU per $m^2$. The first two doses of *B. velezensis* QST713 were 0.1 and 0.2 g per $m^2$ to obtain lower concentrations

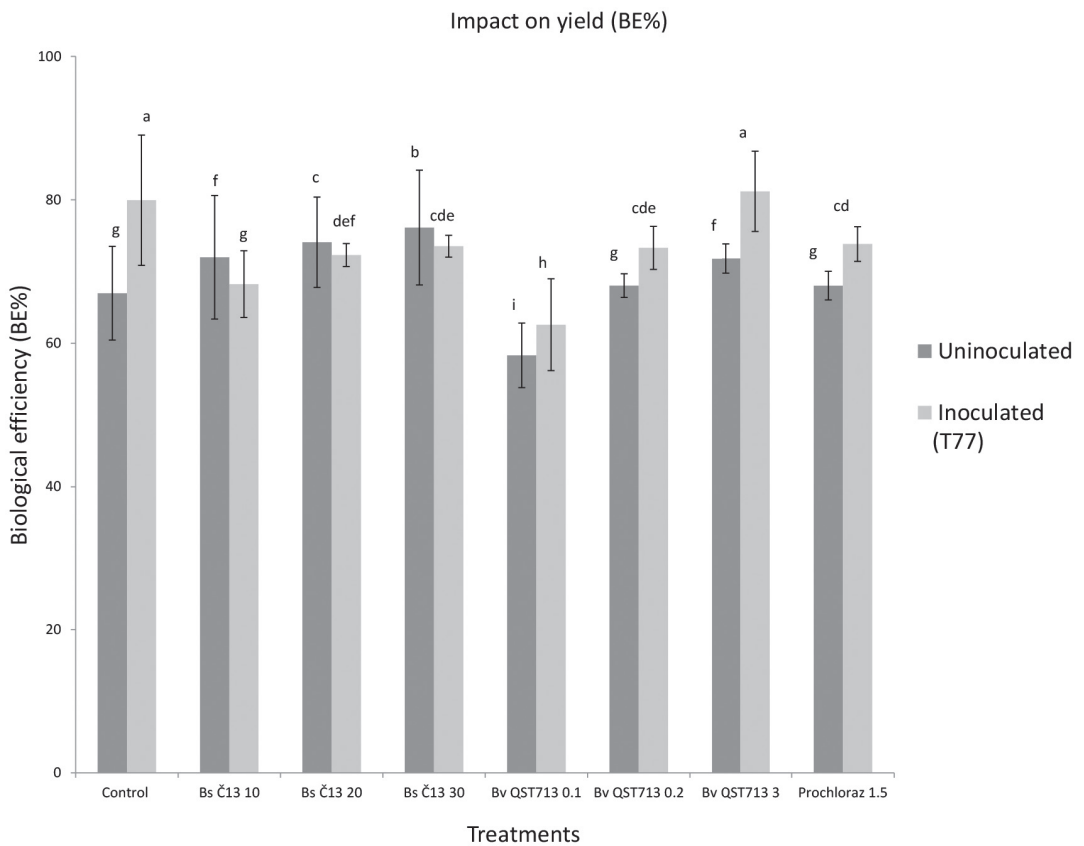


**Figure 1.** Impact on mushroom yield of different bio/fungicides in *in vivo* assays with *Trichoderma aggressivum* f. *euroapeum* T77 on *Agaricus bisporus*. Data are means of six replicates in two trials ± SE, standard error of means; BE% - Biological efficiency = ratio of fresh weight of total mushroom yield and weight of dry spawned substrate; data are means of six replicates in two trials ± SE, SEDs, standard error of differences=32; df, degree of freedom=15; *F*=102.6; *P*-value=0.001. Values within series marked with same letters are not significantly different according to F test (P<0.05).

($5 \times 10^9$ and $1 \times 10^{10}$ CFU per $m^2$) for comparison with the other biofungicide as the available product based on *B. subtilis* Ch-13 could not be more concentrated. The third tested dose of *B. velezensis* QST713 was 3 g per $m^2$ of casing soil as its standard application rate analogous to the concentration of $1.5 \times 10^{11}$ CFU per $m^2$.

The impact on yield in all treatment plots is presented in Figure 1. All three *B. subtilis* Ch-13 treatments showed higher mushroom production in uninoculated plots than in plots inoculated with *T. aggressivum*. Conversely, inoculated plots treated with prochloraz manganese and *B. velezensis* QST713 (at all test doses), as well as the untreated inoculated control, produced higher total yields than the corresponding uninoculated plots. This complies with a previous assumption that the pathogen *T. aggressivum* could enhance the growth and fructification of *A. bisporus* (Mumpuni et al., 1998). It has been noted that the presence of vegetative mycelium is necessary for intensive sporulation of the pathogen (Mamoun et al., 2000). In addition, Mumpuni et al. (1998) suggested the existence of mutual impact of the pathogen and the host. Particularly, the stimulation of *Trichoderma* by metabolites produced by *A. bisporus* and a relatively low level of inhibition of *A. bisporus* by the pathogen facilitates colonization of compost by both fungi. However, as compost colonization reaches its maximum, a change in the competitive balance in favor of *T. aggressivum* f. *europaeum* results in the inhibition of fruiting body production by *A. bisporus* and supports devastating green mould epidemics affecting mushroom production. It is interesting that only *B. subtilis* Ch-13 treatments suppressed that effect of *T. aggressivum*. Hence, the highest yield was found in both inoculated control plots and *B. velezensis* QST treatment at $1.5 \times 10^{11}$ CFU per $m^2$ (standard product application rate). It is noteworthy that the next highest mushroom production was found in uninoculated plots treated with *B. subtilis* Ch-13 at $3 \times 10^8$ CFU per $m^2$ (30 ml per $m^2$), thus enhancing mushroom yield 12% compared to uninoculated control, although a much lower concentration was applied in that treatment than in *B. velezensis* QST713 treatment. Uninoculated plots treated with the biofungicide *B. subtilis* Ch-13 in all tested doses (1, 2, and $3 \times 10^8$ CFU per $m^2$) had higher yields than the uninoculated untreated control. On the other hand, plots treated with *B. velezensis* QST713 applied at the least dose of 0.1 g per $m^2$ ($5 \times 10^9$ CFU per $m^2$) had a significantly lower yield than the uninoculated

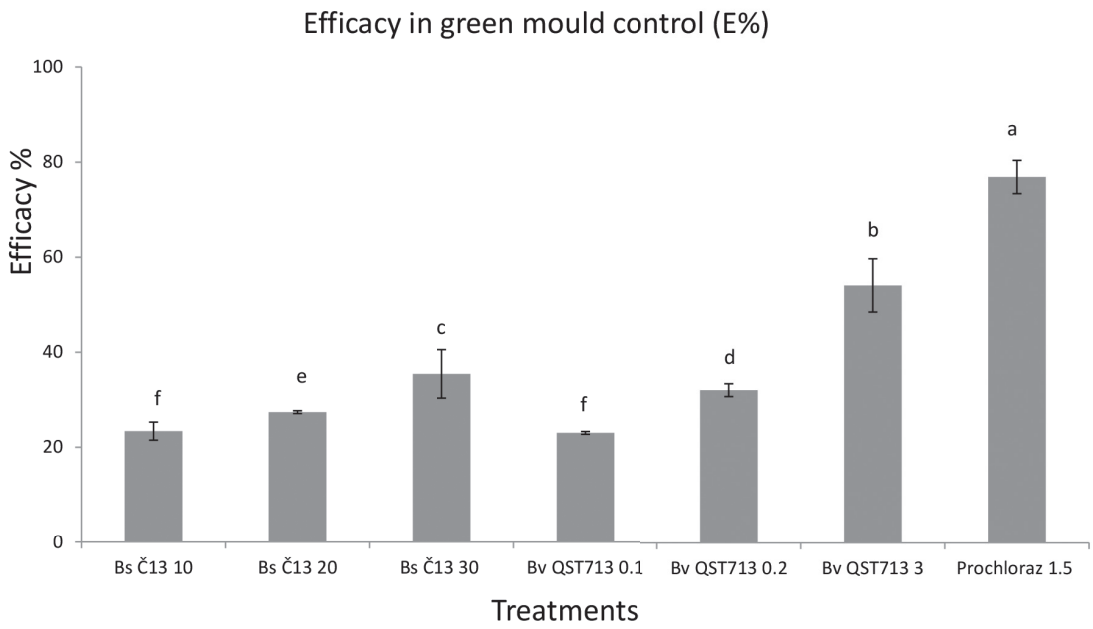


**Figure 2.** *In vivo* efficacy of bio/fungicides in the control of *Trichoderma aggressivum* f. *europaeum* T77 of *Agaricus bisporus*; fungicide efficacy % = [(Ic-It)/Ic] × 100, Ic – disease incidence in inoculated control, It – disease incidence in treated plots; data are means of six replicates in two trials ± SE, standard error of means; SEDs, standard error of differences = 14; df, degree of freedom = 6; $F = 1186.1$; $P$-value = 0.001. Values within series marked with same letters are not significantly different according to F test ($P<0.05$).

untreated control. Again, the ability of *B. subtilis* Ch-13 to enhance the yield of *A. bisporus* was much better compared to the higher concentration of *B. velezensis* QST713. Moreover, uninoculated plots treated with all doses of the biofungicide *B. subtilis* Ch-13 showed higher yields than plots treated with the fungicide prochloraz manganese, while plots treated with *B. velezensis* QST713 had higher yield than those treated with the chemical fungicide only when the highest dose was applied. All three tested doses of *B. subtilis* Ch-13 and the two highest of *B. velezensis* QST713 improved mushroom yield to a level corresponding to yield reported in the previous study of Milijašević-Marčić et al. (2017). The lowest dose of *B. velezensis* QST713 ($5 \times 10^9$ CFU per $m^2$) did not enhance mushroom production, which is consistent with results reported from a study by Kosanović et al. (2013), where *B. velezensis* QST713 was tested at a dose of $8 \times 10^9$ CFU per $m^2$. Control plots inoculated with *T. aggressivum* exhibited higher *A. bisporus* production than control plots without the pathogen in two trials out of four conducted by Potočnik et al. (2018). In addition, Milijašević-Marčić et al. (2017) reported the highest yield in inoculated plots treated with both QST713 strain and prochloraz manganese in comparison with the matching plots without the pathogen.

The highest efficacy against *T. aggressivum* f. *europaeum* was achieved using the fungicide prochloraz manganese (76.87%) (Figure 2). Similar efficacy of prochloraz had also been found in a previous study by Potočnik et al. (2018) (70.4%), where *T. aggressivum* f. *europaeum* ($10^4$ conidia per $m^2$) was inoculated 10 days after spawning by pouring conidial suspension in holes in the compost. In addition, the results of this study revealed a higher efficacy of prochloraz manganese compared to the similar study of Milijašević-Marčić et al. (2017) (49.4%). These differences may be attributed to different inoculation timing and application rates of the fungicide. In the previous study conducted by Milijašević-Marčić et al. (2017) *T. aggressivum* was added to the surface of compost one day after spawning, and the application rate of prochloraz manganese was 2.4 g per $m^2$, while inoculation in the current investigation was conducted two days after spawned compost was placed into plastic boxes and the standard application rate of the fungicide was 3 g per $m^2$. Those findings have demonstrated that infestation of compost with *T. aggressivum* f. *europaeum* at spawning is significantly more devastating than later inoculation. The second highest efficacy was noted for treatment with *B. velezensis* QST713 at standard application rate ($1.5 \times 10^{11}$ CFU per $m^2$) (54.08%). It is noteworthy that *B. subtilis* Ch-13 applied at the concentration of $3 \times 10^8$ CFU per $m^2$ showed better efficacy (35.45%) than *B. velezensis* QST713 (32.05%) applied at the higher concentration ($10^{10}$ CFU per $m^2$). Also, *B. subtilis* Ch-13 applied at the concentration of $2 \times 10^8$ CFU per $m^2$ showed a significantly higher efficacy (27.4%) than *B. velezensis* QST713 at $5 \times 10^9$ CFU per $m^2$ (23.03%). The results with *B. subtilis* Ch-13 tested doses suggested that it could be applied at a much lower concentration than *B. velezensis* QST713 to achieve satisfactory efficacy against *T. aggressivum*. An explanation of better *B. subtilis* Ch-13 characteristics, including both mushroom yield promotion and suppression of the pathogen, compared with *B. velezensis* QST713, could be in faster activation of useful bacteria in suspension concentrate and/or in their own features. Further investigation of different modes of application of *B. subtilis* Ch-13 is recommended as it showed beneficial features in both promoting *A. bisporus* production and suppression of growth of the aggressive compost pathogen *T. aggressivum,* the causal agent of devastating green mould disease.

## CONCLUSION

The biofungicide based on *B. subtilis* Ch-13 showed the highest positive impact on mushroom production. The highest yield in uninoculated plots was found after treatment with *B. subtilis* Ch-13 at $10^8$ CFU per $m^2$, higher than after *B. velezensis* QST713 treatment with its higher concentration ($1.5 \times 10^{11}$ CFU per $m^2$). The significantly better ability of *B. subtilis* Ch-13 (applied at $10^8$ CFU per $m^2$) to enhance *A. bisporus* yield was obvious as it exceeded the yield in uninoculated control, compared to the higher concentration of *B. velezensis* QST713 of $5 \times 10^9$ CFU per $m^2$, which negatively affected the yield. *B. subtilis* Ch-13 may be assumed to suppress the effect of *T. aggressivum* on mushroom yield, and significantly enhance mushroom production (12 %). The fungicide prochloraz manganese may be expected to have the highest efficacy in green mould control, and *B. velezensis* QST713 to follow it with its standard application concentration of $1.5 \times 10^{11}$ CFU per $m^2$. However, it is interesting that *B. subtilis* Ch-13, although applied at lower concentration ($3 \times 10^8$ CFU per $m^2$), demonstrated better efficacy than *B. velezensis* QST713 applied at concentrations of $5 \times 10^9$ and $10^{10}$ CFU per $m^2$. Different modes and timing of application of the biofungicide based on *B. subtilis* Ch-13 should be further investigated to obtain both better yield and efficacy of disease control.

## ACKNOWLEDGEMENT

This study received funding from the Ministry of Education and Science of the Republic of Serbia as part of Grant TR 31043.

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# Uticaj treatiranja pokrivke biofungicidom na bazi *Bacillus subtilis* Ch-13 na suzbijanje zelene plesni i prinos šampinjona

**REZIME**

Biofungicid na bazi *Bacillus subtilis* Ch-13 odabran je za procenu uticaja na prinos šampinjona i efikasnost u suzbijanju *Trichoderma aggressivum* f. *europaeum* T77 iz Srbije u poređenju sa sličnim mikrobiološkim fungicidom na bazi *Bacillus velezensis* QST713 i fungicidom prohloraz manganom. Biofungicid *B. velezensis* QST713 je registrovan u šampinjonima i drugim usevima u mnogim državama, ali nije dostupan na tržištu Sribje. Testirani *B. subtilis* Ch-13 je povećao prinos šampinjona 12% u poređenju sa neinokulisanom kontrolom i u značajno većoj meri od *B. velezensis* QST713 primenjenog u većim koncentracijama. U određivanju efikasnosti biofungicida u suzbijanju kompostnog patogena *T. aggressivum* f. *europaeum*, *B. subtilis* Ch-13 primenjen u koncentraciji $3 \times 10^8$ CFU po $m^2$, ispoljio je veću efikasnost od *B. velezensis* QST713 primenjenog u većim koncentracijama ($5 \times 10^9$ i $1 \times 10^{10}$ CFU po $m^2$). Biofungicid na bazi *B. subtilis* Ch-13 bi trebalo dalje testirati i proučiti različite načine njegove primene da bi se uspostavila veća efikasnost u suzbijanju patogena jer je pokazao značajne osobine u pospešivanju prinosa *A. bisporus* i zaštiti od agresivnog patogena iz komposta *T. aggressivum*, prouzrokovača zelene plesni šampinjona.

**Ključne reči**: šampinjon; *Trichoderma aggressivum*; *Bacillus subtilis*; biofungicidi