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Original scientific paper

Improvement of procedure for casing treatment with a *Bacillus subtilis* Ch-13-based biofungicide to control green mould disease of mushrooms

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SUMMARY

A previous study had confirmed good selective features of a biofungicide based on *Bacillus subtilis* Ch-13 regarding green mould disease control and increase in white button mushroom (*Agaricus bisporus*) production. The recommended application rate of the biofungicide in three split doses (30 + 15 + 15 ml m^{-2}) enhanced mushroom yield by 8.41% and control of green mould disease (*Trichoderma aggressivum*) by 53.57%. A different application procedure of the biofungicide *B. subtilis* Ch-13 was then further investigated, involving six split doses (6 × 10 ml m^{-2}) of the same total dose of 60 ml which was previously tested in three split doses. The impact of the biofungicide on fruiting body yield and its effectiveness in disease control were evaluated on white button mushroom artificially infected with *Trichoderma aggressivum* f. *europaeum* in the experimental mushroom growing room (*in vivo*). No statistically significant differences in disease control efficacy were found between the fungicide prochloraz (71.08%) and the biofungicide applied either in six split doses (63.05%) or three (58.43%). As for the fungicide/biofungicide positive impact on mushroom production, no statistically significant differences were detected among treatments. The *B. subtilis* Ch-13-based biofungicide may be applied in three split doses, as well as in six split applications, depending on the watering or picking schedule during mushroom cultivation, as both schemes showed satisfactory efficacy in disease control and positive effects on mushroom yield.

Keywords: *Bacillus subtilis*; *Trichoderma aggressivum*; mushroom; biofungicide

INTRODUCTION

Many studies have been dedicated to the control of green mould disease (*Trichoderma aggressivum* Samuels & W. Gams), which causes considerable losses in yields of *Agaricus bisporus* L. both in Serbia and worldwide (Seaby, 1996; Samuels et al., 2002; Kosanović et al., 2013; O'Brien et al., 2017). In some countries, the use of certain fungicides in white button mushroom farms has been officially recommended. Thus, thiabendazole

is commonly used in Australia, South Africa and North America, chlorothalonil in France, Poland, and North America (Chakwiya et al., 2015; Grogan & Gaze, 2000; Carrasco et al., 2017), prochloraz in the EU, Australia and South Africa (Grogan et al., 2000; Gea et al., 2005), while metrafenone is used in France, Spain and the USA (Carrasco et al., 2017; USEPA, 2019). However, some of these fungicides are not registered in Serbia, while thiabendazole and prochloraz have been withdrawn from the Serbian market (Kljajić [Ed.], 2020). Decreased sensitivity of mycopathogens to prochloraz (Grogan et al., 2000; Gea et al., 2005) and chlorothalonil (Beyer & Kremser, 2004) has already been noted. Furthermore, chlorothalonil has shown negative influence on mushroom mycelial growth (Challen & Elliott, 1985; Fletcher, 2002). Disease control in cultivated mushrooms in Serbia thus relies solely on the fungicide metrafenone, which is registered in the EU only against the mushroom pathogens *Cladobotryum mycophilum* (Oudemans) W. Gams and Hooz. (cobweb disease) and *Lecanicillium fungicola* (Preuss) Zare & W. Gams (dry bubble disease) (Luković et al., 2021).

Biofungicides based on the antagonistic *Bacillus* spp. bacteria have also been successfully used for mushroom disease control (Savoie et al. 2001; Védie & Rousseau, 2008). The commercial Canadian strain of *Bacillus amyloliquefaciens* (Ehrenberg) Cohn QST713, registered against many plant pathogens and mycopathogens in the EU and worldwide (Pandin et al., 2018; Potočnik et al., 2018), was not accessible on the Serbian market in the past. The preceding study confirmed good selective features of a biofungicide based on a Russian strain of *Bacillus subtilis* (Ehrenberg) Cohn, named Ch-13, which has been introduced in Serbia recently for control of green mould disease and increase in white button mushroom production (Potočnik et al., 2019). The recommended biofungicide application rate of three split doses (30 + 15 +15 ml m^{-2}) was found to enhance mushroom yield by 8.41%, and achieved effective inhibition of *T. aggressivum* of 53.57% (Potočnik et al., 2019).

The biofungicide *B. subtilis* Ch-13 was further investigated by testing a different application procedure consisting of six split doses (6 × 10 ml m^{-2}) of the same total dose of 60 ml per m^2 as previously tested in three split applications. The impact on yield and effectiveness in green mould disease control by the biofungicide were evaluated on white button mushroom using artificial infection with *Trichoderma aggressivum* f. *europaeum* Samuels & W. Gams in the experimental mushroom growing room (*in vivo*).

MATERIAL AND METHODS

Fungal inoculum

The pathogenic fungus *T. aggressivum* f. *europaeum* T77, deposited in a culture collection of the Institute of Pesticides and Environmental Protection, Belgrade, Serbia, was maintained on potato dextrose agar (PDA) medium (fresh-peeled potatoes; dextrose, Torlak, Serbia; agar, Torlak, Serbia) at 4°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%), (REANAL Finomvegyszergyár Rt., Hungary, No.: 805383) and then filtrated through double layers of cheesecloth. Spore concentration was determined by counting on a hemocytometer and the suspension was diluted to achieve the final concentration of 1×10^6 conidia ml^{-1}.

Antifungal agents

The efficiency of the new application mode of Ekstrasol F SC (BioGenesis d.o.o., Belgrade, Serbia), a biofungicide based on *Bacillus subtilis* Ch-13 (1×10^8 CFU ml^{-1}), against *T. aggressivum* f. *europaeum* T77 was tested in a mushroom growing room by treating the casing soil laid over mushroom substrate. Two application rates of the biofungicide were compared. Biological efficiency (impact on yield) of the biofungicide and its effectiveness against the pathogen were evaluated by comparison with the commercial fungicide prochloraz (Table 1).

Test in the 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 a biofungicide based on *B. subtilis* Ch-13 and a commercial prochloraz-based chemical fungicide. Mushroom substrate was provided by the compost producer Uča d.o.o. Vranovo, Smederevo, Serbia. Plastic boxes sized 0.340 x 0.215 x 0.130 m (*l* x *w* x *h*) contained 1.5 kg of compost mixed with 15 g of grain spawn of *A.bisporus* A15 (Sylvan, Hungária zRt) for preparing 1% spawned substrate. Soil surface in 12 plastic boxes represented a plot of 1 m^2 for treatment calculation. Inoculation with *T. aggressivum* f. *europaeum* T77 was performed two days after the spawned compost was placed into boxes by pipetting 1 ml of spore suspension and 9 ml of tap water (10^6 conidia ml^{-1} per m^2) down the inner wall

of each box. The boxes with substrates 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. Casing soil was poured out in 50 mm layers and incubated at 22°C for 8 days (case-run). The day of casing was regarded as day one. Over the following seven days, air temeperature was reduced in stages to 17°C. The fungicide prochloraz was applied at standard product application rate in two split doses: 1.5 ml of product in 1.8 l H_2O per 1 m^2 of casing soil surface on the fourth day after casing and another 1.5 ml m^{-2} after the first flush, 22 days after casing. The biofungicide *B. subtilis* Ch-13 was applied in two different application procedures, using the same total dose of 60 ml m^{-2} split in either three or six doses: *a*) 30 ml m^{-2} (on the second day after casing) + 15 ml m^{-2} (two weeks after casing) + 15 ml m^{-2} (after the first flush, 22 days after casing); *b*) 10 ml m^{-2} (on the second day after casing) + 5 × 10 ml m^{-2} (in seven days intervals). All treatments were applied by spraying the appropriate water suspensions onto mushroom bed areas prepared for six plots, i.e. a total area of 0.5 m^2, in a completely random design with six replicates per treatment. The trial consisted of two groups, uninoculated plots and plots inoculated with *T. aggressivum* f. *europaeum* T77. Control plots within both groups were sprayed with tap water.

The mushroom fruiting bodies were hand-picked in three successive production flushes: the first from day 16 to 28 after casing time, the second from day 30 to 42, and the third from day 43 to 56. 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. The effect of fungicides on mushroom productivity was evaluated as biological efficacy (BE) (Chrysayi-Tokousbalides et al., 2007), calculated as the ratio of fresh weight of total fruiting body yield and the weight of dry spawned substrate, and expressed as %:

$$BE = (\text{fresh total fruiting body yield} / \text{dry spawned substrate mass}) \times 100$$

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 the percentage of fruiting bodies with symptoms compared to those without symptoms.

Statistical analyses

Data were examined using the one-way analysis of variance (ANOVA), including the comparison of means by *F*-test. The test was used to compare the significance of differences among data on the average biological efficacy and effectiveness of different biofungicide/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 by the software Statistica for Windows 6.0 (Stat Soft Italia, 1997).

RESULTS AND DISCUSSION

The first spot symptoms were noticed on *A. bisporus* caps on day 16 after casing time, and it was in plots inoculated with *T. aggressivum* f. *europaeum* T77. A few days later, larger necrotic lesions were noted. Dark green colonies, a few centimeters in diameter, appeared on the casing soil surface 28 days after casing, corresponding to those described by Milijašević-Marčić et al. (2017).

The effectiveness of biofungicide/fungicide in suppression of symptoms of green mould disease is shown in Figure 1. No statistically significant differences were found in disease incidence between the fungicide prochloraz and the biofungicide based on *B. subtilis* Ch-3

Table 1. Fungicide products used in the study

Trade name	Active ingredient	Concentration of active ingredient	Manufacturer
Mirage® EC	Prochloraz	450 ml l^{-1}	ADAMA Agricultural Solutions UK Ltd., UK
Ekstrasol F SC	*Bacillus subtilis* Ch-13	1 × 10^8 CFU ml^{-1}	BioGenesis d.o.o., Serbia

applied either three or six times in the same total dose of bacterial units. The effectiveness in disease control was presented in two ways: by setting the standard fungicide prochloraz (E_{st}) performance as 100%, and also by relating the results to the untreated control (E_k) (Table 2). The biofungicide *B. subtilis* Ch-13, applied in both application procedures of three or six split doses, significantly decreased disease incidence, compared to untreated control, after artificial infection of cultivated mushrooms with *T. aggressivum*. No statistically significant difference was found in the effectiveness of the biofungicide, applied in three (58.43%) or six (63.05%) split doses, and the fungicide prochloraz (71.08%) against *T. aggressivum*. Nevertheless, the biofungicide used in three split applications demonstrated an effectiveness which was 17.8% lower than the standard chemical fungicide, and its six split doses had a 11.3% lower result than the efficacy of prochloraz. In the preceding small-scale experiment, Potočnik et al. (2019) reported that *B. subtilis* Ch-13 achieved the effectiveness of 23% when used in the amount of 10 ml m^{-2}, 27% in 20 ml m^{-2} dose, and 35% in 30 ml m^{-2}, respectively, against *T. aggressivum*. In another large-scale evaluation, Potočnik et al. (2021) found the efficacy of *B. subtilis* Ch-13 dose of 60 ml m^{-2} to be: 46.45% when applied twice (2 × 30 ml m^{-2}), and 53.57% when applied three times (30 + 15 + 15 ml m^{-2}). In the current small-scale study, the biofungicide effectiveness was 58.43% when it was applied three times (30 + 15 + 15 ml m^{-2}), and 63.05% after six split doses (6 × 10 ml m^{-2}) were applied to suppress green mould disease.

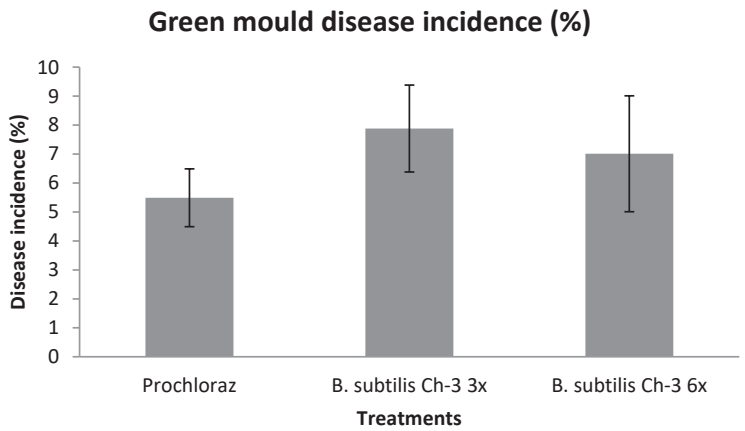

Figure 1. Suppression of disease incidence on *Agaricus bisporus* artificially infected with *Trichoderma aggressivum* by using biofungicide/fungicides in a small-scale assay; data are means of six replicates in experimental plots ± SE, standard error of means; standard error of differences = 14.66; df, degree of freedom = 2; *F* = 0.26; *P*-value = 0.77. Values within series marked with the same letter are not significantly different according to F-test (P<0.05).

Table 2. Effectiveness of biofungicide/fungicides in disease control of *Agaricus bisporus* artificially infected with *Trichoderma aggressivum* in a small-scale assay, compared to a standard fungicide (E_{st}) and untreated control (E_k)

Treatments	Biofungicide/fungicide application rate (ml m^{-2})	E_{st} (%)	E_k (%)	SE
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1}	1 × 30 + 2 × 15	82.20 b	58.43 a	11.91
Bacillus subtilis Ch-13 1 × 10^8 CFU ml^{-1}	6 × 10	88.70 c	63.05 a	22.32
Prochloraz 450 ml a.i. l^{-1}	2 × 1.5	100.00 a	71.08 a	9.76

Data are means of six replicates of experimental plots ± SE, standard error of means; Effectiveness (E)% in disease symptoms control, when standard fungicide effectiveness (E_{st}) is set to 100% or the effectiveness is compared to untreated control (E_k); SE, standard error; SEDs, standard error of differences=14.66; df, degrees of freedom=2; *F*=0.26; *P*-value=0.77. Values with series marked with the same letters are not significantly different according to *F*- test (P<0.05).

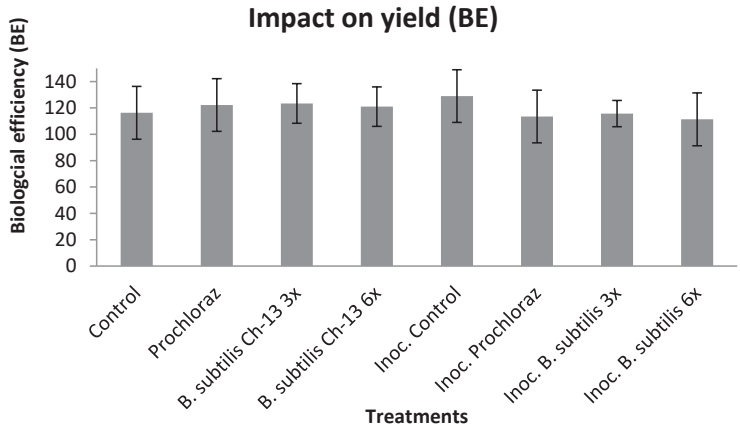

Figure 2. Impact of different biofungicide/fungicides on the yield of *Agaricus bisporus* artificially infected with *Trichoderma aggressivum* in small-scale assays. Data are means of six replicates per experimental plot ± SE, standard error of means; BE% - Biological efficiency = ratio of the fresh weight of total mushroom yield and the weight of dry spawned substrate; SEDs, standard error of differences=1.5; df, degrees of freedom=7; *F*=0.37; *P*-value=0.34. Values with series marked with same letters are not significantly different according to F-test (P<0.05).

No statistically significant difference was noted in increased mushroom yield after using the fungicide prochloraz and the biofungicide *B. subtilis* Ch-13 in three or six split doses, compared with the untreated control (Figure 2). The biofungicide increased mushroom yield 6.11% when it was used three times and 4.04% after six split doses. Inoculation with *T. aggressivum* enhanced yield in inoculated control (10.88%), compared with uninoculated control as recorded before (Potočnik et al., 2019). Mumpuni et al., (1998) noted that the pathogen *T. aggressivum* was able to enhance the growth and fructification of *A. bisporus.* The somatic mycelium of *A. bisporus* was crucial for colonization of the pathogen (Mamoun et al., 2000). The findings confirmed an interrelation between the pathogen and the host (Mumpuni et al., 1998). When compost colonization by the green mould agent reaches its peak, *T. aggressivum* f. *europaeum* suppresses the production of *A. bisporus* fruiting bodies. It is interesting that only *B. subtilis* Ch-13 treatments suppressed the effect of *T. aggressivum* to increase the (infected) mushroom yield as plots inoculated with *T. aggressivum* yielded less than uninoculated plots, correspondingly to our previous findings (Potočnik et al., 2019). The earlier small-scale experiment showed that treatments with *B. subtilis* Ch-13, used at concentrations of 10, 20 and 30 ml m^{-2}, resulted in considerably enhanced mushroom yield (respectively 3.03, 9.09 and 12.12%) in comparison with uninoculated control (Potočnik et al., 2019). In the previous large-scale experiment (Potočnik et al., 2021), the biofungicide *B. subtilis* Ch-13 dose of 60 ml m^{-2} improved yield with its two split doses (30 + 30 ml m^{-2}) by 5.07%, and with three split applications (30 + 15 + 15 ml m^{-2}) by 8.41%. Generally, an uncommonly lower yield than in foregoing investigations was recorded in the current study using both application procedures of the biofungicide dose of 60 ml m^{-2} (6.11 and 4.04%) (Potočnik et al., 2019; 2021). It was perhaps due to lower cultivation temperature during the winter months.

The mode of action of *Bacillus* spp. biofungicides is based on: competition for nutrients, substrate colonization (Chen et al., 2013), antifungal activity of volatile organic metabolites (Stanojević et al., 2016), various antibiotics (dipeptides and cyclic peptides) (Loeffler et al., 1990), iron chelators, and hydrolytic enzymes production (Maarten et al., 2000; Manjula & Podile, 2005). Competition could also be responsible for the inhibition of *T. aggressivum* growth. *B. subtilis* strains are generally recognized as safe (GRAS) organisms to the environment and human health (FDA, 1999). Endospores produced by *Bacillus* species enable their attainable formulation, persistence in the environment and successful application (Cawoy et al., 2011). Evaluation of different application procedures of *B. subtilis* Ch-13 showed benefits of using either three or six split doses (depending of watering and picking schedule during the production cycle), suppressing the spreading of green mould pathogen *T. aggressivum*, and showing positive effects on mushroom yield.

CONCLUSION

Two frequencies of application of *B. subtilis* Ch-13 showed similar benefits, indicating that both could be used in practice, depending on the watering or picking schedule during mushroom cultivation. Symptoms of green mould disease were prevented and mushroom production improved regardless of whether three doses were used: 30 ml m^{-2} (on the second day after casing) + 15 ml m^{-2} (two weeks after casing) + 15 ml m^{-2} (after the first flush, approximately 20-25 days after casing) or six split applications: 10 ml m^{-2} (on the second day after casing time) + 5 × 10 ml m^{-2} (at seven days interval).

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Poboljšanje postupka primene biofugicida na bazi *Bacillus subtilis* Ch-13 u suzbijanju prouzrokovača zelene plesni tretiranjem pokrivke šampinjona

REZIME

Prethodna ispitivanja su potvrdila dobre selektivne osobine biofungicida na bazi *Bacillus subtilis* Ch-13 u suzbijanju prouzrokovača zelene plesni i povećanju prinosa šampinjona (*Agaricus bisporus*). Preporučena primena biofungicida u tri podeljene doze primene (30 + 15 + 15 ml m^{-2}) povećala je prinos šampinjona 8,41 % i ispoljila efiksanost u suzbijanju *Trichoderma aggressivum*, prouzrokovača zelene plesni 57%. Biofungicid na bazi *B. subtilis* Ch-13 je dalje testiran da bi se ispitao drugi način njegove primene, u šest podeljenih doza (6 × 10 ml m^{-2}) u istoj ukupnoj količini od 60 ml kao kod prethodne doze primene u tri tretmana. Uticaj na prinos i efikasnost u suzbijanju prouzrokovača zelene plesni je ispitana veštačkom inokulacijom patogena *Trichoderma aggressivum* f. *europaeum* u oglednom gajilištu šampinjona (*in vivo*). Nisu utvrđene statistički značajne razlike u efikasnosti između fungicida prohloraza (71,08 %), biofungicida primenjenog u šest podeljenih doza (63,05 %) i primenjenog tri puta (58,43 %). U odnosu na uticaj na prinos fungicida i biofungicida, takođe nisu zabeležene statistički značajne razlike u njihovom pozitivnom uticaju. Primena biofungicida na bazi *B. subtilis* Ch-13 u šest podeljenih doza se može preporučiti jer je ispoljio zadovoljavajući učinak u suzbijanju prouzrokovača zelene plesni i nije pokazao negativan uticaj na prinos šampinjona.

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