Impact of *Bacillus subtilis* QST713 mushroom grain spawn treatment on yield and green mould control

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SUMMARY

A biofungicide based on *Bacillus subtilis* QST713 was tested for impact on yield and efficacy against a *Trichoderma aggressivum* f. *europaeum* T77 strain from Serbia by coating mushroom grain spawn and comparing the results with the chemical fungicide prochloraz manganese in a mushroom growing room. The tested *B. subtilis* QST713 strain did not inhibit mycelial growth of *Agaricus bisporus* in plots free of the pathogen, showing an impact on yield of 91.95%, which was not significantly different from an untreated control. As for the efficacy of the fungicides used against *T. aggressivum* f. *europaeum* T77, there were no significant differences between a prochloraz manganese casing treatment, and *B. subtilis* QST713 coating on mushroom grain spawn, as the efficacy was 70.37 and 53.09%, respectively. These results implied that the biofungicide based on *B. subtilis* could serve as a harmless alternative to synthetic fungicides in mushroom production, especially during serious compost green muold outbreaks caused by *T. aggressivum*. Furthermore, the biofungicide should be applied alone because an antagonistic reaction was detected between the fungicide prochloraz and *B. subtilis* QST713.

Keywords: Mushroom; *Bacillus subtilis*; *Trichoderma*; Fungicides

INTRODUCTION

Green mould of mushrooms remains the most devastating disease causing great losses in edible mushroom production. In serious outbreaks, the disease is characterised by fast-growing dark green colonies in the substrate that prevents the production of mushroom frutiting bodies (Seaby, 1996). Its causal agents belong to the soil-inhabiting genus *Trichoderma* (teleomorph *Hypocrea,* Ascomycota, Hypocreales). Of more than 200 analysed species, several have been found to belong to the widely cultivated beneficial micromycetes, plant symbionts or antagonist phytopathogenic fungi and antibiotic and enzyme producers (Druzhinina & Kubicek, 2005). Initially, green mould appeared simultaneously in 1985/86 in the British Isles (Doyle, 1991) and North America (Rinker, 1993), and spread rapidly to other parts of Europe and other continents (Geels, 1997; Hermosa et al., 1999; Mamoun et al., 2000; Hatvani et al., 2007; Romero-Arenas et al., 2009; Clift & Shamshad, 2009; Hatvani et al., 2012; Kosanović et al., 2013). Aggressive biotypes were discribed as

Trichoderma aggressivum f. *aggresivum* in North America and *T. aggressivum* f. *europaeum* Samuels & W. Gams in Europe (Samuels et al., 2002), and they were phylogenetically closely related to *T. harzianum* Rifai and emerged through population adaptation to environmenal conditions (Kredics et al., 2010).

A regular method of pathogen control on mushroom farms worldwide is the application of various fungicides. Only a few fungicides have been officially recommended in mushroom production, prochloraz and metraphenone in Europe and worldwide, and chlorothalonil, thiabendazol and tiophanate-methyl in North America (Grogan & Gaze, 2000). Fungicides are usually applied to mushroom casing, but the introduced compost pathogen *T. aggressivum* has made disease control much more complicated. Benzimidazole and prochloraz were the first fungicides applied to compost, but they could not stop *T. aggressivum* (Abosriwil & Clancy, 2004). Fungicide application to grain spawn was found to provide better control of green mould colonization than compost treatment with the fungicide. It seems that mushroom grain spawn alone provides an available carbohydrate source from which compost colonization of green mould can proceed and be established (Grogan & Fletcher, 1993). However, *Trichoderma* growth was not supported in unspawned compost. After thorough investigation of mushroom grain spawn treated with benzimidazoles, benomyl and carbendazim, these fungicides received registration as spawn grain treatments in England and Pennsylvania, USA (Grogan et al., 1996). In addition, Rinker and Alm (1997a, b) found that green mould management is possible only by unifrom coating of spawn grains with a fungicide. However, its effectiveness may be reduced when supplements are mixed into the compost at spawning. Considering that only 1.15 g of fungicide is needed to treat the spawn for 1 tonne of compost, spawn treatment represents a more economical use of the fungicide, compared to compost treatment, which would require 70 g of product per tonne of compost. Over time, *Trichoderma* spp. have developed resistance to benzimidazoles (Romaine et al., 2005; Grogan, 2008). Also, fungicides such as benomyl, carbendazim and prochloraz have shown to be susceptible to microbial degradation in soils (Fletcher et al., 1980; Yarden et al., 1990). However, none of these fungicides is currently approved for spawn treatment. Registered fungicides are applied at a much later stage of crop cycle, on casing soil, when disease outbreaks are more likely.

As fungicides may also reduce the mycelial growth of *A. bisporus*, there must be a balance between the benefit from limitation of undesirable contaminants and a possible reduced vigor of a mushroom crop. Furthermore, many chemicals are no longer approved for use. In addition, there is an increased demand for reduced pesticide use and growers will increasingly have to depend on other measures to control outbreaks rather than resort to chemicals (Grogan, 2008). An alternative to chemical control of *Trichoderma* green mould is the application of microorganisms as biocontrol agents. Savoie et al. (2001) reported inhibitory effects of *Bacillus* species to *T. aggressivum* growth. Furthermore, Védie & Rousseau (2008) reported the effectiveness of lactonase-producing bacteria, *Bacillus subtilis* (Ehrenberg) Cohn, in preventing green mould disease, revealing data on the application rates but providing neither results of biofungicide efficacy in green mould control nor its impact on mushroom yield. The most studied *Bacillus subtilis* strain used as a biofungicide, the QST713, has been recently renamed as *Bacillus velezensis* (Pandin et al., 2018a,b). This biocontrol strain was chosen for tests of biological efficiency and efficacy against Serbia-originating *T. aggressivum* f. *europaeum* by coating mushroom grain spawn, in comparison with the fungicide prochloraz manganese in the mushroom growing room.

MATERIAL AND METHODS

Fungal species and culture conditions

The pathogenic fungus used in this study was obtained from a culture collection of the Institute of Pesticides and Environmental Protection. The isolate *Trichoderma aggressivum* f. *europaeum* T77 was obtained from mushroom compost in the Serbian mushroom farm Lisovići in 2010. The fungus was identified based on morpho-physiological characteristics and ITS1/ITS4 sequence analyses (Kosanović et al., 2013). Fungal cultures were maintained on potato dextrose agar (PDA) at 4°C. For inoculum preparation, agar discs of fungal isolates were grown on PDA for three days at 22°C. In preparation for *in vivo* trials, conidia from three-day old cultures of both fungi were flooded with 10 ml of sterile distilled water and Tween 20 (v/v 0.01%), followed by filtration through double layers of cheesecloth.

Antifungal agents

The biofungicide Serenade® WP (AgraQuest, Canada) based on *Bacillus subtilis* (*B. subtilis* QST 713 [$5.13 \cdot 10^{10}$ CFU/g] 15.7%; other ingredients 84.2%) was tested as a potential antifungal agent for treatment of spawn grain of *A. bisporus* Sylvan A15 strain against the isolate

of *T. aggressivum* f. *eurpaeum* T77 in the mushroom growing room. The biological efficacy and effectiveness of the biofungicide was evaluated by comparison with the commercial 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

Plastic bags sized 0.60 x 0.4 x 0.25 m (*l* x *w* x *h*) with 18 kg of compost (Uča & Co. Vranovo, Smederevo, Serbia) spawned with *A.bisporus* A15 (Sylvan, Hungária zRt) were incubated at 24ºC for 18 days. Compost surface of each bag was divided by a wooden barrier into two equal sections, making total area of each experimental plot to be 0.12 m². Compost was cased with 50 mm layer of black peat casing soil Terahum (Treset d.o.o., Veliko Gradište, Serbia), disinfected with peracetic acid 0.02% (Peral-S 15%, Vetprom, Belgrade, Serbia), 90 ml per m² of casing, and incubated at 21ºC for 8 days (case-run), and after that air tememperature was reduced to 16ºC. The casing soil was drenched with the tested fungicide prochloraz manganese by split application on the fourth and twenty-third day after casing (after the first flush). Ten days after spawning, the tested plots were inoculated using conidial suspension of the pathogen at a concentration of 10^4 conidia/ml per m² of casing soil by pouring 125 ml in holes of 2.5 cm depth in the middle of the experiental plot surface (upper side). Spawn grain of *A. bisporus* A15 were coated using doses for one bag of mushroom compost of 18 kg: 252 g spawn grain *A. bisporus*, 15 g gypsum, 1.5 g of *B. subtilis* QST713. Spawn, gypsum and *B. subtilis* were thoroughly mixed before spawning. The fungicide prochloraz manganese was applied at the standard product application rate: 0.6 g of active ingredient (a.i.) in 1.8 l H_2O per 1 m² of casing surface. The treatments were: *(1)* compost + *A. bisporus* spawn (control); *(2)* compost + *A. bisporus* spawn grain + biofugicide *B. subtilis* QST 713; *(3)* compost + *A. bisporus* spawn grain + *T. aggressivum* f. *europaeum* inoculum; *(4)* compost + *A. bisporus* spawn grain + biofugicide *B. subilis* QST 713 + *T. aggressivum* f. *europaeum* inoculum; *(5)* compost + *A. bisporus* spawn grain + prochloraz manganese; *(6)* compost + *A.*

bisporus spawn grain + biofungicide *B. subtilis* QST713 + prochloraz manganese; *(7)* compost + *A. bisporus* spawn grain + biofungicide *B. subtilis* QST713 + prochloraz manganese + *T. aggressivum* f. *europaeum* inoculum; *(8)* compost + *A. bisporus* spawn grain + prochloraz-Mn + *T. aggressivum* f. *europaeum* inoculum.

Inoculated and uninoculated bags without fungicide treatments were used as controls. The plots were arranged in a completely randomized block design with four replicates per treatment. The fruiting bodies were hand-picked in three successive production flushes: the first from day 14 to 23 after casing, the second from day 24 to 34, and the third from day 35 to 45. 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 as biological efficacy (BE), calculated as the ratio of fresh weight of total fruiting body yield and the weight of dry spawned substrate, and expressed as %:

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

(Chrysayi-Tokousbalides et al., 2007). Fungicide effectiveness was calculated by Abbott's formula:

$$\% \text{ effectiveness} = [(Ic - It)/Ic] \cdot 100$$

where Ic - disease incidence in the 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 analyses

Data were examined using one-way analysis of variance (ANOVA), including comparisons of means by F-test. The test was used to compare the significance of differences in 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 using the software Statistica for Windows 6.0 (Stat Soft Italia, 1997).

Table 1. 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 subtilis* QST 713	15.7% (5.13×10^{10} CFU g^{-1})	AgraQuest, Canada

RESULTS AND DISCUSSION

Small 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 measuring a few centimeters were observed several days later. Small white colonies were noted on the casing surface 27 days after casing. A few days later, the colonies become emerald green after profuse sporulation.

The tested *B. subtilis* QST713 did not inhibit the growth of *A. bisporus* mycelia in plots without the pathogen, which is consistent with the results of experiments conducted by Milijašević-Marčić et al. (2017) in button mushroom, and Nagy et al. (2012) in oyster mushroom (*Pleurotus ostreatus*). Conversely, Kim et al. (2008) reported slightly inhibited mycelial growth of edible mushrooms, enoki (*Flammulina velutipes*), shiitake (*Lentinula edodes*) and oyster mushrooms in Korea, as did Kosanović et al. (2013) in *A. bisporus*, too.

The productivity of mushroom fruiting bodies was the highest in two controls, inoculated and uninoculated, and in the uninoculated plot treated with prochloraz manganese (Table 2). There were no significant differences between those plots and the rest of treatments, except the inoculated plots treated with *B. subtilis* QST713 which had the lowest yield in the experiment. Furthermore, there were no significant differences between the previous and all other treatments with *B. subtilis* QST713, and those inoculated and treated with prochloraz manganese. Both Kosanović et al. (2013) and Milijašević-Marčić et al. (2017) also reported the least yield from inoculated *B. subtilis* treatments, i.e. different strains applied on casing soil, QST713 and B-38, although all treatments had lower BE in these experiments, perhaps because of the lower *A. bisporus* inoculation (0.7%) in both previous experiments, whereas it was double in our trial, i.e. we used 1.4% mycelia of *A. bisporus* spawning in the compost. Milijašević-Marčić et al. (2017) noted the highest yield in uninoculated control and uninoculated prochloraz manganese treatment, which corresponded to the results in our experiment. We found the lowest yield in inoculated plots supplemented with mushroom grain spawn coated with *B. subtilis* QST713 which was consistent with findings reported by Kosanović et al. (2013), where a *Bacillus* strain was applied on casing soil. Milijašević-Marčić et al. (2017) also found the highest yield in the same treatment, i.e. plots treated with *B. subtilis* QST713 and applied to casing. Uninoculated plots treated with prochloraz manganese gave also the highest yields in all compared experiments.

As for the fungicide efficacy in controlling *T. aggressivum* f. *europaeum* T77, there were no significant differences between treatment with the fungicide prochloraz manganese, applied on casing, and *B. subtilis* QST713 coated on spawn grains of *A. bisporus* (Figure 1). Furthermore, there were no significant differences between the two treatments with *B. subtilis* QST713, with or without prochloraz manganese. However, prochloraz showed higher efficacy when it was applied alone than together with the biofungicide. There is a possible antagonistic reaction between the fungicide and the biofungicide in their simultaneous application. Milijašević-Marčić et al. (2017) found no differences between treatments with prochloraz and *B. subtilis* QST713 applied alone, which corresponds to our results. Our data showed 70.4% efficacy of prochloraz manganese. Similarly, Kosanović et al. (2013) reported 71.6% efficacy against *T. harzianum*, while Milijašević-Marčić et al. (2017) found a much lower efficacy of that fungicide (49.4%) against *T. aggressivum*. It is noteworthy that in the experiment of Kosanović et al. (2013), *T. harzianum* was a casing pathogen and the soil was inoculated six days after casing. In our trials, inoculation with *T. aggressivum* was done 10 days after casing, while in the investigation of Milijašević-Marčić et al. (2017), *T. aggressivum* was added on the surface of compost on the very same day of spawning, at least 20-24 days earlier than in the two previous experiments. These experiments have revealed that infestation of compost at spawnig is significantly more devastating than when spawn run compost is infested at casing time, which is consistent with the findings of Rinker and Alm (1997a, b). In our experiment, the efficacy of *B. subtilis* QST713 coating mushroom grain spawn against *T. aggressivum* was 53.1%, which was slightly higher than the data from Kosanović et al. (2013) with *B. subtilis* QST713 applied to casing soil against the casing pathogen *T. harzianum* (44%), and Milijašević-Marčić et al. (2017) when *Bacillus subtilis* strain was used against the compost pathogen *T. aggressivum* (48%). Kosanović et al. (2013) also detected antagonism between prochlroaz manganese and *B. subtilis* QST713, when they were applied together at 80% and 20% of the respective standard product application rates, which showed an efficacy of 29.2% against *T. harzianum*, while we found 43.21% efficacy with simultaneous application of both biofungicide and fungicide at full standard application rates.

It could be inferred that the fungicide prochloraz had satisfactory effectiveness when *T. aggressivum* inoculation occurred later, while the biofungicide

Table 2. Impact of different treatments on total yield (BE%) of mushrooms (*Agaricus bisporus*) artificially inoculated with *Trichoderma aggressivum* f. *europaeum* T77

Treatment	Trial I	Trial II	Trial III	Trial IV	Mean BE%	SE
Uninoculated Control	114.88	108.44	100.62	109.99	108.48 a	7.91
Inoculated Control	88.08	106.39	103.09	108.71	101.57 a	9.28
Inoculated *Bacillus subtilis* QST713	68.87	63.01	91.16	84.56	76.9 b	13.16
Uninoculated Prochloraz-Mn	103.83	101.72	93.33	99.97	99.71 a	4.54
Inoculated *Bacillus subtilis* QST711 + Prochloraz-Mn	69.55	70.23	112.05	106.39	89.55 ab	22.82
Inoculated Prochloraz-Mn	100.53	86.22	100.42	93.99	95.29 ab	6.78
Uninoculated *Bacillus subtilis* QST713	91.22	68.57	122.49	85.51	91.95 ab	22.52
Uninoculated *Bacillus subtilis* QST713 + Prochloraz-Mn	75.25	89.06	82.86	106.58	88.44 ab	13.35

Data are means of four replicates±SE, standard error of means;
BE% - Biological efficiency = ratio of fresh weight of total mushroom yield and weight of dry spawned substrate;
SEDs, standard error of differences=370.6; df, degrees of freedom=7; F=1.9; P-value=0.1.

showed similar efficacy as the chemical fungicide under an early presence of the pathogen. These results imply that the biofungicide based on *B. subtilis* could serve as a harmless alternative to synthetic fungicides in mushroom production, especially in serious compost green mould outbreaks caused by *T. aggressivum*. Also, the biofungicide should be applied alone because an antagonistic reaction was found between prochloraz and *Bacillus*. Certainly, similar experiments are required to confirm these findings and to investigate the efficacy of mushroom grain spawn coated with different strains of beneficial microorganisms against early pathogen infection.
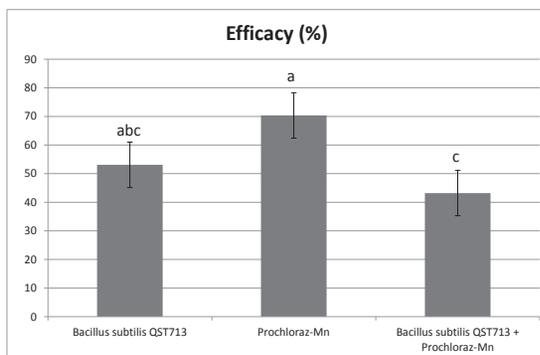

Figure 1. Efficacy (%) of fungicides in the control of *Trichoderma aggressivum* f. *europaeum* T77 on *Agaricus bisporus* mushroom; data are means of four replicates±SE, standard error of means; SEDs, standard error of differences = 113.88; degrees of freedom = 2; F = 3.2; P-value = 0.1.

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Uticaj tretmana micelije („semena") šampinjona (*Agaricus bisporus* L.) bakterijom *Bacillus subtilis* QST713 na prinos i zaštitu od zelene plesni

REZIME

Biofungicid na bazi *Bacillus subtilis* QST713 je odabran za testiranje uticaja na prinos i efikasnosti u suzbijanju *Trichoderma aggressivum* f. *europaeum* T77 iz Srbije, kada je primenjeno za tretiranje micelije („semena") šampinjona u poređenju sa fungicidom prohloraz manganom u oglednom gajilištu. Testirani soj *B. subtilis* QST713 nije inhibirao rast micelije *Agaricus bisporus*, u tretmanima bez prisustva patogena, sa uticajem na prinos 91.95% i nije se statistički značajno razlikovao od neinokulisane kontrole. U pogledu efikasnosti fungicida u suzbijanju *T. aggressivum* f. *europaeum* T77, nije bilo statistički značajne razlike među tretmanima sa fungicidom prohloraz manganom primenjenim na pokrivku i tretmanom *B. subtilis* QST713 primejenim na miceliju (»seme«) šampinjona, sa odgovorajućim vrednostima 70.37 i 53.09%. Ovi rezultati ukazuju da se biofungicid na bazi *B. subtilis* može primenjivati kao alternativa sintetičkom fungicidu u proizvodnji šampinjona, posebno kod značajne pojave zelene plesni u kompostu za gajenje šampinjona koju izaziva *T. aggressivum*. Takođe, preporuka je da se biofungicid primenjuje samostalno jer je uočena antagonistička reakcija između prohloraz mangana i *B. subtilis* QST713.

Ključne reči: Šampinjon; *Bacillus subtilis; Trichoderma*; Fungicidi