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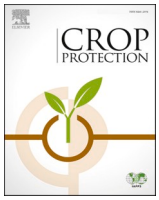

Response of the mushroom pathogen *Cladobotryum mycophilum* to the fungicides prochloraz and metrafenone and two *Bacillus*-based biological control agents in mushroom crop trials

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ABSTRACT

Cobweb disease caused by members of the *Cladobotryum* genus is a major problem for growers of the white button mushroom (*Agaricus bisporus*). Synthetic fungicides such as prochloraz and metrafenone have been very successful at targeting and eliminating the pathogens that cause mushroom disease. However, prochloraz can no longer be used in the European Union (EU) from June 2023 and over-reliance on metrafenone has resulted in putative resistant pathogenic strains emerging. Prochloraz still showed good control of two different isolates of *Cladobotryum mycophilum* with efficacy values consistently reaching 70%. Metrafenone inhibited the growth of *C. mycophilum* isolate 618, which was isolated before metrafenone was introduced (efficacy 96%), but it failed to control *C. mycophilum* 1546, which was isolated after metrafenone was introduced, and which should now be classified as resistant. Two further *C. mycophilum* isolates from mushroom farms in 2019 also showed metrafenone resistance *in vitro*. In this work two biological control agents (BCAs) were investigated as potential environmentally sustainable alternatives to the fungicides prochloraz and metrafenone. The BCA *Bacillus velezensis* QST 713 was unsuccessful in controlling cobweb disease caused by *C. mycophilum* isolate 1546 while the BCA *Bacillus velezensis* Kos showed moderate control over two trials reaching 30–40% efficacy. Lower inoculum concentrations resulted in slightly lower but not significantly different disease levels across all treatments. Future trials with BCAs need to look at alternative methods to evaluate efficacy.

1. Introduction

Mushrooms have become the focus of attention as a future source of protein and commercial production and this is likely to continue to increase (Bell et al., 2022; Scholtmeijer, 2023). The cultivation of *Agaricus bisporus* (Lange) [Imbach] is an important commercial practice for many countries around the world, including Asia, Europe and America, and it accounted for 11% (4.7 million tonnes) of the total world production of mushrooms in 2018–19 (Singh et al., 2020). Common fungal diseases of *A. bisporus,* such as dry bubble disease (*Lecanicillum fungicola*), wet bubble disease (*Mycogone perniciosa*), green mould disease (*Trichoderma aggressivum*) and cobweb disease (*Cladobotryum* spp.), are considered as a serious threat to this industry (Fletcher and Gaze 2007) as disease has a direct and negative impact on both yield and quality of mushrooms, resulting in economic loss.

Cobweb disease is caused by several members of the *Cladobotryum* genus, the most important of these being *Cladobotryum mycophilum* (teleomorph: *Hypomyces odoratus*) and *Cladobotryum dendroides* (teleomorph: *Hypomyces rosellus*) (Gams and Hoozemans, 1970). *Cladobotryum mycophilum* has been reported as affecting several mushroom species, including *A. bisporus, Flammulina velutipes, Ganoderma lucidum, Pleurotus eryngii* and *Pleurotus ostreatus* (Grogan and Gaze, 2000; Back et al., 2010; Gea et al., 2011, 2017, 2019; Kim et al., 2012). *Cladobotryum dendroides* has been reported on *Lentinula edodes* (Gea et al., 2018). Spores from these pathogens are dry and air-borne and are easily disturbed by crop watering, and then dispersed within growing rooms through the air-handling systems (Adie and Grogan, 2000; Adie et al., 2006). Mushroom spotting will occur when the *Cladobotryum* spores land and germinate on the cap of the mushroom fruiting body. The pathogen can grow over the casing layer and colonise developing mushrooms with a thick, white mycelium, causing them to discolour and rot. Cobweb disease is controlled on mushroom farms through a

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combination of very strict hygiene practices and the application of fungicides. Patches of cobweb that are detected early should be covered carefully with damp paper and salt as soon as they appear on the mushroom bed in order to limit conidial dispersion and disease spread (Adie et al., 2006; Grogan and Gaze, 2008). Fungicides can also be applied.

Synthetic fungicides have given the farming community, including mushroom growers, support when dealing with outbreaks of difficult-to-control diseases, thereby safeguarding their livelihoods. However, there has been a steady withdrawal of synthetic active substances in the EU in recent years, down from 320 in 2017 to 234 in 2022, and it is projected that up to half of those remaining may be further withdrawn or unsupported in the next ten years (Marchand, 2023). This is due largely to enhanced regulation, concern about potential toxic effects on non-target organisms in the wider environment and the emergence of fungicide-resistant strains. Although fungicides can be effective at controlling disease, over-reliance on a few active substances has led to fungicide resistant strains emerging over time within different mushroom pathogen populations including *Lecanicillium fungicola, Cladobotryum mycophilum* and *Trichoderma aggressivum* (Fletcher and Yarham, 1976; McKay et al., 1998; Grogan and Gaze, 2000; Grogan, 2006; Romaine et al., 2008; Gea et al., 2021). This has limited the range of fungicides available as a treatment option. Up to recently, two synthetic fungicides have been widely used in the EU and worldwide to control mushroom pathogens – prochloraz and metrafenone - however, approval for the use of prochloraz (and other demethylation inhibitor fungicides) within the EU was withdrawn in 2021, with use-up dates of June 2023 (EC, 2021), leaving only metrafenone in many cases. Anecdotal evidence from across the mushroom sector in Europe however suggests that metrafenone is no longer effective against cobweb disease but there is no documented evidence to support this.

Given the continued downward pressure on synthetic chemical use worldwide, the European Commission (EC) outlined a more sustainable approach to pest management in its Sustainable Use of Pesticides Directive (SUD) 2009/128/EC (Anon, 2009), where integrated pest management (IPM) strategies are recommended to combat over-reliance on chemicals. Barzman et al. (2015) describe in detail the eight principles of IPM and list biological control agents (BCAs) as an important non-chemical method to be considered when intervention is needed to control a pest or disease outbreak but they note that BCAs may be less effective in comparison to chemicals. *Bacillus* species are commonly used as protective BCAs in agriculture (Borriss, 2015) as they can produce anti-fungal compounds such as lipopeptide antibiotics, lytic enzymes and biofilm siderophores which can all contribute to the destruction of the pathogen (Stein, 2005; Yang et al., 2012; Zhou et al., 2012; Abo-Elyousr et al., 2019). Increasing the population of naturally occurring antagonists in the mushroom growing environment should also result in competition for space and nutrients, which may reduce the growth of the pathogen. Serenade® is a commercially available biocontrol product that is used to protect against many plant diseases. It uses *Bacillus velezensis* QST 713 as its active ingredient, which has been studied as a potential BCA of a mushroom pathogen (Pandin et al., 2018). *Bacillus velezensis* Kos, was isolated from a mushroom crop by Kosanovic et al. (2021) and it has been shown to be effective *in vitro* at inhibiting the growth of *T. aggressivum*, *L. fungicola* and *C. mycophilum* (Clarke et al., 2022a, 2022b; Kosanovic et al., 2021) offering potential as a BCA.

The aim of this study was to investigate the *in vitro* resistance levels of *Cladobotryum* isolates collected between 1995 and 2019 to prochloraz and metrafenone and to evaluate their efficacy *in vivo* in crop trials against two contrasting *C. mycophilum* isolates. We also wanted to evaluate the performance of two BCAs, *B. velezensis* QST 713 and *B. velezensis* Kos, in crop trials in conjunction with a recently isolated *C. mycophilum* isolate. Different inoculum concentrations were also studied to determine if BCAs might perform better under lower levels of disease pressure. The results from this work provide data on the *in vitro* and *in vivo* response of *Cladobotryum* isolates to prochloraz and metrafenone as well as data on what level of disease control can be obtained by two BCAs in comparison with standard fungicide treatment. This provides important information for the mushroom sector on an IPM approach to cobweb disease control.

2. Materials and methods

2.1. Fungal cultures

Seven *C. mycophilum* isolates (202 A, 235, 618, 1545, 1546, 1583 and 1588) and one *C. dendroides* isolate (1571) were evaluated for their *in vitro* response to two fungicide active ingredients (a.i.): prochloraz and/or metrafenone (Table 1). All were isolated from cobweb-infected mushroom crops between 1995 and 2019 and are stored in liquid nitrogen and −80 °C in the Teagasc Ashtown culture collection (Dublin, Ireland). Four *C. mycophilum* isolates (1545, 1546, 1583 and 1588) were obtained in 2019 from mushroom farms with severe cobweb disease and that had been using the product metrafenone since it had been approved for use against cobweb mould in Ireland in 2017. Three *C. mycophilum* isolates had been isolated prior to metrafenone introduction: two (202 A and 235) had been obtained from mushroom farms with cobweb disease in the UK in 1995 (Grogan and Gaze, 2000), and one (618) had been isolated from a mushroom farm in Ireland in 2010. One *C. dendroides* isolate (1571) was collected from the Teagasc Mushroom Unit in 2019 as an isolated patch in an experimental crop that was not associated with a severe outbreak. At that time, the mushroom unit was relatively new and metrafenone had never been used. It was considered to be a wild strain contaminating the crop from the environment.

2.2. Fungicides and biological control agents (BCAs)

The chemical fungicides prochloraz (Sporgon® 50 W P) (460 g a. i. kg^{-1}) and metrafenone (Vivando®) (500 g a. i. L^{-1}) were supplied by BASF Ireland Ltd. The commercially available biocontrol product Serenade® ASO (*B. velezensis* QST 713) was supplied by Bayer CropScience Ltd. and contains a minimum of 1×10^{12} colony forming units (CFUs) per litre (=1×10^{9} CFUs per ml). A bacterial strain *B. velezensis* was originally isolated from mushroom casing by Kosanovic et al. (2021) (designated here as *B. velezensis* Kos) and was obtained for this work from liquid nitrogen stores at Maynooth University (Kildare, Ireland). Culture filtrate (CF) from this bacterium was collected by inoculating 4 L of sterile nutrient broth (NB) with 140 h B. *velezensis* Kos liquid culture. Flasks were grown for 96 h (30 °C at 120 rpm) and the CF was collected by centrifugation (1792×*g*, 10 min). The CF was filtered using Miracloth (Merck) into sterile flasks (Duran). This strain has previously been shown to be inhibitory toward *C. mycophilum in vitro* (Clarke et al., 2022a).

2.3. In vitro analysis of fungicide resistance

Two independent *in vitro* experiments were conducted (Table 1). A preliminary experiment with metrafenone only was conducted initially for *C. mycophilum* isolates 1545, 1546 and 1583, all obtained in 2019 from farms with serious cobweb disease, despite using metrafenone. Two *C. mycophilum* isolates were also included for comparison (202 A and 618) which had been obtained prior to metrafenone use and one *C. dendroides* isolate (1571). Cultures were grown in 90 mm Petri dishes on malt extract agar (MEA) (Merck 105,398, www.merckmillipore.com) amended with metrafenone at concentrations of 0 (Control), 0.001, 0.01, 0.1, 1, 10 and 100 mg a. i. kg^{-1}. Three replicate cultures were prepared for each concentration by placing a 6 mm plug approximately 10 mm from the margin of the Petri dish. Growth was measured after 4 days incubation at 25 °C when control cultures had almost filled the Petri dish. To confirm the results for three selected isolates (618, 1546 and 1571), and to determine their response to prochloraz, another fungicide approved at the time, an additional experiment was conducted

Table 1
Cladobotryum isolates used in two *in vitro* experiments.

Isolate Number	Species	Year of isolation	Country of origin	Preliminary Experiment metrafenone only	Expt. 1 prochloraz & metrafenone
618	*C. mycophilum*	2010	Ireland	X	X
1546	*C. mycophilum*	2019	Ireland	X	X
1571	*C. dendroides*	2019	Ireland	X	X
202 A	*C. mycophilum*	1995	United Kingdom	X	
1545	*C. mycophilum*	2019	Ireland	X	
1583	*C. mycophilum*	2019	Ireland	X	
235[a]	*C. mycophilum*	1995	United Kingdom		X
1588	*C. mycophilum*	2019	Ireland		X

[a] Isolate 235 was originally identified as *C. dendroides* Type II in Grogan and Gaze (2000) and was later re-identified as *C. mycophilum*.

(Experiment 1). Two additional isolates were included – a pre-metrafenone isolate (235) and another culture recently isolated from a commercial farm (1588). Cultures were grown as before in 90 mm Petri dishes on MEA amended with either prochloraz, or metrafenone, at concentrations of 0 (Control), 0.01, 0.1, 1, 10 and 100 mg a. i. kg^{-1}. Five replicate cultures were prepared for each isolate/fungicide/fungicide concentration combination. Radial growth was measured after 5 days incubation at 25 °C when control cultures had almost filled the Petri dish Means were calculated and the data were converted to percentage growth of the control so that the ED50 could be estimated. Following on from these *in vitro* tests, two isolates were selected for crop inoculation experiments: a metrafenone-resistant *C. mycophilum* isolate (1546) and a metrafenone-sensitive isolate (618).

2.4. Mushroom cultivation

Two independent crop trials were carried out in industry-standard environmentally controlled mushroom growing rooms at the Mushroom Research Unit at Teagasc Ashtown Research Centre (Dublin, Ireland). Plastic crates (external l x b x h dimensions of 400 mm × 600 mm x 300 mm) with a 0.2 m^2 internal crop surface area were filled with 16 kg (equivalent fill rate of 80 kg m^{-2}) of commercially-sourced Phase III substrate (Carbury Compost Ltd., Carbury, Co. Kildare, Ireland), fully colonised with *A. bisporus* strain Sylvan A15. The crates of substrate were covered with a 50 mm layer of commercial peat-based mushroom casing (Harte Peat Ltd., Clones, Co. Monaghan, Ireland) on day 1 of the crop cycle and then placed onto shelves in the growing room. Crops and growing rooms were managed following standard operating procedures for mushroom crops using the Fancom environmental control system for mushroom cultivation (https://www.fancom.com/system/mushroom-growing-phase) at the Teagasc Mushroom Unit. Air temperature was set at 21 °C, compost temperature to 25 °C and relative humidity (RH) to a range of 96–100 %, for 7 days (case run). After 7 days, fresh air was introduced at 50% and the air temperature and compost temperature were dropped gradually over 72 h to 20 °C and 21 °C respectively (cool down pinning). This change in growing conditions triggers *A. bisporus* reproductive cycle, resulting in mushroom production. These conditions were maintained for a further 5 days then air temperature was reduced to 18 °C for the remainder of the crop. Six replicate crates were prepared for each treatment combination. Healthy mushrooms were harvested as predominantly 'closed cups' of 40–60 mm diameter, over 2–3 days for each of two flushes and recorded as kg $plot^{-1}$. Diseased or spotted mushrooms were recorded separately. Any patches of cobweb that were visible at the end of the first flush were covered with damp paper and salt to prevent disease spread during crop watering, following industry best practice. Trials were stopped after two flushes due to high levels of cobweb disease in inoculated plots. It is worth noting that the yields from the uninfected control treatments would be higher if the trials were taken to a third flush.

2.5. Fungicide and BCA application

Commercial fungicides and BCAs were applied to the relevant plots on day 7 after casing according to the approved rates on the label and using a calibrated knapsack sprayer. Prochloraz was applied at a rate of 1 g of product (Sporgon® 50 W P) m^{-2}, metrafenone was applied at a rate of 1 ml of product (Vivando®) m^{-2} and *B. velezensis* QST 713 was applied at the label rate of 8 L of product (Serenade® ASO) $hectare^{-1}$, equivalent to 0.8 ml of product m^{-2} (0.8×10^9 cfu m^{-2}). *B. velezensis* Kos 96 h culture filtrate was prepared fresh on the morning of treatment application. All prepared treatment solutions were applied at a rate of 1 L m^{-2}. Water (1 L m^{-2}) was applied to control plots. After the first flush of mushrooms had been harvested, a second application of the two BCA treatments was applied. Water was applied to control and fungicide plots.

2.6. Crop inoculation and disease data collection

Inoculum was prepared for selected isolates for each crop trial experiment: metrafenone resistant *C. mycophilum* isolate (1546) and metrafenone sensitive isolate (618). Subcultures of isolates were grown on MEA at 25 °C for 72 h. Plate cultures were washed with phosphate buffered saline (PBS) to collect a concentrated spore suspension and the concentration was determined using a haemocytometer. Inoculum for the crop trials was prepared by dilution to give a spore concentration of 1×10^6 ml^{-1}. This was further diluted to give a final working concentration of 1×10^4 ml^{-1}. In crop trial 1 inoculum was prepared for both isolates and a 50 ml aliquot was applied to each 0.2 m^2 plot to give a final application rate of 1×10^6 spores m^{-2}. In crop trial 2 inoculum was prepared for isolate 1546 only. In this trial two inoculum concentrations were included: the same rate of 1×10^6 spores m^{-2} as in trial 1 and a reduced rate of 5×10^5 spores m^{-2}. Inoculation of plots took place on day 11 of the crop cycle.

A disease assessment of cobweb growth on plots was carried out at the end of the first and second flushes. Cobweb patches were roughly circular in shape therefore two diameters were measured and an average diameter/radius was calculated for each patch. The area of each patch was calculated according to the formula πr^2 where $\pi = 3.1416$ and $r =$ radius of the patch and then the total area of all disease patches for each plot was calculated. As the patches merged and were no longer circular, a square template measuring 10% was used to estimate the area of larger coalesced patches. The average percentage of diseased area per treatment was calculated as Disease Incidence (DI), where: DI = [(Average area of disease in cm^2/total area of plot (2000 cm^2)) x 100]. Treatment efficacy was calculated using Abbotts formula (Abbott 1925) given as % efficacy = [(Ic -It)/Ic] x 100, where Ic = disease incidence in the inoculated control; It = disease incidence in treated samples (Stanojević et al., 2019). Images of randomly chosen plots which represented each treatment were taken during each disease analysis.

2.7. Crop trials

Two independent crop trials were conducted to evaluate the efficacy of different fungicides and BCAs to control cobweb disease. In crop trial 1 there were 13 treatments included and in crop trial 2 there were 12 treatments included, summarised in Table 2. Eight treatments were

Table 2
Details of treatments in crop trials 1 and 2.

Crop Trial	Treatment	Fungicide/BCA treatment	Inoculum treatment
Crop Trial 1	1. Control uninoculated[a]	None	None
	2. Control 1546[a]	None	Isolate 1546
	3. Control 618	None	Isolate 618
	4. Prochloraz uninoculated[a]	Prochloraz	None
	5. Prochloraz 1546[a]	Prochloraz	Isolate 1546
	6. Prochloraz 618	Prochloraz	Isolate 618
	7. Metrafenone uninoculated	Metrafenone	None
	8. Metrafenone 1546	Metrafenone	Isolate 1546
	9. Metrafenone 618	Metrafenone	Isolate 618
	10. QST 713 uninoculated[a]	QST 713 (*B. velezensis*)	None
	11. QST 713 1546[a]	QST 713 (*B. velezensis*)	Isolate 1546
	12. Kos uninoculated[a]	Kos (*B. velezensis*)	None
	13. Kos 1546[a]	Kos (*B. velezensis*)	Isolate 1546
Crop trial 2	1. Control uninoculated[a]	None	None
	2. Control 1546 1×10^6[a]	None	Isolate 1546
	3. Control 1546 5×10^5	None	Isolate 1546
	4. Prochloraz uninoculated[a]	Prochloraz	None
	5. Prochloraz 1546 1×10^6[a]	Prochloraz	Isolate 1546
	6. Prochloraz 1546 5×10^5	Prochloraz	Isolate 1546
	7. QST 713 uninoculated[a]	QST 713 (*B. velezensis*)	None
	8. QST 713 1546 1×10^6[a]	QST 713 (*B. velezensis*)	Isolate 1546
	9. QST 713 1546 5×10^5	QST 713 (*B. velezensis*)	Isolate 1546
	10. Kos uninoculated[a]	Kos (*B. velezensis*)	None
	11. Kos 1546 1×10^6[a]	Kos (*B. velezensis*)	Isolate 1546
	12. Kos 1546 5×10^5	Kos (*B. velezensis*)	Isolate 1546

[a] = treatments repeated in both trials.

repeated in both trials.

2.8. Statistical analysis

The results from the two *in vitro* fungicide resistance experiments were analysed independently. There were three replicates per treatment combination in the preliminary experiment and five replicates in experiment 1. *In vitro* growth data were converted to % growth so that ED50 values could be determined. Raw *in vitro* growth data were analysed by ANOVA. The results from the two crop trials were analysed independently. In both, there were six replicates per treatment combination and treatment plots were arranged in a randomized block design. Crop trial data were analysed by ANOVA. Prior to ANOVA, normal probability plots of residuals were produced in Minitab (version 20.04.00) to determine if residuals were normally distributed. Significant differences between treatments were determined using Turkey's method and 95% confidence for pairwise comparisons. An f-value was reported for each ANOVA test. All data analyses can be found in Supplementary Tables 1–5.

3. Results

3.1. In vitro analysis of fungicide resistance in Cladobotryum isolates

The *in vitro* responses of eight *Cladobotryum* isolates to the fungicides metrafenone and prochloraz are shown in Fig. 1 and Supplementary Tables 1A and 1B. Four *C. mycophilum* isolates 1545, 1546, 1583 and 1588 all grew significantly better at concentrations of >0.1 mg kg^{-1} compared to other isolates (Supplementary Tables 1A and 1B) and had ED50 values of between 0.1 and 1 mg kg^{-1} (Fig. 1). Three *C. mycophilum* isolates, 618, 202 A and 235 and *C. dendroides* isolate 1571 were more sensitive and had ED50 values of <0.01 mg kg^{-1}. None of the five isolates tested grew at 10 mg kg^{-1} prochloraz but the responses over the range 0.01–1 mg kg^{-1} were more variable, indicating a degree of tolerance in some isolates. Isolate 618 was more tolerant to prochloraz compared to *C. mycophilum* isolate 1546. Contrasting *C. mycophilum* isolates 1546 and 618 were taken forward to crop trials.

3.2. Efficacy of fungicides and BCAs to control cobweb disease: crop trial 1

Yield. The average yield of healthy mushrooms produced for each treatment over two flushes is shown in Fig. 2. There was no statistically significant difference in yields across all treatments in the first flush, with yields ranging from 2.00 to 2.56 kg $plot^{-1}$. Total yield over two flushes for the uninoculated controls across all treatments ranged from 6.13 to 6.43 kg $plot^{-1}$. By this time, yields from the untreated inoculated controls for *C. mycophilum* isolates 1546 and 618 were significantly lower, while there was no significant reduction in yield caused by either isolate when treated with prochloraz. Metrafenone treatment was ineffective against the metrafenone-resistant isolate 1546, which caused a significant reduction in yield while no yield reduction occurred when the metrafenone-sensitive isolate 618 was used. For the BCA treatments, which were only done in conjunction with isolate 1546, *B. velezensis* QST 713 treatment did not prevent a significant yield reduction while treatment with *B. velezensis* Kos CF gave a reduced yield but which was intermediate between the control and the inoculated control. The average yield of each treatment at the end of trial 1 and ANOVA results can be found in Supplementary Table 2.

Cobweb disease. There was no cobweb disease present in any plot at the beginning of the first flush. A few mushrooms with cobweb 'spotting' symptoms were present in all inoculated treatments but not in the uninoculated controls. The highest average number of spotted mushrooms was present in metrafenone treated plots inoculated with *C. mycophilum* isolate 1546 (4 per plot), (Supplementary Fig. 1). An assessment of cobweb growth was taken at the end of flush 1 (Supplementary Fig. 2). All uninoculated control plots remained free of cobweb with just a few

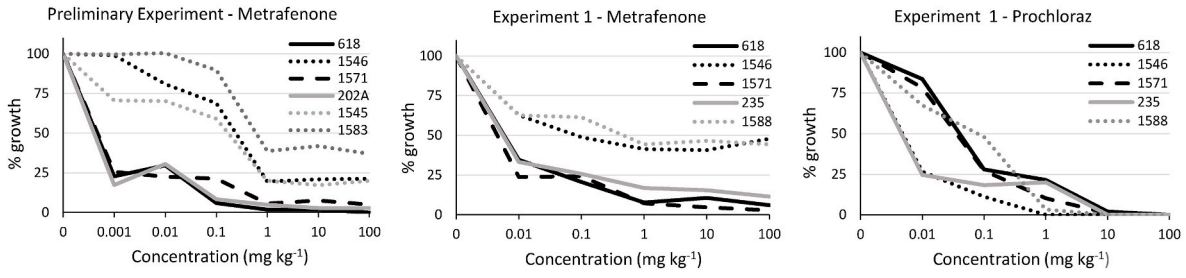

Fig. 1. *In vitro* response of six *Cladobotryum* isolates to metrafenone (Preliminary Experiment, n = 3) and five *Cladobotryum* isolates to metrafenone and prochloraz (Experiment 1, n = 5). Values are mean % growth at each concentration. ANOVA data in Supplementary Tables 1A and 1B

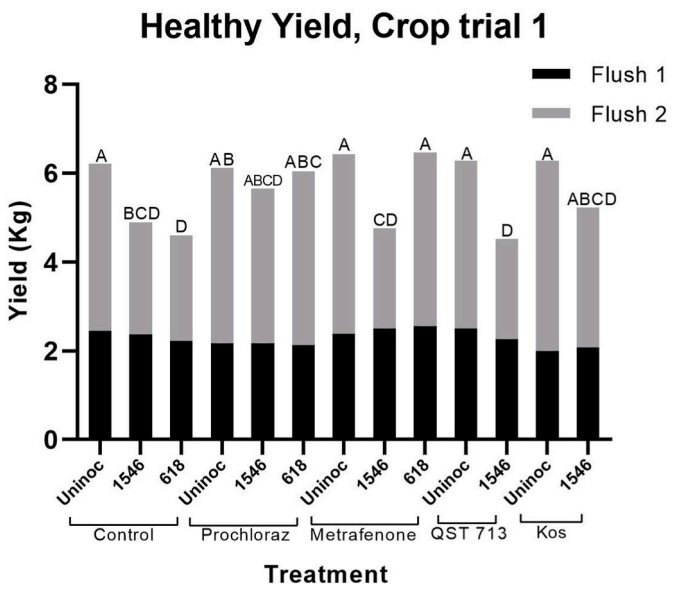

Fig. 2. Yield of healthy mushrooms over two flushes following treatment with the fungicides prochloraz or metrafenone or the BCAs QST 713 or Kos, followed by inoculation with one of two different *C. mycophilum* isolates, 1546 or 618. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukey's pairwise comparisons test (Supplementary Table 2).

small patches developing by the end of the second flush, with average disease incidence levels of <3% (Fig. 3).

By the end of the second flush the untreated inoculated controls for both isolates had developed a high incidence of cobweb at 52% and 66% for isolates 1546 and 618, respectively (Fig. 3). Disease incidence in response to prochloraz treatment was reduced to 14% and 20% for *C. mycophilum* isolate 1546 and 618, respectively, relative to the controls, with corresponding efficacy values of 73% and 70% (Fig. 3, Supplementary Table 3). Efficacy of metrafenone against isolate 618 was very high at 96%, but it failed to control cobweb caused by isolate 1546, which had a disease incidence level of 69%, similar to the inoculated control (Fig. 3). Similarly, *B. velezensis* QST 713 failed to inhibit isolate 1546 with disease incidence levels of 57%, similar to the untreated inoculated control. *B. velezensis* Kos efficacy was intermediate at 30% at the end of flush 2, but this was not significantly different to the control, with disease incidence levels of 36% still occurring (Fig. 3, Supplementary Table 3). Images of representative plots at the end of flush 2 are presented in Fig. 4.

3.3. Effect of inoculum concentration on the efficacy of fungicides and BCAs to control cobweb disease: crop trial 2

Yield. The yields from crop trial 2 were lower than those for crop trial 1, in particular the second flush. This would have been compensated for in the third flush, but as disease levels in inoculated plots were very high, no third flush was taken. The average yield of healthy mushrooms produced for each treatment over two flushes is shown in Fig. 5 and were broadly in agreement with the results for similar treatments in Crop trial 1. Total yield for the uninoculated controls across all treatments ranged from 3.03 to 3.49 kg plot^{-1} after two flushes. By this time, yields from the untreated inoculated controls for *C. mycophilum* isolate 1546 at both inoculum concentrations were significantly lower than the uninoculated control, while there was no significant reduction in yield for either inoculum concentration when treated with prochloraz ($P < 0.05$). For the two biocontrol treatments, there was a significant reduction in yield in conjunction with the higher inoculum concentration but yields for the lower inoculation concentration were not significantly different to the controls. The average yield data and ANOVA results at the end of trial 2 can be found in Supplementary Table 4.

Cobweb disease. Similar to crop trial 1, there was no evidence of cobweb disease found at the beginning of flush 1, but a few spotted mushrooms were present in all inoculated plots except for prochloraz inoculated at 5×10^5 spores m^{-2}. *B. velezensis* QST713 had the highest average number of spotted mushrooms (Supplementary Fig. 3). Cobweb growth was only detected in three treatments at very low levels (<0.5%) by the end of the first flush, the two Control inoculated treatments and the QST 713 1546 1×10^6 treatment (Supplementary Fig. 4) (Supplementary Table 5).

By the end of the second flush significant cobweb growth had developed in all inoculated treatments at levels that were higher than in crop trial 1 while all uninoculated control plots remained free of cobweb (Fig. 6). The untreated inoculated controls at both inoculum concentrations had developed a high incidence of cobweb by the end of the second flush at 91% and 85%, for *C. mycophilum* 1546 at 1×10^6 and 5×10^5, respectively (Fig. 6). Disease incidence in response to prochloraz was significantly lower than for the inoculated controls at 28% and 23% for *C. mycophilum* 1546 at 1×10^6 and 5×10^5 ($P < 0.05$), respectively, with corresponding efficacy values of 69% and 73%. *B. velezensis* QST

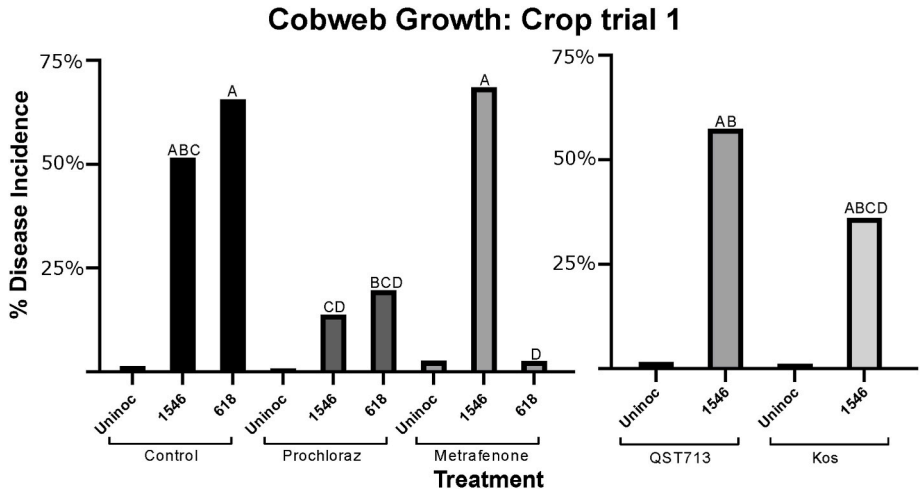

Fig. 3. Cobweb disease incidence (%) developing after two flushes following treatment with the fungicides prochloraz or metrafenone or the BCAs QST 713 or Kos, followed by inoculation with one of two different *C. mycophilum* isolates, 1546 or 618. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Turkey's pairwise comparisons test (Supplementary Table 3).

Fig. 4. A representative plot from each fungicide/BCA/inoculum treatment showing cobweb growth at the end of flush 2 in crop trial 1.

713 performed poorly, as in trial 1, with disease incidence for the 1×10^6 treatment at 95% and the 5×10^5 treatment at 82%, neither of which were significantly different to the inoculated controls. The efficacy of *B. velezensis* Kos was again intermediary, as in crop trial 1, but this time the reduction in cobweb growth levels was significant compared with the controls ($P < 0.05$) (Fig. 6, Supplementary Table 5). Disease incidence levels of 56% and 46% were recorded for the two inoculation treatments, 1×10^6 and 5×10^5, respectively, with corresponding efficacy values of 38 % and 46%. Images of representative plots at the end of flush 2 can be seen in Supplementary Fig. 5.

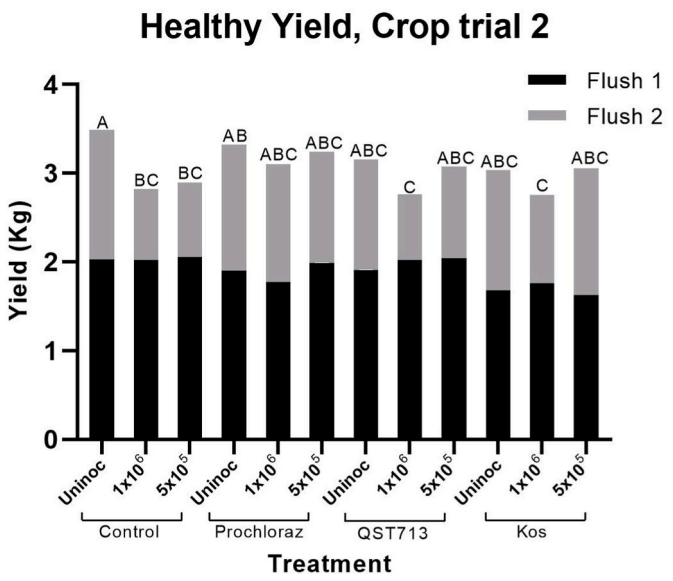

Fig. 5. Yield of healthy mushrooms over two flushes following treatment with the fungicide prochloraz or the BCAs QST 713 or Kos, followed by inoculation with *C. mycophilum* isolate 1546 at either 1×10^6 or 5×10^5 spores m^{-2}. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukey's pairwise comparisons test. (Supplementary Table 4).

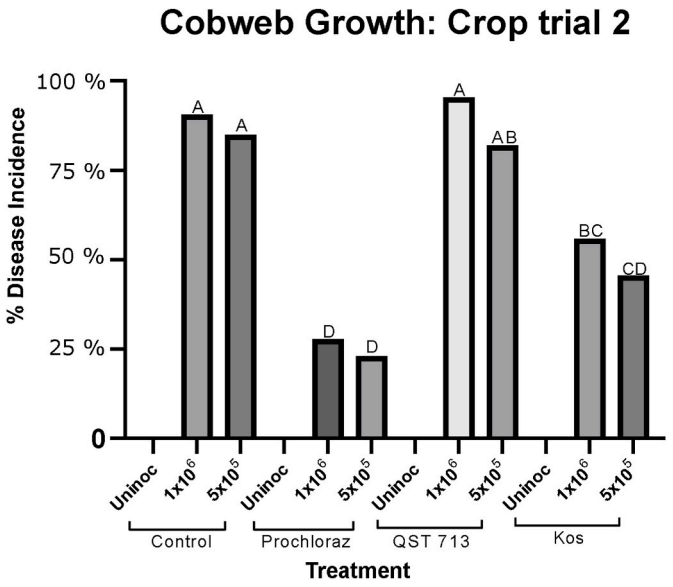

Fig. 6. Cobweb disease incidence (%) developing after two flushes following treatment with the fungicide prochloraz or the BCAs QST 713 or Kos, followed by inoculation with *C. mycophilum* isolate 1546 at either 1×10^6 or 5×10^5 spores m^{-2}. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukey's pairwise comparisons test (Supplementary Table 5).

4. Discussion

C. mycophilum, causal agent of cobweb disease, is a major pathogen of the cultivated mushroom *A. bisporus* and results in significant yield and revenue losses. The aim of this work was to evaluate the resistance levels of *C. mycophilum* isolates to metrafenone and prochloraz, and their efficacy. The two fungicides have, up until now, been widely used in the mushroom industry (Gea et al., 2021). Two BCAs were also evaluated for efficacy. The results have shown that the synthetic fungicide prochloraz was more consistent at controlling high levels of cobweb disease, compared to metrafenone and BCAs. Prochloraz was effective at significantly ($P < 0.05$) controlling cobweb disease caused by two different isolates, over two trials, even at extremely high disease pressure (1×10^6 spores m^{-2}). No resistance or significant yield reductions were observed following prochloraz treatment of *Cladobotryum* inoculated plots. This supports the findings of Stanojević et al. (2019), who also found prochloraz performed better than tested BCA in green mould and dry bubble disease trials *in vivo*. However, approval for the use of prochloraz (and other demethylation inhibitor fungicides) within the EU was withdrawn in 2021 (EC, 2021), with use-up dates of June 2023, therefore controlling *Cladobotryum* will be a challenge into the future without this product.

Metrafenone is a fungicide that was approved for use to control cobweb mould in various European countries between 2014 and 2016. Carrasco et al. (2017) showed that it was highly effective against *C. mycophilum* in growth trials and suggested it could be used as an alternative to prochloraz to treat cobweb disease. The results of *in vitro* testing of a number of *Cladobotryum* isolates in this study, collected either before or after the introduction of metrafenone to control cobweb disease, illustrates clearly how tolerance to metrafenone has emerged rapidly since its introduction. Four *C. mycophilum* isolates, 1545, 1546, 1583 and 1588, collected from farms with severe cobweb disease in 2019, were highly tolerant to metrafenone while three *C. mycophilum* isolates, 202 A and 235 collected in 1995, and 618 collected in 2010, before the introduction of metrafenone, were more sensitive (Fig. 1). A *C. dendroides* wild type isolate that had been collected in 2019 on the Teagasc mushroom unit, which had no history of metrafenone use, was also sensitive to metrafenone. In the crop trials, metrafenone was able to significantly ($P < 0.05$) control the growth of *C. mycophilum*, isolate 618, but it was unable to prevent the growth of *C. mycophilum* isolate 1546, confirming the *in vitro* tolerance data. Metrafenone should therefore no longer be used routinely for cobweb control and *Cladobotryum* isolates should be tested for their sensitivity to metrafenone before deciding if it is appropriate to use it, as metrafenone still has an 'ongoing extension of approval period' at EU level (Marchand, 2023). With prochloraz no longer available to use as an alternating chemical as an anti-resistance measure, BCAs and enhanced hygiene measures may be all that will be available to growers into the future.

In these trials two BCAs were evaluated for controlling cobweb disease, *B. velezensis* QST 713 and *B. velezensis* Kos. *B. velezensis* QST 713 is a commercially available biocontrol product, which was shown to significantly reduce the effects of mushroom compost green mould, *Trichoderma aggressivum,* following two applications (Pandin et al., 2019), and to significantly reduce the fungal propagule count in the substrate. In crop trial 1, *B. velezensis* QST 713 did not prevent significant yield reductions or disease incidence caused by *C. mycophilum* 1546. There are no reports in the literature reviewing the activity of *B. velezensis* QST 713 against *Cladobotryum* however, crop trials were conducted as part of an EU funded project that indicated that *B. velezensis* QST 713 did not significantly reduce cobweb disease (MushTV, 2016). Kosanović et al. (2013) reported that a casing application of *B. velezensis* QST 713 could reduce green mould disease but found prochloraz performed better. Potocnik et al. (2018) described how *B. velezensis* QST 713 coated on *A. bisporus* spawn grain could inhibit green mould disease and that there was no statistical difference between it and prochloraz casing treatment. Stanojević et al. (2019) reported that *B. velezensis* QST 713 was able to significantly reduce both green mould and dry bubble disease compared with untreated controls, but it was generally out performed by a prochloraz fungicide treatment. It is worth noting that some of the trials mentioned above were often done using small quantities of compost (1–1.5 kg plots) and high inoculum concentrations. More recently, Navarro et al. (2023) indicated the low effect of biocontrol agents *B. velezensis* QST 713 and *B. amyloliquefaciens* on the control of wet bubble disease caused by *Hypomyces perniciosa*, even at a relatively low inoculum concentration.

In the work described here over two trials, QST 713 had no impact on cobweb disease levels compared to the inoculated controls whereas prochloraz significantly reduced disease levels by about 70–75% ($P < 0.05$). Results were similar when the inoculum concentration was reduced by half, suggesting that QST 713 is unlikely to be useful against cobweb disease. Perhaps a new approach is needed to evaluate BCAs to better evaluate their potential, particularly in a disease prevention capacity, where pathogen inoculum loads may be quite low to start with.

In contrast to *B. velezensis* QST 713, *B. velezensis* Kos BCA was able to considerably reduce cobweb disease levels by 30–40% over two trials, compared to the untreated controls, but it was not as effective as prochloraz. Results were similar when the inoculum concentration was reduced by half, suggesting that *B. velezensis* Kos is worth investigating further as a potential biocontrol option for cobweb disease. It is important to note however that the Kos treatment consisted of an application of a culture filtrate (CF), containing a cocktail of metabolites, rather than an application of live cells and that the mode of action of the two products may be different as a result. This finding agrees with Kosanovic et al. (2021) who first discussed the antagonistic potential of *B. velezensis* Kos as a BCA for pathogens of *A. bisporus*. We have previously demonstrated that the CF from *B. velezensis* Kos can inhibit the *in vitro* growth of *C. mycophilum* and *L. fungicola* (Dry bubble disease) (Clarke et al., 2022a, 2022b). The proteomic response of these two pathogens when exposed to *B. velezensis* Kos CF was characterised and it was demonstrated that proteins associated with growth were significantly reduced in abundance compared to an untreated control, while proteins associated with stress response were significantly increased in abundance. Subtilisin and several other proteases, were identified within the inhibitory fraction of the Kos CF, which may play a role in the growth suppression of the pathogen within the substrate (Clarke et al., 2022a). *B. velezensis* Kos is not a commercial product but these results demonstrate that further research is needed to evaluate different formulations of BCAs that may be more effective.

5. Summary and conclusions

The results presented here demonstrate that tolerance to metrafenone has emerged in *C. mycophilum* isolates, the only remaining synthetic fungicide approved for mushroom disease control in many European countries. Crop trials demonstrated that recent isolates of *C. mycophilum* from mushroom farms in Ireland showed increased tolerance to metrafenone both *in vitro* and *in vivo,* compared to isolates collected before metrafenone was approved. These results highlight the urgent need for more research into biological alternatives to synthetic fungicides due to the emergence of fungicide-resistant pathogen strains as well as withdrawals of product approval over environmental, health and safety concerns.

With so few fungicides approved for use to control mushroom pathogens, it is inevitable that fungicide resistance in pathogen populations will continue to rise, leaving them ineffective in controlling disease outbreaks; this has been seen in the past with the benzimidazoles, and now with metrafenone (McKay et al., 1998; Grogan, 2006; Romaine et al., 2008). Although prochloraz has remained an effective a.i., despite some shifts in sensitivity, it is no longer approved for use in the EU from June 2023. The sector must now rely heavily on their own disease management strategies and embrace the principles of IPM, especially (1) prevention and suppression through good crop management and hygiene and (2) monitoring and recording so as to detect and treat early occurrences. Ongoing work by us suggests that early detection and salting of disease in a mushroom crop can be as effective as fungicides at controlling the spread of disease. BCAs will have a role to play in future disease control and IPM strategies but, as demonstrated here for two of them, getting a good level of efficacy is challenging. More data are needed to characterise how BCAs work in the mushroom environment and whether or not there are synergies to be had by combining several BCAs rather than relying on one (Barzman et al., 2015). Furthermore, the way crop inoculation trials are conducted to test product efficacy needs to be reconsidered as these protocols were developed with synthetic fungicides in mind (EPPO, 2010). The inoculum doses used to test efficacy of synthetic fungicides can cause severe levels of disease that really test a control agent. Effective synthetic chemicals usually perform well unless a fungicide resistant strain is used. In this study we also tested a lower inoculation rate of 0.5×10^6 spores m^{-2} and the data showed a small reduction in disease expression. It may be that lower inoculation rates offer a more realistic scenario to on-farm conditions and future work with BCAs will explore this hypothesis. However, it is also likely that the responses of different pathogens to BCAs may vary as Navarro et al. (2023) have recently reported two BCAs to be ineffective against *Hypomyces perniciosus* at 1×10^3 conidia m^{-2}.

In conclusion, several cobweb-causing *C. mycophilum* isolates have developed tolerance to the recently approved fungicide, metrafenone. Coupled with the loss of prochloraz as an approved product, this means that the control of cobweb disease of mushrooms into the future will be challenging. Two *B. velezensis*-based BCAs differed in their ability to control cobweb disease under high disease pressure. One product, based on *B. velezensis* Kos, reduced disease symptoms consistently by 30–40% over two crop trials and offers promise in terms of its potential as a future BCA for the sector. This level of efficacy however is not enough to control serious outbreaks of disease therefore future disease control strategies will have to fully embrace the IPM principles of prevention, monitoring and early detection so that early interventions can be made to prevent outbreaks getting out of control.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2023.106530.

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