Evaluation of the efficacy of fungicide and biocontrol treatments for the control of disease on *Agaricus bisporus* mushroom crops

A thesis submitted to Maynooth University for the degree of Doctor of Philosophy

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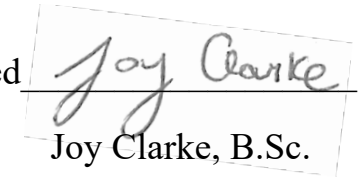
Declaration

This thesis has not been submitted whole or in part to this or any university for any other degree and is the original work of the author except where otherwise stated.

Signed 

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Publications (first author)

Clarke, J., Grogan, H., Fitzpatrick, D. and Kavanagh, K., 2022. Analysis of the effect of *Bacillus velezensis* culture filtrate on the growth and proteome of *Cladobotryum mycophilum. Fungal Biology*, 126(1), pp.11-19.

Clarke, J., Grogan, H., Fitzpatrick, D. and Kavanagh, K., 2022. Characterising the proteomic response of mushroom pathogen *Lecanicillium fungicola* to *Bacillus velezensis* QST 713 and Kos biocontrol agents. *European Journal of Plant Pathology*, 163(2), pp.369-379.

Clarke, J., McGuinness, B., Fitzpatrick, D., Kavanagh, K. and Grogan, H., 2024. Response of the mushroom pathogen *Cladobotryum mycophilum* to the fungicides prochloraz and metrafenone and two *Bacillus*-based biological control agents in mushroom crop trials. *Crop Protection*, 177, p.106530.

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Oral Presentations

Analysis of the effect of *Bacillus velezensis* culture filtrate on the growth and proteome of *Cladobotryum mycophilum.* **Clarke, J**., Grogan, H., Fitzpatrick, D., Kavanagh, K. Irish fungal society annual meeting, virtual event, June 2021.

Analysis of the effect of *Bacillus velezensis* culture filtrate on the growth and proteome of *Cladobotryum mycophilum.* **Clarke, J**., Grogan, H., Fitzpatrick, D., Kavanagh, K. The International Society of Mushroom Science conference, virtual event, June 2021.

Analysis of the effect of *Bacillus velezensis* culture filtrate on the growth and proteome of *Cladobotryum mycophilum* **Clarke, J**., Grogan, H., Fitzpatrick, D., Kavanagh, K. The Society of Irish Plant Pathologists annual meeting, virtual event, November 2021.

Biological control of cobweb disease with *Bacillus*. **Clarke, J**., Grogan, H., Fitzpatrick, D., Kavanagh, K. Teagasc Mushroom Stakeholder seminar, virtual event, February 2022.

Controlling cobweb disease on mushroom farms using *Bacillus* biocontrol agents**. Clarke, J**., Grogan, H., Fitzpatrick, D., Kavanagh, K. Department of Biology Seminar series, Maynooth University, Ireland, March 2022.

Treatment of cobweb disease on white button mushrooms (*Agaricus bisporus*) using biocontrol agents. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. The Society of Irish Plant Pathologists annual meeting, Kildare, Ireland, July 2022

The biological control of cobweb disease on mushroom farms. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. The British Society of Plant Pathologists annual meeting, Newcastle, UK, September 2022.

The mushroom crisis. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. Three-minute thesis competition, Maynooth University, Ireland, October 2022.

Analysis of the effect of *Bacillus velezensis* culture filtrate on the growth and proteome of *Cladobotryum mycophilum.* **Clarke, J**., Grogan, H., Fitzpatrick, D., Kavanagh, K. 8th Conference on Physiology of Yeasts and Filamentous Fungi, Cork, Ireland, June 2023.

The treatment of dry bubble disease on *Agaricus bisporus* crops using fungicide and biocontrol treatments. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. Irish fungal society annual meeting, Dublin, Ireland, June 2023

The management of dry bubble disease on white button mushrooms (*Agaricus bisporus)* with fungicide and biocontrol treatments. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. Department of Biology Seminar series, Maynooth University, Ireland, November 2023.

The control of cobweb disease in *Agaricus bisporus* mushroom crop trials. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. Department of Biology Seminar series, Maynooth University, Ireland, January 2024.

The effect of metrafenone, prochloraz and *Bacillus* based biological control agents on the control of cobweb disease in *Agaricus bisporus* mushroom crop trials. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. The 20th International Society of Mushroom Science conference, Nevada, USA, February 2024.

Investigating the treatment of dry bubble disease during *Agaricus bisporus* crop trial studies. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. Department of Biology annual research day, Maynooth University, Ireland, January 2024.

Characterisation of the microbial population within the *Agaricus bisporus* casing layer and how it is impacted by crop cycle progression and biological treatment application. **Clarke, J**., Grogan, H., Kavanagh, K., Fitzpatrick, D. Irish fungal society annual meeting, Belfast, UK, June 2024.

Poster Presentations

Characterising the proteomic response of mushroom pathogen *Lecanicillium fungicola* to *Bacillus velezensis* QST 713 and Kos. **Clarke, J**., Grogan, H., Fitzpatrick, D., Kavanagh, K. Microbiology Society Annual Conference, Belfast, UK, April 2022.

Characterising the proteomic response of mushroom pathogen *Lecanicillium fungicola* to *Bacillus velezensis* QST 713 and Kos. **Clarke, J**., Grogan, H., Fitzpatrick, D., Kavanagh, K. Department of Biology annual research day, Maynooth University, Ireland, May 2022.

Comparing the use of fungicides and biological control agents for the treatment of cobweb disease. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. Microbiology Society Annual Conference, Newcastle, UK, April 2023.

Comparing the use of fungicides and biological control agents for the treatment of cobweb disease. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. Department of Biology annual research day, Maynooth University, Ireland, May 2023.

Comparing the use of fungicides and biological control agents for the treatment of cobweb disease. **Clarke, J**., Fitzpatrick, D., Kavanagh, K., Grogan, H. Dutch mushroom days event, s-Hertogenbosch, the Netherlands, May 2023.

Characterisation of the microbial population within the *Agaricus bisporus* casing layer and how it is impacted by crop cycle progression and biological treatment application. **Clarke, J**., Grogan, H., Kavanagh, K., Fitzpatrick, D. Microbiology Society Annual Conference, Edinburgh, UK, April 2024.

Characterisation of the microbial population within the *Agaricus bisporus* casing layer and how it is impacted by crop cycle progression and biological treatment application. **Clarke, J**., Grogan, H., Kavanagh, K., Fitzpatrick, D. Irish fungal society annual meeting, Belfast, UK, June 2024.

Awards and Achievements

2nd place winner of Three-minute thesis competition, Maynooth University, October 2023.

Best postgraduate student presentation at Society of Irish Plant Pathologists (SIPP) meeting, July 2022.

Highly commended oral presentation prize at the Irish fungal society (IFS) annual meeting, June 2023.

Awarded a Microbiology Society external event grant to attend the International Society of Mushroom Science (ISMS) congress 2024 (USA) and Microbiology society events grant 2022 (Belfast), 2023 (Birmingham) and 2024 (Edinburgh).

Awarded a British Society of Plant Pathology (BSPP) travel fund to attend the BSPP 2022 annual conference (Birmingham, UK).

Awarded Ken Haynes travel award from the Irish Fungal Society to attend the International Society of Mushroom Science Congress (ISMS), 2024 (USA).

First runner up in the Walsh Scholar of the Year competition in the Crops, Environment and Land Use Programme.

Highly commended oral presentation prize at the Irish fungal society (IFS) annual meeting, June 2024

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Abbreviations

°C	Degrees Celsius
AAA	ATPases Associated with diverse cellular Activities
ACN	Acetonitrile
AI	Active ingredient
a.i.kg	Active ingredient/kilogram
a.i.L	Active ingredient/Litre
AMBIC	Ammonium Bicarbonate
ANOVA	Analysis of variance
APS	Ammonium persulphate
ASV	Amplicon Sequence Variants
ATP	Adenosine triphosphate
BCAs	Biological control agents
CAC	Compost at casing
CF	Culture filtrate
CFU	colony forming units
cm	Centimetre
ddH2O	Deionised water
dH2O	Distilled water
DI	Disease incidence
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide 5'-triphosphate
DTT	Dithiothreitol
EU	European Union
EC	European commission
g	Grams
g	g-force

GO	Gene ontology
GOBP	Gene ontology biological process
GOCC	Gene ontology cellular component
GOMF	Gene ontology Molecular function
GRAS	Generally regarded as safe
GTP	Guanosine-5'-triphosphate
h	Hours
HIS	Hyperspectral imaging
hr	Hours
HCl	Hydrochloric acid
IAA	Iodoacetamide
IPM	Integrated pest management
ISWI	Imitation SWItch
ITS	Internal transcribed spacer
Kg	Kilogram
kDa	Kilodaltons
LFQ	Label free quantification
L	Litre
LC-MS	Liquid chromatography mass spectrometry
M	Molar
m	metres
MBC	methylbenzimidazole carbamate
MEA	Malt extract agar
Mg	milligram
min	Minute
mL	Millilitre
mM	Millimolar
mm	Millimetre

MS	Mass spectrometry
MT	Million tonnes
NA	Nutrient agar
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Nutrient broth
ns	not significant
ng	Nanogram
nl	Nanolitre
nm	Nanometre
PBS	Phosphate buffered saline
PCA	principal component analysis
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
PDA	Potato dextrose agar
PMSF	Phenylmethylsulphonyl fluoride
pH	Potential hydrogen
PRIDE	PRoteomics IDEntifications
RH	Relative humidity
ROS	Reactive oxygen species
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDB	Sabouraud dextrose liquid broth
S.E.	Standard error
SD	Standard deviation
Sec	Seconds
SEM	Scanning electron microscopy

SSDA	Statistically significantly differentially abundant
STRING	Search Tool for the Retrieval of INteracting Genes/Proteins
SUD	Sustainable use of pesticides directive
TFA	Trifluoroacetic acid
U	Unit
v	Volume
v/v	Volume per volume
w/v	Weight per volume
µg	Microgram
µl	Microliter
µM	Micromolar

Abstract

Disease control within the mushroom industry has become a significant challenge. Diseases of *Agaricus bisporus* were once controlled with the use of preventative chemical fungicides. However, the number of approved fungicides has significantly reduced. There is an urgent need to find viable alternative treatments, which is the primary aim of this thesis. Two biocontrol strains (*Bacillus velezensis* QST 713 & Kos) were investigated for their ability to control cobweb disease (*Cladobotryum* spp.) and dry bubble disease (*Lecanicillium fungicola)*, two major pathogens of cultivated mushrooms.

B. velezensis Kos was able to significantly reduce the growth of *Cladobotryum* and *L. fungicola* in liquid/plate cultures and resulted in structural damage to fungal hyphae. Lytic enzymes such as subtilisin were identified within the inhibitory component of the *B. velezensis* culture filtrate (CF). The CF also triggered changes to protein abundance from both pathogens. Proteins associated with stress were increased in abundance compared to the control, while proteins associated with growth were decreased. Similar *in vitro* responses were recorded for the *L. fungicola* pathogen in response to *B. velezensis* QST 713. Biocontrol strains were investigated at a crop level and their efficacy was compared to conventional fungicide treatments. A *C. mycophilum* isolate highly tolerant to metrafenone, was identified. Metrafenone was shown to be capable of controlling dry bubble disease but not cobweb disease caused by tolerant isolates. Biocontrol treatments based on *B. velezensis* were shown to significantly control dry bubble disease when disease pressure was low to moderate. However, biocontrol treatments struggled to control both dry bubble and cobweb disease under extreme disease levels. It was determined that the application of biocontrol treatments did not significantly impact casing microbiome dynamics. The lack of persistence of biocontrol strains within the *A. bisporus* casing may explain the reduced antagonistic abilities of both strains at a crop level. Other integrated pest management strategies such as salting, and disease monitoring were shown to be effective at limiting disease symptoms.

Results suggest that biocontrol agents can form part of the future mushroom disease control strategies in combination with increased hygiene and integrated pest management strategies.

Chapter 1
Introduction

1.1 Overview of mushroom species

1.1.1 Kingdom Fungi

The kingdom of fungi contains eukaryotic organisms which can be either unicellular or multicellular and live as heterotrophs (Whittaker, 1969). Although they were originally classified in the same kingdom as plants, we know now that fungi are more closely related to the animal kingdom (Baldauf & Palmer, 1993). In nature, the main role of fungi is as saprophytic decomposers, recycling carbon, nitrogen, and other essential mineral nutrients back to the environment from non-living organic matter. However, fungi may be parasitic in nature and cause harm to their hosts (Volk, 2013). Fungi may also be mycorrhizal which grow in association with plant species. There are also endophytic fungi, that form complex relationships with plant species, which may be either parasitic or mutualistic (Nair *et al*., 2014). Fungal species can be divided into several phyla, with the subkingdom Dikarya containing the 'higher fungi' which include the Ascomycota, and the Basidiomycota.

The division of Basidiomycota include about one third of all fungal species, this diverse group of fungi include both aquatic and terrestrial species (Taylor *et al.*, 2015). Basidiomycota is further divided into three sub-phyla, Pucciniomycotina (rusts and others), Ustilaginomycotina (smuts and others) and Agaricomycotina (mushroom-forming fungi) (Taylor *et al.*, 2015). Within the sub-phyla Agaricomycotina is the class Agaricomycetes which contains the orders Agaricales (gill bearing mushrooms). The macroscopic fungus *Agaricus bisporus* (J. E Lange) [Imbach] (white button mushroom) which is the focus of this thesis belongs to the order Agaricales within the Agaricomycetes sub-phylum of the Basidiomycota (Margulis & Chapman, 2009; Schoch *et al.*, 2020) (**Figure 1.1**). The term 'mushroom' refers to the characteristic fruiting body produced by many Basidiomycete fungi.

The members of the division of Ascomycota have been found living in extreme environments but they are also commonly found in forest and agricultural habitats (Wijayawardene *et al.*, 2021). Ascomycota are defined by having an ascus, which is a spore producing reproductive structure, from which the haploid ascospores are produced during sexual reproduction. Asexual reproduction or propagation without sexual fusion can also be achieved by Ascomycota with the production of asexual

conidia spores (Margulis & Chapman, 2009). The three subphyla of Ascomycota are Taphrinomycotina (leaf curl fungi), Saccharomycotina (yeasts) and Pezizomycotina (all other ascomycetes). Within the subphyla Pezizomycotina is the class Sordariomycetes which contains the genera *Lecanicillium* and *Cladobotryum*, both of which are studied in this thesis (Schoch *et al.*, 2020) (**Figure 1.1**).

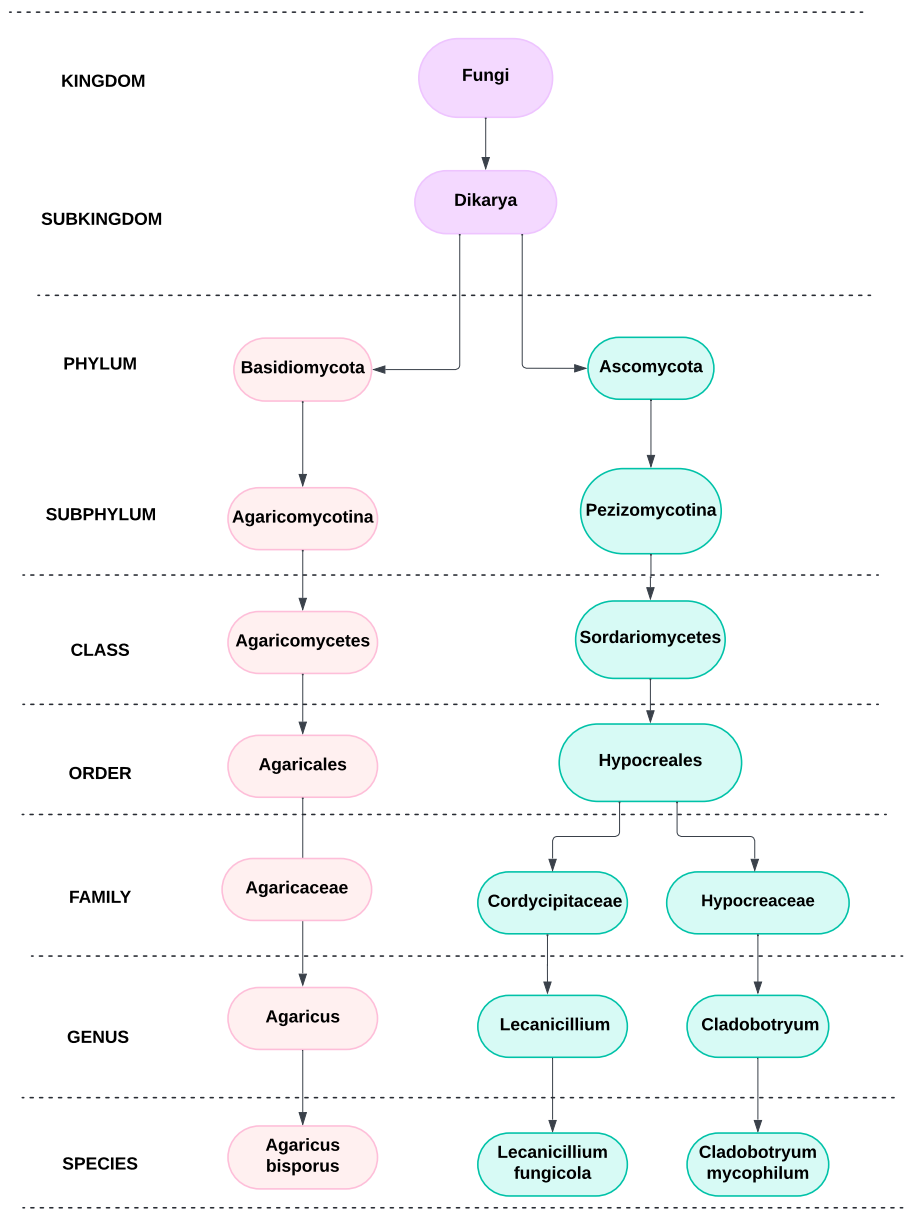

Figure 1.1 Taxonomy of fungal species investigated during this thesis (Schoch *et al.*, 2020). Figure created using Lucid (lucid.com)

1.1.2 The history of mushroom use

The importance of mushrooms to human civilisation can be traced back to the first hunter gathers who depended on foraging in their environment for survival. Evidence of mushroom diets in humans can be found as early as the upper Palaeolithic era. Researchers analysed microremains from the 'Red Lady of Paviland' and found fungal spores belonging to *Boletus* and *Agaricus* species (Power *et al.*, 2015). The mummified remains of a male person, who lived more than 5000 years ago who is now referred to as 'Otzi' or 'the Iceman' was discovered with the fruiting body of *Piptoporus betulinus* in his possession. It is theorized that Otzi used this mushroom for medicinal purposes, to treat an intestinal parasite also found in his body (Peintner *et al.*, 1998). Another study has reported that during the early 20th century, indigenous peoples of Northern America used the saprophytic wood-inhabiting fungus *Halploporus odorus* as an object of great spiritual importance (Blanchette, 1997). It was worn around the necks of older people to protect them from illness. In ancient Egypt, mushrooms were 'plants of immortality' and were only consumed by Egyptians with significant nobility (Niksic *et al.*, 2016; Ahmed *et al.*, 2023). Similarly, ancient Romans perceived mushrooms as 'God's food' which were considered a luxury food which was reserved only for those with great wealth (Niksic *et al.*, 2016).

1.1.3 Benefits of mushrooms

Mushrooms are highly nutritious and can provide significant health benefits to consumers. They are low in calories (27–30 kcal/100 g), fat and cholesterol but have a high content of carbohydrates, vitamins, minerals, fibre, protein, and polyunsaturated fatty acids (Mattila *et al.*, 2002; Krishnamoorthi *et al.*, 2022). Their unique 'umami' taste and advantageous therapeutic properties have meant that mushrooms are a core ingredient in the cuisines of many different cultures. Mushrooms are a popular ingredient in vegetarian/vegan diets. Plant-based ingredients blended with mushrooms can mimic many meat-based products such as sausages and burger patties to provide a meat-free alternative. Mushrooms have also been used as additives/substitutions in meat products to reduce their sodium, salt and fat content (Wang *et al.*, 2019; Wong *et al.*, 2019; Rangel-Vargas *et al.*, 2021). Another beneficial nutritional quality of mushrooms is that they are high in vitamin D, most significantly in vitamin D_2, but also vitamin D_3 and D_4 (Cardwell *et al.*, 2018). Vitamin D is an important nutrient for

the human diet. It increases absorption of calcium, helping to prevent osteomalacia in adults and rickets in children (Lips, 2006). Vitamin D_3 is mainly obtained in the diet through the consumption of animal-based proteins such as oily fish. Mushrooms are the best source of vitamins D_3 in vegetarian and vegan diets. The level of vitamin D in mushrooms can be enhanced even further by exposing the mushrooms to ultraviolet light prior to consumption (Hu *et al.*, 2020).

The antioxidant effects of mushrooms have also been widely reported (Obodai *et al.*, 2014; Boonsong *et al.*, 2016; Gąsecka *et al.*, 2018; Ramos *et al.*, 2019). This antioxidant effect is due to mushrooms being rich in bioactive compounds (phenolic compounds, polyketides, terpenes and steroids). Mushrooms have been used in Chinese medicine for centuries. An encyclopaedic collection of Chinese herbology called 'Compendium of Materia Medica' lists over twenty mushrooms species. This book was written by Li Shizhen during the Ming Dynasty (Niksic *et al.*, 2016). Generally, the use of mushrooms in modern medicine is referred to as 'complementary or alternative medicine', meaning it can be taken alongside conventional medicines and therapies. There are many studies which investigate the direct link of *A. bisporus* with the treatment of cancer, cardiovascular and inflammatory diseases (Chen *et al.*, 2002; Twardowski *et al.*, 2015; Atila *et al.*, 2017). However sometimes conflicting evidence can be seen in these reports and studies are often limited to cell-line or animal model studies (Rizzo *et al.*, 2021). Therefore, caution is needed when stating the medical effects of mushrooms and further research and human clinical trials are required in this area.

The production of meat and dairy based products are known to contribute to greenhouse gas emissions and global warming. The livestock industry contributes 12-18% of total greenhouse emission (González *et al.*, 2020). The cultivation of *A. bisporus* and other mushroom species is known to be one of the most environmentally sustainable cultivation practices. Waste products from different sectors of agriculture and forestry are the foundation of the mushroom industry's compost or substrate. After a crop has been grown, the used compost or 'spent mushroom substrate' (SMS) can be further recycled and re-used in different agricultural areas (see section 1.2.8). Therefore, mushroom cultivation can be described as a circular economy (Grimm & Wösten, 2018). One of the main components of casing used during mushroom

cultivation is peat, a non-renewable, fossil-based material (see section 1.2.5). More sustainable alternatives for casing materials are under investigation (Noble *et al.*, 2023).

Recent studies into the structural properties of fungal mycelium have made it possible to engineer novel biomaterials from fungal mycelium sources (Islam *et al.*, 2017; Jones *et al.*, 2017; Appels *et al.*, 2018; Sun *et al.*, 2019). An exciting new area which is emerging involves growing mushroom mycelium to produce eco-friendly materials which can mimic plastics, leather, and textiles. Ecovative is a company established in 2007 which specialises in the production of 'Myco-Materials' such as footwear, apparel and packaging.

1.1.4 The white button mushroom – *Agaricus bisporus* (J.E Lange) [Imbach]

Approximately 11% of the cultivated mushrooms crops produced globally are *Agaricus bisporus*, more commonly known as the white button mushroom (Singh *et al.*, 2020). The structure of the white button comprises of a stipe (stalk) and a hemispherical, fleshy pileus (cap), under which, gills/lamellae can be found. A ring of tissue called the annulus can be found around the stipe (Ramos *et al.*, 2019). Gills function in the production and dispersion of fungal spores when the mushroom reaches maturity. A veil or velum is defined as a thin layer of tissue which completely covers the cap and stipe of an immature mushroom body. The veil of the mushroom gradually opens as it matures (Umar & Van Griensven, 1997). White button mushrooms are generally sold as fresh produce but can also be canned or frozen. *A. bisporus* has two different colour varieties, white (white button) and brown (cremini). A cremini mushroom which has grown to full maturity is referred to as a portobello mushroom. The lifecycle of *A. bisporus* differs from the characteristic heterothallism lifecycle of other homobasidiomycetes. Rather than producing four spores per basidium, each containing one haploid nuclei after meiosis, *A. bisporus* predominately produces two spores per basidium. Each contain a pair of non-sister nuclei and so are diploid from the start and capable of reproduction without the need to mate (Sonnenberg *et al.*, 2011). Spores will develop into hyphae and form heterokaryotic mycelium networks which, under the right conditions, can produce fertile mushroom fruitbodies (Savoie *et al.*, 2013). This life cycle is classified as secondary homothallism or pseudo-homothallism (**Figure 1.2**) (Savoie *et al.*, 2013). *A. bisporus* strains with tetracyclic

(four spored) basidia have also been observed, although very rarely. These spores are haploid and must anastomose with a complimentary haploid mycelium to form a fertile diploid mycelium that can produce a fruitbody (Callac *et al.*, 1993). Another distinguishing feature of *A. bisporus* is that it lacks clamp-connections, which is a characteristic feature of other basidiomycetes (Flegg *et al*., 1985). The purpose of clamp-connections is to maintain the binucleate nature of the mycelium. Microscopic filaments or hyphae will extend and branch to form mycelium which is the vegetative phase of the *A. bisporus* lifecycle. *A. bisporus* is said to be in the sexual phase when it has developed the spore-bearing structure (fruiting body). The spores produced by *A. bisporus* are chocolate-brown in colour (Flegg *et al*., 1985).

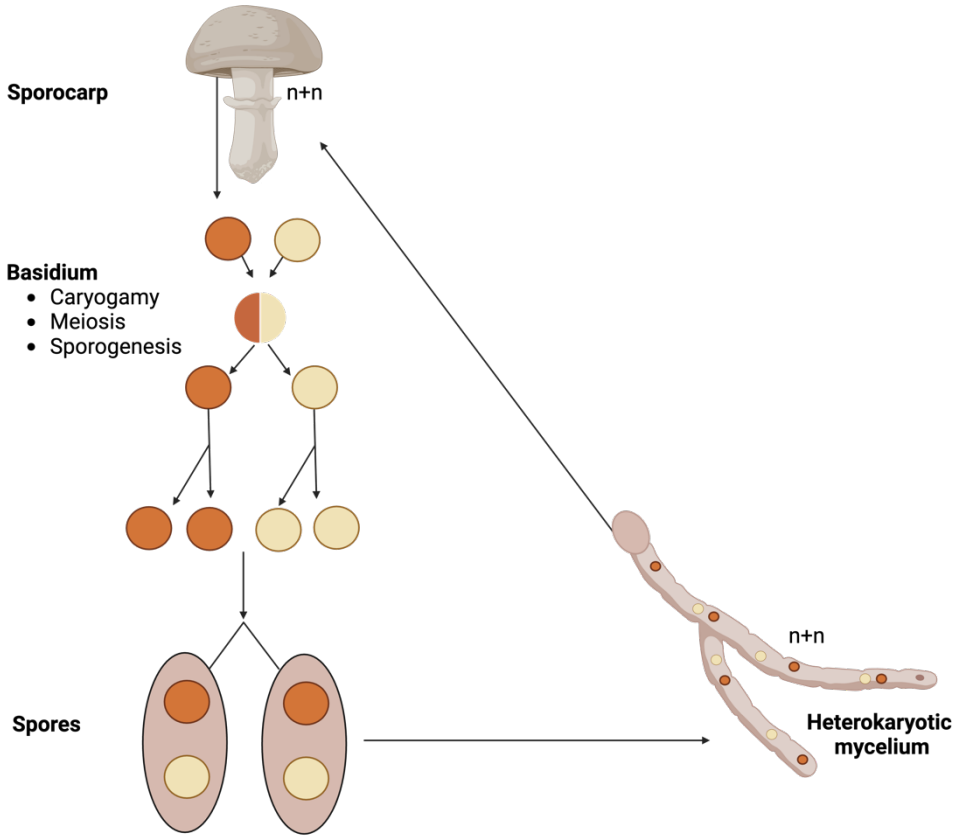

Figure 1.2 Secondary homothallism lifecycle which is characteristic of many *Agaricus bisporus* species. Adapted from Savoie *et al.*, 2013. Created with BioRender.com

1.1.5 Early *Agaricus bisporus* cultivation

One of the earliest records of growing mushrooms was believed to be described by the royal academy of science in 1707, when it was observed that *Agaricus bisporus* could be grown on horse manure in France (Smith, 1993). Although quite primitive, this first description of mushroom cultivation became highly significant, as horse manure is still a key component of mushroom cultivation today. Mushroom cultivation in the early years was very unreliable, but gradually new developments and techniques contributed to a more stable production. Mushroom cultivation became a reliable process which was undertaken in several countries. At the beginning of the 19th century, France was one of the first countries which grew *Agaricus bisporus* at a relatively large scale, this began in abandoned quarries located underneath Paris. These quarries provided an ideal environment for mushroom growing. France and the UK held onto a monopoly for mushroom production up until World War II, when countries, such as the USA, Canada, Sweden and Switzerland were able to develop mushroom production. In the 1960/70s, more countries like Australia, Spain, Hungary, Poland and Ireland began to produce mushrooms. Today, these countries are still growing mushrooms but production in recent times has been dominated by Asian countries, led by China which is the premier mushroom producer worldwide (Royse *et al.*, 2017).

1.1.6 Global mushroom production

Commercial mushroom production is an important horticulture sector for many countries. The world production of cultivated mushrooms was estimated to be 43 million tonnes (MT) in 2018/2019 (Singh *et al.*, 2020). China is by far the largest producer of mushrooms and has seen the most significant increase in production in the past 30 years. It is estimated that China produces 88% of the mushrooms grown commercially in the world (Singh *et al.*, 2020). The cultivation of *Agaricus bisporus* was most popular in the 20th century and remains the most common mushroom produced and consumed in Europe, North America and Australia. Globally production of *A. bisporus* has been surpassed by mushroom species such as *Lentinula edodes* (Shiitake mushroom), *Auricularia spp* (wood ear mushroom), *Pleurotus* ostreatus (Oyster mushroom) and *Flammulina velutipes* (Enoki mushroom) (Royse *et al.*, 2017). China is the largest producer of *A. bisporus*, at 2.48 million tonnes, followed by Europe (1.32 million tonnes), the Americas, Oceania and Africa (Singh *et al.*, 2020) Within

Europe, Poland and the Netherlands are the largest producers of mushrooms, followed by Spain, the UK, France, Germany, Italy and Ireland (**Figure 1.3**) (Singh *et al.*, 2020). Many of the mushroom species which are cultivated on a global scale are saprophytic species which require non-living, organic matter for substrate. The large-scale cultivation of mycorrhizal mushroom species such as chanterelle, truffle and porcini has been achieved but requires much more complicated approaches. Fruit body production is dependent on complex fungi-plant interactions and trying to mimic specific growth requirements on a large scale, is challenging, especially compared to saprophytic mushroom species (Hall *et al.*, 2003; Wang & Chen, 2014).

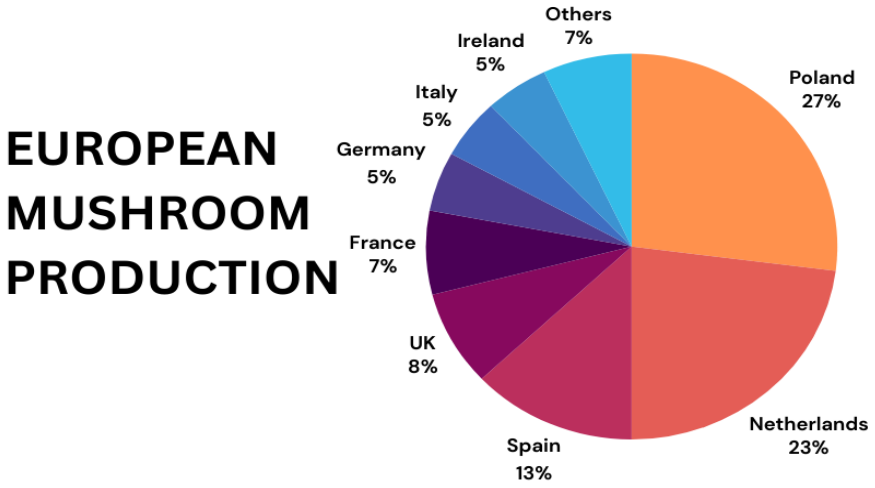

Figure 1.3 Mushroom production from European countries in 2019, FAOStat. Adapted from (Singh *et al.*, 2020) Created with Canva.com

1.2 Modern *Agaricus bisporus* cultivation

Generally, the procedure for cultivating *A. bisporus* is similar between all mushroom-producing countries and involves a sophisticated multi-step process (**Figure 1.4**). *Agaricus bisporus* can be grown on shelves or in trays, bags, blocks, or troughs, depending on the preferences of the individual producer or location. The composition of the compost or substrate used during *A. bisporus* cultivation may be subject to the local variations, but in general, an animal-manure nitrogen source (e.g. horse or poultry manure) is mixed with a cellulose-rich material like hay or cereal straw, a compost activator material like urea and an inorganic conditioner like gypsum (Grimm & Wösten, 2018). Mushroom farming consists of several production steps with one common goal, to facilitate the production of a high yield of healthy *A. bisporus* mushrooms. The first steps of this process involve biologically composting the substrate so it will have the optimum nutrient level for *A. bisporus* to grow but will not support the growth of unwanted microbes which may cause disease to the crop. This is achieved through a two-phase fermentation process (Phase I and II compost) first described by Sinden and Hauser in 1950 (Sinden & Hauser; 1950).

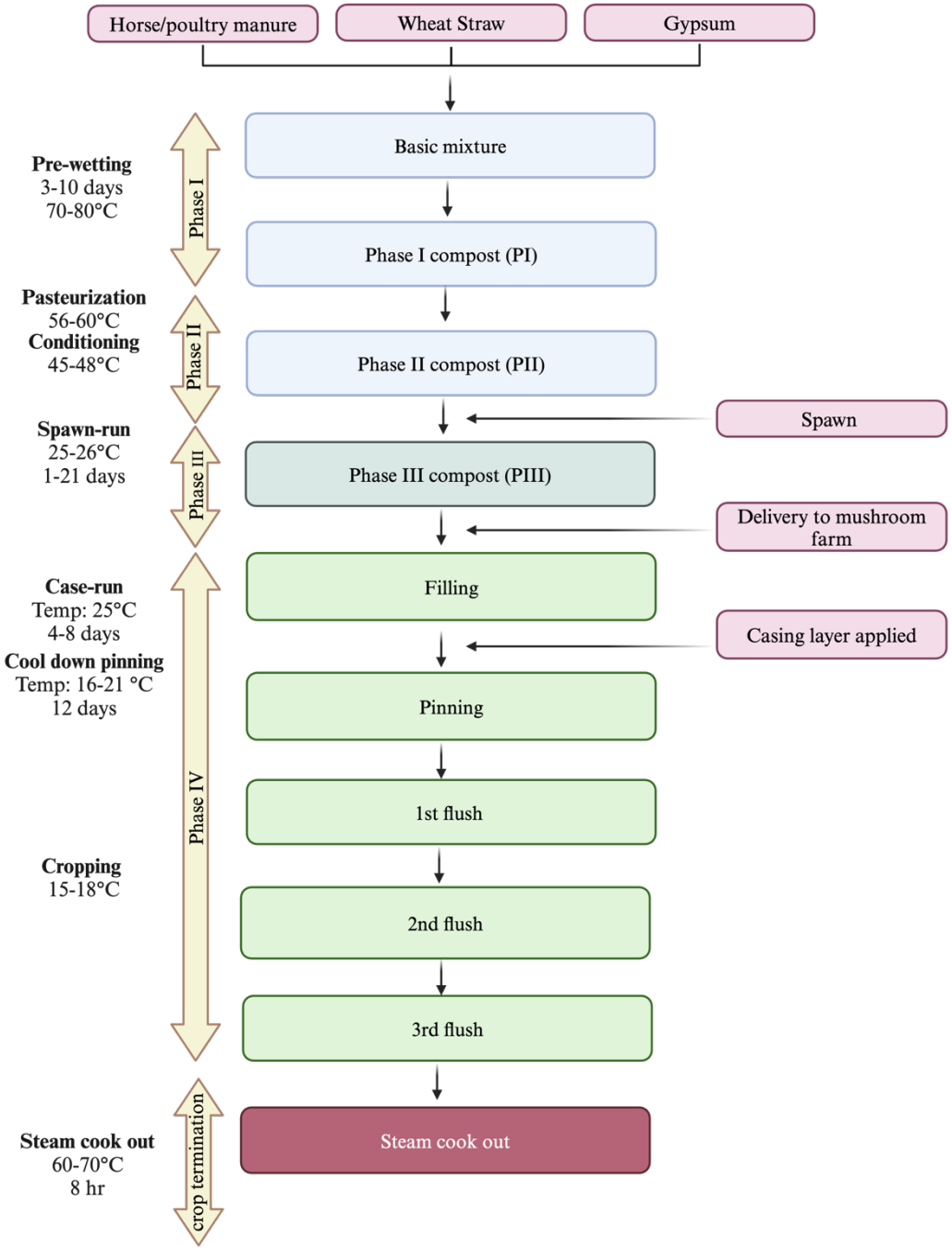

Figure 1.4 A schematic overview of the modern white button mushroom (*Agaricus bisporus*) cultivation growth cycle. Adapted from (Kabel *et al.*, 2017). Created with BioRender.com

1.2.1 Phase I compost

Phase I is the first step of mushroom substrate production. The raw materials are first wetted and mixed for 3-10 days (Beyer, 2024). This step is carried out in either an open yard or covered bunker. It is then stacked into piles which are left for several days to soften (Beyer, 2024). At this point aerobic fermentation will commence. The microbial metabolism which occurs within the stacks cause the release of heat, ammonia, and carbon dioxide. The temperature within the centre of the stacked substrate can reach 70-80°C, this high temperature will remove many microorganisms from the substrate, leaving mainly thermophilic species (Beyer, 2022; Thai *et al.*, 2022). The moisture, oxygen, nitrogen, and carbohydrate levels must be monitored carefully during this process. Most producers use aerated floor systems to ensure adequate aerobic conditions throughout the pile and reduce odour emissions. Stacks are turned to ensure consistent degradation and to maintain aerobic conditions throughout the entire stack. Phase I is complete once the substrate is pliable, can hold water and has a strong smell of ammonia (Smith, 1993; Beyer, 2024).

1.2.2 Phase II compost

The Phase I substrate is filled into a tunnel to initiate Phase II composting. The main aim of this process is to pasteurize the substrate, which is done in an enclosed temperature-controlled room under aerobic conditions (Noble & Gaze, 1996). The substrate is heated to a pasteurization temperature of between 56-60°C for 8 to 12 hours (den Ouden, 2016). Pasteurization will selectively sterilise the substrate of microorganisms or insects which may contribute to disease later in the crop or compete with *A. bisporus* growth (Beyer, 2024; Beyer, 2023). Once the pasteurisation stage is complete a conditioning phase commences where the temperature is maintained between 45-48°C for 4-5 days (den Ouden, 2016). Conditioning involves favouring the conditions that will sustain the survival of the beneficial microbes that will convert the Phase I compost into a complex lignocellulosic substrate that favours *A. bisporus* growth (Thai *et al.*, 2022). Phase II substrate should be completely free of ammonia which is toxic to mushroom growth (Noble & Gaze, 1996; Beyer , 2022). Ammonia is removed by microorganisms which are not affected by the previous sterilisation process. Ammonia is converted to amine and other nitrogen compounds by these microorganisms and these compounds are used by the developing *Agaricus* mycelium

to grow. The substrate is cooled when ammonia can no longer be detected. The substrate should also have a high moisture content between 70-75% (Smith, 1993; Mcgee, 2018).

1.2.3 Spawn-run

During the next stage, the *A. bisporus* mycelium is introduced into the Phase II compost in the form of mushroom 'spawn' or inoculum. Spawn is made by dedicated commercial companies. In principle, pure, contaminant free cultures of *A. bisporus* mycelium are added to sterilised spawn grains, either rye, millet, barley or other cereal grain. Once the spawn grains are fully colonised, they can be bagged and cold-stored for a short period of time before being used to inoculate Phase II substrate. Colonised spawn grains essentially act as a carrier to introduce the *A. bisporus* mycelium into the Phase II compost where *A. bisporus* can use the nutrients in the spawn grain to initiate colonisation of the Phase II substrate. Spawn grains should be evenly distributed throughout the compost to ensure consistent and uniform development of *A. bisporus* mycelium (den Ouden, 2016). Horst ® U1 and Horst ® U3 were the first varieties developed for *A. bisporus* commercial production (Van Griensven & Van Roestel, 2004) with Horst U1 being the most popular for growing for white varieties. Modern strains are largely derived from hybrids originating from these strains (Sonnenberg *et al.*, 2017). A Horst U1 and Horst U3 hybrid strain (Sylvan A15), has replaced Horst ® U1 as the most popular cultivar for commercial spawn production. Substrate colonization or 'spawn-run' requires a substrate temperature range of 25-26°C and uniform air circulation, a process which usually lasts 16 to 19 days. As the substrate is colonised, delicate white thread-like structures of *Agaricus* mycelia will begin to grow throughout the compost (Van Griensven & Roestel, 2004; Beyer, 2024).

1.2.4 Bulk Phase III

Historically, growers would have carried out spawn run themselves, either by spawning their own substrate, or buying in spawned substrate in bags, blocks or bulk, however it is increasingly more common today for growers to get bulk Phase III compost delivered in bulk from specialised compost companies. These companies carry out Phase I, Phase II and Phase III spawn run in separate purpose-built facilities with very high standards of sanitation. The grower only has to apply the casing layer

to the Phase III compost to begin the cropping cycle on their farm. Despite the increased cost, bulk Phase III allows growers to produce more crops on average per year (Beyer, 2022). The large quantities of Phase III compost produced per batch means that there is considerable vulnerability for the composter in terms of greater risk should a batch become contaminated, as a single batch can be delivered to multiple mushroom farms.

1.2.5 Casing application

A casing layer covering the spawned substrate is required to stimulate the fruitification of the mushroom i.e. to facilitate the *A. bisporus* mycelium switching from a vegetative phase to a reproductive phase (Noble *et al.*, 2003). The specific reasons for why the casing layer is essential for mushroom fruitification have not been fully characterised, but several functions of the casing layer have been identified. It provides structural support for the emerging mushrooms and contains the correct chemical/physical properties which facilitates the correct moisture levels and prevents the substrate from drying out. The microbial population of the casing layer is also very important to promote the fructification of *A. bisporus*. The casing layer is most commonly composed of sphagnum-based peat due to its excellent water retention capacity. However, it is expected that in the future casing will be made of an alternative material. Peat is a non-renewable resource and most countries discourage the use of peat. Research in this area is ongoing, suitable replacement materials are under investigation (Noble *et al*., 2023). A CACing technique (**C**ompost **A**dded at **C**as**ing**) involves adding casing inoculum to the casing layer to improve crop uniformity and quality. This technique was developed in Ireland in 1972 (MacCanna, 1972). Air temperature and compost temperature in the growing room is kept at 25°C for 4-8 days after the casing layer is added (case-run) to encourage growth of the *Agaricus* mycelium into the casing layer (den Ouden, 2016). An appropriate level of water will also be applied during this time. The length of case-run depends of the amount of CAC material applied. Applying larger volumes of CAC at the beginning of case-run means that *Agaricus* mycelium can colonise faster which will shorten case-run (den Ouden, 2016).

1.2.6 Cropping

During case-run, the *A. bisporus* mycelium expands and extends throughout the compost and casing layer absorbing nutrients to aid in its growth. Once case-run is complete, cool-down pinning can commence. Environmental conditions in the growing room need to be changed to initiate fruit body formation. This switch mimics the change in conditions experienced in summer months to conditions of colder autumn months, which is the natural environmental trigger which causes mushroom fruitification in nature (den Ouden, 2016). CO_2 levels are dropped gradually by introducing fresh air and the temperature is gradually dropped to 16-21°C (cool down pinning). When the mycelium begins condensing and growing upon itself, a multi-hyphae formation termed a hyphal knot is formed (Moore, 1995). The hyphal knot then also condenses to become a primordia. Many thousands of primordia can be produced from one mycelium, however only those which grow the fastest and are most productive will develop into a fruiting body which meets the desired criteria for retail (Straatsma *et al.*, 2013). The changes in environmental conditions stimulate the production of *A. bisporus* primordia or mushroom pins to develop from the hyphal knots which have developed on the *A. bisporus* mycelium. The timing of this environmental trigger is very important to the yield and quality of the mushrooms which will develop. Mushroom pins will grow and develop into pre-buttons, which in turn develop into mature mushrooms (Pardo-Giménez *et al.*, 2017). At this point, cropping can commence. Mushrooms can be harvested over 2-4 days for generally 3 flushes, with each flush lasting 7-10 days, temperature is maintained at 15–18°C (Beyer, 2024). The first flush of mushrooms will be the most productive of the crop, accounting for approximately 50% of total yield. The second flush and third flush contribute 35% and 15% respectively (den Ouden, 2016). The maturity of a mushroom crop is determined by how open its veil is. White button mushrooms are generally harvested with a closed cap but a variety of mushroom sizes can be harvested such as buttons, closed cups, opens and flats. Freshly picked mushrooms must be kept refrigerated to prolong their shelf life both before and during shipment.

1.2.7 Steam cook out

Once a mushroom crop is no longer productive, the growing room should be ‘cooked out’ using steam. During steam cook out, the compost in the room should reach a

temperature of 60-70°C for a minimum of 8 hours (den Ouden, 2016). This is done to destroy any pests or pathogens that may be present and avoid diseases or pests from one crop being spread to neighbouring crops (Beyer, 2022). It also clears the room of any pest/pathogens so that the next mushroom crop can be brought in without risking contamination.

1.2.8 Spent mushroom substrate (SMS)

The substrate that has gone through the cook out procedure is referred to as spent mushroom substrate (SMS). SMS has further uses and can be recycled in other areas of agriculture and horticulture. SMS has been re-used for the cultivation of new mushroom crops following the supplementation of the nutrient content. This has been most successful with *Pleurotus* species and is less applicable to *A. bisporus* cultivation (Mamiro *et al.*, 2007). SMS has a high level of organic matter along with nutrients such as nitrogen, phosphorous and potassium, which makes it ideal to act as a soil conditioner or to supplement the nutrition of a range of different crops (Medina *et al.*, 2012; Peregrina *et al.*, 2012; Wuest *et al.*, 2013; Paula *et al.*, 2017; Velusami *et al.*, 2021). SMS can also be used as a dietary supplement for poultry, ruminants and monogastric animals (Fazaeli & Masoodi, 2006; Fazaeli *et al.*, 2014; Martín *et al.*, 2023). All of these uses contribute to how mushroom cultivation can be a part of sustainable agriculture and a circular bioeconomy (Grimm & Wösten, 2018).

1.3 Mushroom disease

There are several diseases which may challenge productivity in *Agaricus bisporus* cultivation (Fletcher & Gaze, 2008). The texture, size and colour of a mushroom is a very important factor that growers must consider when selling their produce to consumers. Generally disease will result in mushrooms of poor quality which are aesthetically unpleasing and must be discarded. This will negatively impact the yield of healthy mushrooms and consequently the revenue that the grower gets from their crop. More revenue will also have to be invested into disease control methods to prevent disease from spreading further. This may involve additional labour costs as disease levels across the farm will have to be monitored carefully (Grogan, 2008). Disease may be caused by viral, bacterial or fungal pathogens and range in severity and symptoms (Fletcher & Gaze, 2008). Disease is a particular issue for mushroom growers as the optimum conditions used to grow the mushrooms also favour the growth and dispersal of pathogens (i.e. warm temperatures, high humidity and low aeration) (den Ouden, 2016). Although every effort is made to make mushroom compost selective for the growth of *A. bisporus* only, it is possible for other fungal species to thrive in this environment. This is why it is critical to take great care to avoid the introduction of pathogens into the compost during its preparation. Once the pathogen is present, it is very likely that disease will develop on the crop. There are four major fungal pathogens which cause significant problems for mushroom growers. They are; green mould disease (*Trichoderma aggressivum*), wet bubble disease (*Mycogone perniciosa*), dry bubble disease (*Lecanicillium fungicola*) and cobweb disease (*Cladobotryum* spp.) (Fletcher & Gaze, 2008). This thesis will focus on dry bubble disease and cobweb disease.

1.3.1 Cobweb disease

Prior to the 1990s, cobweb disease was of little importance to mushroom growers. The disease, caused by species of *Cladobotryum*, was known to affect mushrooms in the wild but generally only caused minor issues on farms which were quickly resolved with fungicide use (Grogan and Gaze, 2008). In the wild, *Cladobotryum* spp. grow on substrates such as leaf litter and decaying wood. They mostly infect the orders Aphyllophorales and Agaricales (Gams & Hoozemans, 1970). This changed drastically in the 1990s when cobweb disease became more prominent on mushroom

farms and began to reach epidemic proportions in the UK and in Ireland, causing severe disease (Grogan & Gaze, 2008). Crop losses of up to 40% were reported during this time (Adie *et al.*, 2006). There were several reasons why this outbreak occurred, including advances in cultivation techniques at this time. Mushrooms were grown in much warmer and moist conditions compared to pre-1990s, which may have facilitated *Cladobotryum* growth (Gaze, 1995). The emergence of resistance to benzimidazole fungicide treatments was also a major factor associated with severe outbreaks (Grogan and Gaze, 2000). Cobweb disease is now considered one of the four most serious fungal diseases of mushrooms that can cause many problems for mushroom growers. It has been reported in all of the major mushroom growing countries, including Ireland (McKay *et al.*, 1999), China (Zuo *et al.*, 2016), UK (Adie *et al.*, 2006), Spain (Gea *et al.*, 2012), and France (Largeteau & Savoie, 2010). Cobweb disease most commonly is reported late in the crop cycle, usually in the second or third flush. However, the earlier cobweb disease appears, the more severe and problematic disease symptoms will be. It can affect crops year-round, but it has been observed that infection on Spanish crops was higher in autumn and winter compared to spring and summer (Carrasco *et al.*, 2016). This correlates with the high incidence of fungal fructifications in woodlands in the autumn time, which can then be infected with *Cladobotryum* spp. Many aspects of cobweb disease have been detailed in a comprehensive review by Carrasco *et al*., (2017).

1.3.1.1 Cobweb disease causative agents: *Cladobotryum* spp.

Cobweb disease is caused by several fungal species belonging to the *Cladobotryum* genus. Disease outbreaks on *Agaricus bisporus* mushroom farms is caused most commonly by *C. dendroides* (teleomorph: *Hypomyces rosellus*), *C. mycophilum* Type 1 and *C. mycophilum* Type 2 (teleomorph: *Hypomyces odoratus*). It was found that *C. mycophilum* Type 2 was responsible for the most recent cobweb epidemic (Grogan & Gaze, 2000). It is known to be a more aggressive strain than either *C. dendroides* or *C. mycophilum* Type 1 (Grogan & Gaze, 2008). Other species such as *C. varium, C. semicirculare, C. asterophorum, C. protrusum, C. multiseptatum* have also been reported as causing cobweb disease on mushroom species (McKay *et al.*, 1999; Carrasco *et al.*, 2017).

The *Cladobotryum* species causing disease should be identified in order to manage cobweb disease effectively as different species may have different sensitivity to fungicide products. *Cladobotryum* isolates can be identified through traditional morphological and taxonomic characterisations as well as more advanced molecular and phylogenetic techniques. A good distinguishing feature to test when differentiating *Cladobotryum* strains is whether or not the species produces a distinct camphor odour or is a producer of aurofusarin which is associated with a distinct red/brown colour (Carrasco *et al.*, 2017). Aurofusarin is a secondary metabolite secreted by some *Cladobotryum* strains in response to stress. The agar growing aurofusarin-producing *Cladobotryum* strains will turn red in colour after approximately 25 days as the nutrients from the agar plates begins to diminish (Põldmaa, 2011). *Cladobotryum* species produce branched conidiophores which branch into 3-4 phialide tips. Initially the conidia are unicellular, but will develop 1-3 septa (Gams & Hoozemans, 1970; Rogerson & Samuels, 1994).

1.3.1.2 *Cladobotryum dendroides*

The *C. dendroides* species (teleomorph *Hypomyces rosellus*) (W. Gams & Hoozem. 1970) has been studied as it was one of the first species commonly reported to cause cobweb disease on *A. bisporus* (McKay *et al*., 1998; Potočnik *et al*., 2008*)*. It has since been shown to also affect other mushroom species like *L. edodes* (Gea *et al.*, 2017b). *C. dendroides* is characterised by a thin-walled phialide extension. The conidia produced are 2-3 septa with a basal hilum (Rogerson & Samuels, 1994; Potočnik *et al.*, 2008). Genomic features and phylogenetic analysis of *C. dendroides* has been recently completed (Xu *et al.*, 2020).

1.3.1.3 *Cladobotryum mycophilum*

C. mycophilum species (teleomorph *Hypomyces odoratus*) (Oudemans) (Gams & Hoozem. 1970) is at present, the species most reported to cause cobweb disease. *C. mycophilum* Type 1 is characterised by an extremely strong camphor odour which increases in intensity as the culture ages. *C. mycophilum* Type 2 was first discussed in the mid 1990s after it was found to be resistant to benzimidazole fungicides (Grogan & Gaze, 2000). *C. mycophilum* Type 2 lacks any camphor odour. *C. mycophilum* mycelia initially grow on plates as cream/white colonies, which turn to a yellow colour

after around 5 days of growth. Mycelia then turn red after 25 days due to the release of aurofusarin. The phialide tips are characterised as being regular, without any rachis present (Rogerson & Samuels, 1994; Carrasco *et al.*, 2016). *C. mycophilum* has been reported as affecting other mushroom species which include *Ganoderma lucidum* (Zuo *et al.*, 2016) and *Pleurotus eryngii* (Gea *et al.*, 2017a).

1.3.1.4 Cobweb disease symptoms

Cobweb disease is characterised by the growth of a dense, white, fungal mycelium over the developing *A. bisporus* mushrooms. Cobweb patches can begin as very small areas on the beds which are difficult to distinguish from *A. bisporus* mycelium growth, cobweb patches will be slightly greyish in colour compared to the white colour of *A. bisporus* (**Figure 1.5A**). These cobweb patches develop into a circular shape and expand in diameter across the casing layer when left untreated, engulfing adjacent mushrooms and spreading disease further (**Figure 1.5.B**). This fungal growth has been compared to spider-web like threads, hence where the name of this disease originates. The quality of infected mushrooms deteriorates rapidly and they succumb to wet-rot. As the *Cladobotryum* mycelium on the surface of the casing layer ages, it can turn a more red/pink colour and acquire a mealy texture as a result of excessive sporulation (Fletcher & Gaze, 2008; Tamm & Põldmaa, 2013). Mushrooms that are covered with the fungal mycelium will eventually become discoloured and rot (**Figure 1.5C**). It is recommended to growers that infected mushrooms and the adjacent areas surrounding the infected mushrooms are treated immediately as these infected mushrooms become a source of masses of *Cladobotryum* spores. Spores of *Cladobotryum* are characterised as being light-weight and dry (Fletcher & Gaze, 2008; Adie *et al.*, 2006). This contributes to the ease in which these spores can be distributed with any sort of movement as air flow currents are sufficient to carry the spores around the mushroom house. Spore masses are likely to be disturbed and distributed during the watering of the crop, harvesting the crop or even when treating diseased areas on the crop, if it is not done carefully (Adie & Grogan, 2000). Spores can then land on the caps of developing mushrooms within the growing room which will result in a spotting symptom, where small, well defined, brown circular spots appear on mushroom caps (Fletcher & Gaze, 2008).

Figure 1.5 Cobweb disease symptom development. **A**. Early cobweb patch development. **B.** Late cobweb patch development. **C.** Infected *A. bisporus* mushrooms

As spores are easily dislodged and transferable, dealing with cobweb disease needs to be well managed and always carried out carefully in a controlled manner (Adie & Grogan, 2000). If a cobweb patch is identified, the crop should not be watered, doors should remain sealed and air conditioning within the room should be switched off while treatment is occurring (Adie *et al.*, 2006). It is important to avoid touching the cobweb patch directly as this could dislodge spores and cause secondary colonies to develop (Adie & Grogan, 2000). The recommended treatment is to gently apply a layer of damp tissue paper to cover the entirety of the cobweb patch, plus the surrounding area beyond the patch. Salt (NaCl) should then be applied to the tissue paper. To avoid the release of spores from the patch during this treatment, it is recommended to apply salt first to the edges of the tissue paper, rather than directly in the centre. Once the edges have been covered, NaCl should be applied to the centre of the tissue paper. As an extra precaution, an additional layer of damp tissue can be applied on top of the salted patch to prevent salt splashing on adjacent mushrooms during watering (Grogan & Gaze, 2008) (**Figure 1.6**). Salting may not be recommended, in some cases, if the disease patches are too large. If disease cannot be controlled, it will result in termination of the crop early (Fletcher & Gaze, 2008), which will have significant financial impacts on the growers.

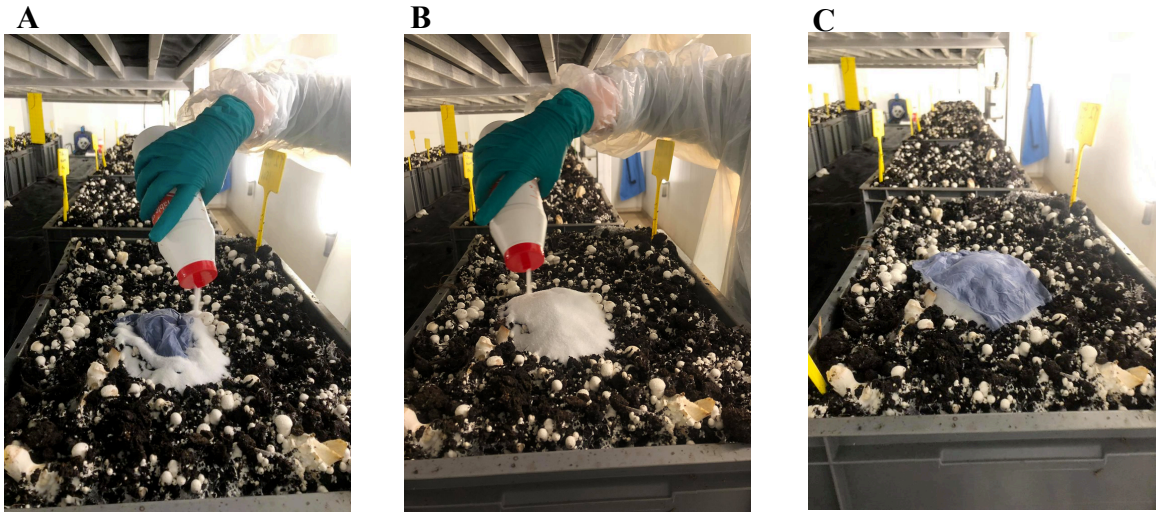

Figure 1.6 Salting regime steps for the treatment of cobweb disease on white button mushroom (*A. bisporus*) crops. **A.** Identify disease patch at an early stage, cover with damp tissue paper, apply salt to the perimeter of tissue. **B.** Cover centre of tissue with salt. **C.** Add another layer of damp tissue carefully over salted patch.

1.3.1.5 **Sources of *Cladobotryum* infection on mushroom farms**:

One of the best ways to prevent disease on farms, is to avoid the introduction of spores to the growing rooms completely. It is therefore important to identify potential sources of pathogenic spores. There are several possibilities for the primary source of *Cladobotryum* spores on a mushroom farm. It is unlikely that the spores of *Cladobotryum* will survive the high temperatures which are used during the pasteurization process during compost preparation. Therefore compost delivered to mushroom farms is generally not considered as a potential reservoir for *Cladobotryum* spores (Beyer, 2023). Casing material on the other hand has been suspected as a potential primary source for infection. Studies have shown that the presence of *Cladobotryum* spores in the casing layer will result in the development of cobweb disease symptoms on both *A. bisporus* and *P. eryngii* crops (Carrasco *et al.*, 2016; Gea *et al.*, 2017a). Casing material is generally prepared off site in a separate facility and delivered to mushroom farms. Therefore it is essential for these facilities to avoid contamination of casing material prior to its arrival at the mushroom farms. Similarly, growers need to ensure that fresh casing supplies are protected from contamination prior to its application to a mushroom crop. *Hypomyces* species with *Cladobotryum* anamorphs have been identified growing on wild *Agaric* species (Rogerson & Samuels, 1994). It is also suspected that *Cladobotryum* spores from wild specimens in close proximity to the farms may be responsible for introducing disease to mushroom farms (Fletcher & Gaze, 2008). *Cladobotryum* produce highly resistant microsclerotia structures in periods of low relative humidity (RH) which can survive until they reach the high relative humidity conditions in growing rooms which facilitates their germination (Carrasco *et al.*, 2017). Mushroom growing facilities should be fitted with high quality dust filtration systems to exclude the entry of dust and materials from outside to avoid the introduction of *Cladobotryum* spores from the environment.

1.3.2 Dry bubble disease

Dry bubble disease is a very serious disease which has extremely negative consequences for commercial *A. bisporus* production. It is caused by the fungal pathogen *Lecanicillium fungicola* (Preuss) Zare and Gams [synonyms: *Verticillium fungicola* (Preuss) Hassebrauk]. The taxonomic history and overview of this disease has been well defined in a review by Berendsen *et al*., (2010). In 1851, Preuss identified the fungus we now know to cause dry bubble disease, growing on the cap of an unidentified wild mushroom in woodlands (Gams, 1971). This fungus was named *Acrostalagmus fungicola*. Dry bubble disease was officially first reported in 1982 in France, when it was referred to as 'la môle' disease. It is assumed that the name was derived from the Latin word for mass, which is 'moles' (Berendsen *et al.*, 2010). The researchers suggested the disease was caused by *Hypomyces perniciosae* which is the fungus responsible for wet bubble disease, another major disease which affects mushrooms. Costantin and Dufour believed that this fungus appeared in two distinct forms producing either large or small *Verticillium*-like spores.

In 1924, it was discovered that the fungus which produced small *Verticillium*-like spores and caused dry bubble disease was not the same species as the fungus which caused wet bubble disease. It was renamed to *Cephalosporium constantinii* (Smith, 1924). The symptoms of both dry bubble and wet bubble disease are often mistaken for one another due to their similarities (Fletcher & Gaze, 2008). Dry bubble disease was also described in 1933, but it was suggested that *Verticillium malthousei* was the fungus responsible for the disease rather than *Cephalosporium constantinii* (Ware, 1933). In 1936, Hassebrauk also characterised an unidentified fungus which would eventually be named *Verticillium fungicola* (Hassebrauk, 1936). In 1971, Gams discovered that *Cephalosporium constantinii, Verticillium malthousei* and *Verticillium fungicola* were actually all the same species but separated them into three varieties; *Verticillium fungicola: var. fungicola, var. aleophilum and var. flavidum* (Gams, 1971). Finally, molecular advancements determined that the species was actually more closely related to the genus *Lecanicillium*, than *Verticillium*, and the species was re-classified to *Lecanicillium* in 2008. This was discovered by sequencing the internal transcribed spacer region (ITS) and small subunit rDNA sequences (SSU rDNA) (Zare & Gams, 2008).

Dry bubble disease has been reported in most countries growing *A. bisporus* at a large scale including Spain, (Gea *et al*., 2003) Australia (Nair & Macauley, 1987), France (Costantin & Dufour, 1892), UK (Smith, 1924) and Ireland (Gaze & Grogan, 2008). *Verticillium fungicola:* var. *flavidum* was discovered to be its own species due to differences in ITS sequence, optimum growth conditions and morphology. It was renamed to *Lecanicillium flavidum*, leaving two varieties of *Lecanicillium fungicola*, var. *fungicola* and var. *aleophilm* (Zare & Gams, 2008).

1.3.2.1 Dry bubble disease causative agent: *Lecanicillium fungicola*

L. fungicola var. *aleophilm* is more common in North America, while *Lecanicillium fungicola*, var. *fungicola* causes disease in Europe (Collopy *et al.*, 2001; Gea *et al.*, 2003; Largeteau *et al.*, 2004). The molecular homogeneity between European isolates of *L. fungicola*, var. *fungicola* is dependent upon the cultivation practices used in the countries and the type of fungicide treatments used which can contribute to fungicide selective pressure (Bonnen & Hopkins, 1997).

The *Lecanicillium* spp are categorised as hyphomycetes, which are hyaline and phialidic (Zare & Gams, 2008). Hyphomycete fungi lack the ability to produce complex asexual fruiting structures - instead growth consists of either hyaline, or dark hyphae which produce spore-bearing phialides. Growth is filamentous. Phialidic fungi have phialide structures which are specialised conidiogenous cells which produce conidia/spores. The genus contains species which are both fungicolous (associated with other fungi) and entomogenous (associated with insects). *L. fungicola* specifically is characterised by erect, verticillate conidiophores. Conidia are produced from the tip of the phialide and accumulate in mucilage, which will hold the conidia together until they are dispersed (Zare & Gams, 2008).

1.3.2.2 Dry bubble disease symptoms

The symptoms of dry bubble disease are highly dependent upon the timepoint and developmental stage at which the infection of *A. bisporus* occurs (North & Wuest, 1993). Holmes, (1971) found that when crops were infected with *L. fungicola* early in the crop cycle, during casing application, disease severity and symptoms were low. It is believed that this was due to a lack of nutrients at this time due to soil fungistasis (Berendsen *et al.*, 2012). Severity of disease was highest when infection took place 14

days after casing. At this point *A. bisporus* had not yet formed primordia, but the mycelium was well established in the casing layer. It is suggested that in order for *L. fungicola* germination to occur, nutrients from the *A. bisporus* mycelium are required (Fletcher & Gaze, 2008; Berendsen *et al.*, 2012). Disease severity then dropped when infection took place on days 21 and 28, when *A. bisporus* mushrooms were fully matured. These results suggest, that the *A. bisporus* mushrooms are most susceptible to dry bubble disease prior to reaching full maturity.

As mentioned previously, *Cladobotryum* generally affects *A. bisporus* fruiting bodies which have already developed and are present on the surface of the casing layer. *L. fungicola* on the other hand, is able to infect the developing *A. bisporus* primordia within the casing layer, prior to it reaching full maturity (North & Wuest, 1993). As a consequence, when this infected pin develops, it will emerge as an undifferentiated mass of mushroom tissue, lacking any of the distinguishing features known of *A. bisporus*. The infection prevents the mushroom from developing and differentiating into a stipe and cap (Gaze & Grogan, 2008). This symptom is referred to as bubble mushroom, which is where the name of this disease originates (**Figure 1.7.A, Figure 1.7.C**). Bubble mushrooms are also known to ooze small amounts of amber drops as they age (Gaze & Grogan, 2008) (**Figure 1.7D**). The development of a bubble mushroom can be identified at an early stage with adequate training, but requires very intense monitoring of the crop. Salting these small pieces of bubble could be enough to prevent a significant outbreak. Generally the bubble mushroom is more often identified when it is medium sized and when it may already be sporulating. New discoveries provide promise that easier and early detection of *L. fungicola* infection in the future will be possible. For example, Hayes *et al.*, (2024) found that there are distinct changes in volatile compounds from mushrooms infected with *L. fungicola* such as increased levels of β-barbotine and a diterpene which could be exploited to aid in early detection. Split stipe or stipe blow-out is another symptom associated with dry bubble disease (Gaze & Grogan, 2008). Split stipe can occur if infection occurs after the primordia has developed. Infection is localised at the tissue of the stipe, causing it to split or rupture on one side of the mushroom, while the cap grows normally. Finally, spotting symptoms are also associated with dry bubble disease (Gaze & Grogan, 2008). Similar to cobweb disease, spotting occurs when spores land and germinate on the cap of developing mushrooms. Theses spots are often greyish in the centre with a darker

brown outline (**Figure 1.7.B**). 'Symptomless mushrooms' have also been previously described (North & Wuest, 1993). This is particularly troublesome as some mushrooms will only develop spotting symptoms of dry bubble disease after they have been harvested or even after they have been shipped to retailers. The reason this causes such problems is because growers will handle these seemingly normal mushrooms, not knowing they are carrying *Lecanicillium* spores and unknowingly spread these spores to the healthy mushrooms.

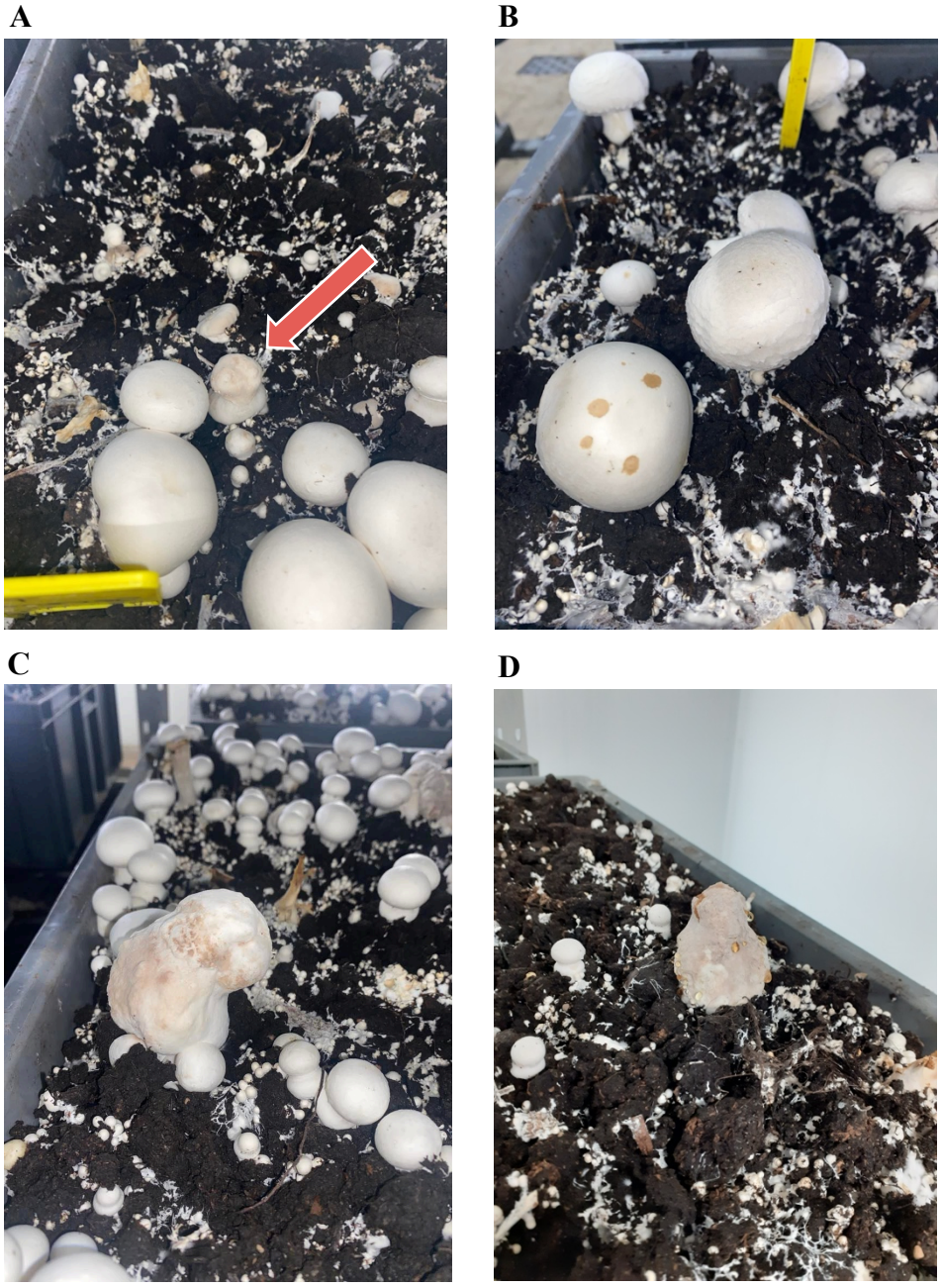

Figure 1.7 Dry bubble disease symptom development. **A.** Early bubble development. **B.** Spotted mushroom symptom. **C.** Late bubble development. **D.** Bubble mushroom oozing amber liquid and showing grey colour due to sporulation.

1.3.2.3 Sources of *Lecanicillium* infection on mushroom farms

Identifying the primary source of dry bubble disease infection can help prevent disease occurrence on the farm. Similar to *Cladobotryum*, it is not suspected that infected compost material will act as the primary source of infection for dry bubble disease as the *Lecanicillium* would not survive the high temperatures used during compost preparation. *Lecanicillium* spores originating from the wild may also act as a primary source of infection, however there is little evidence in the literature to support this. Infected casing material may also introduce disease to the growing rooms (Berendsen *et al.*, 2012). *Lecanicillium* spores can survive for long periods of time in dry conditions, meaning once spores are present in the farm, regardless of how they got there, it is very difficult to fully eradicate them (Gaze & Grogan, 2008). The spores accumulate in debris and dust around the farm which act as reservoirs for the inoculum (Grogan, 2001). Contamination of casing materials from these on-farm reservoirs is often the beginning of large outbreaks on mushrooms farms (Fletcher & Gaze, 2008).

Infected bubble mushrooms produce millions of *L. fungicola* spores which are covered in a sticky mucilage or gelatinous material (Berendsen *et al.*, 2010; Berendsen *et al.*, 2012). These spores can attach very easily to the hands of pickers during harvesting or onto the equipment used during crop watering or monitoring (McGuinness *et al.*, 2021). Insects have long been associated with *L. fungicola* transmission, especially flies (Ware, 1933). Phorid flies (*Megaselia halterata)* and sciarid flies (*Lycoriella castanascens*) can be a very problematic pest for the mushroom industry (Tibbles *et al.*, 2005; Jess *et al.*, 2007; Navarro *et al.*, 2021). Disease dispersal can be limited by controlling the population of mushroom flies, either through chemical or integrated pest management strategies (Shamshad, 2010). Physical barriers such as screens and filters are also very important to prevent flies from entering the units. *Lecanicillium* spores are known to survive for many months in water. Water is therefore another common dispersal method for this disease (Fletcher & Gaze, 2008). This can occur for example if the crop is watered when diseased mushrooms are present, dispersing the spores across the room creating secondary points of infection (Fletcher & Gaze, 2008). Ironically, excessively cleaning the mushroom growing rooms when dry bubble disease is present will often exacerbate disease severity. For crops experiencing severe disease levels, large pieces of bubble mushroom should be removed by experienced

personal, using a plastic bag and gloves. The bag is turned inside out and disposed of appropriately along with the workers gloves. Salt is then added to the area of the bed where the diseased mushroom was taken. For dry bubble disease, a damp paper towel is not required as with cobweb as the spores are not as likely to become airborne (Gaze & Grogan, 2008).

1.4 Disease treatment

Both dry bubble disease and cobweb disease pose significant economic challenges to those working in the mushroom industry. Both result in substantial financial losses which can either be due to loss of yield due to diseased mushrooms that are not suitable for retail or the losses which result from crops being terminated early due to excessive disease. Furthermore, growers will need to invest more for the treatment of disease and implementing strategies for crop protection. The recorded annual cost which growers spent trying to deal with dry bubble disease was 2-4% of their total revenue (Berendsen *et al.*, 2010). To prevent significant disease outbreak, and consequential financial losses, growers have relied upon the use of chemical fungicides for the past number of decades.

The mushroom growers are in a unique position when it comes to fungicide use. Unlike many of the other horticulture crops, both the pathogen that growers wish to target, and the crop they are trying to grow are fungal species. This complicates the use of fungicides as growers are restricted in the number of products that they can use which will selectively target the disease, but not the mushroom. For this reason, the mushroom industry has had very limited options for fungicide treatments.

1.4.1 Fungicide use

Applying solutions to crops of plants to prevent disease occurring is a practice which has been carried out since 1807. Prévost, a Swiss scientist discovered that the disease ‘wheat bunt’ could be prevented by wetting the wheat kernels in a copper sulfate solution (Leadbeater, 2014). Fungicides have been an essential component to the agriculture sector ever since and have been an important tool used to prevent and control crop diseases.

In the early twentieth century, very simple fungicidal solutions, which were mainly made of sulphur, lime and copper sulphate mixtures, were used by growers. In those early days of fungicide use, there was no concept of appropriate applications with most solutions being applied at extraordinarily high and toxic rates (Leadbeater, 2014). The 1940s saw the introduction of more complex, chemical products and the idea of using measured application rates to get the best results from the product. Between 1940s-1970s there were many advances in fungicide development with new chemical classes being introduced to the market, including benzimidazoles (Leadbeater, 2014). During the 1970s-1980s, there was more focus on product research and development which contributed to the major expansion of the fungicide market and the introduction of broad spectrum fungicides. During the 1980s-2000s, although agriculture and crop production was booming, the toxic effects of the fungicides were becoming apparent (Beckerman *et al.*, 2023). This lead to places such as the USA (Environmental Protection Agency) and Europe (European Directive, 91/414) introducing strict restrictions and legislations for the use of fungicides. Despite the development of important classes of fungicides during this time such as triazoles and strobilurins, there was a significant decrease in fungicide developments as a result of these restrictions. Development of new fungicide products became very expensive and time consuming and this has slowed down the market drastically (Leadbeater, 2014).

1.4.2 Chemical treatments for mushroom disease

1.4.2.1 Benzimidazoles:

The benzimidazoles group is a class of fungicides which were used in the mushroom industry from 1960-1970 (Fletcher and Gaze., 2008). After their introduction, they became very popular due to their high effectiveness even at low use rates and broad spectrum of activity making them functional for several different crops such as grains, fruits and vegetables (Leadbeater, 2014). Major fungicides in this group include benomyl, carbendazim (MBC), thiophanate-methyl, thiabendazole and fuberidazole (Chung *et al.*, 2023; Bai *et al.*, 2024). Benomyl was one of the first benzimidazoles fungicides to be developed by an American company. Later, carbendazim (a metabolite of benomyl) and thiabendazole (an intermediate of benomyl) were also developed (Bai *et al.*, 2024). Benomyl, carbendazim and thiabendazole are characterised as having low acute toxicity levels while fungicides thiophanate-methyl

and fuberidazole have moderate toxicity. Benzimidazoles activity is based on the inhibition of β-tubulin polymerization in fungal pathogens (Leadbeater, 2014). Basidiomycete species seem to be more tolerant to benzimidazoles and hence *A. bisporus* growth was not reduced when this fungicide was applied on mushroom crops (Bollen & Fuchs, 1970).

Benzimidazole fungicides like benomyl were of huge importance to mushroom growers and were very effective at controlling dry bubble disease and wet bubble disease (Bollen & Zaayen, 1975; Fletcher *et al.*, 1975). Unfortunately, within a year of the registration of benzimidazoles for use on mushroom crops, tolerance from *Lecanicillium* isolates were observed in the Netherlands and the UK (Bollen & Zaayen, 1975). Benzimidazole continued to have good control of cobweb disease until the 1990s when growers started to notice an increase in disease severity despite using the benzimidazole fungicide treatments. As discussed in section 1.3.1, cobweb disease reached epidemic proportions in the UK and Ireland and mushroom farms suffered tremendous losses (Adie & Grogan, 2000). Development of resistance of *Cladobotryum* isolates to the fungicide class benzimidazoles, which were used heavily during this time was believed to be a contributing factor to this epidemic (Grogan, 2006). Benzimidazoles only have one active target site which meant that they were vulnerable to resistance developing against them. Due to limitations in the fungicide products that could be used, mushroom growers used benzimidazoles excessively and almost exclusively on their crops. Resistance towards benzimidazoles developed and spread extremely quickly and became a very serious problem for mushroom growers (Grogan & Gaze, 2000). Many publications have discussed the development of resistance to benzimidazoles from pathogens impacting mushroom cultivation but also from other important crop pathogens (Bollen & Fuchs, 1970; Wuest *et al.*, 1974; Bollen & Zaayen, 1975; Grogan & Gaze, 2000; Grogan, 2006).

1.4.2.2 Chlorothalonil

Chlorothalonil is a broad spectrum, non-systemic fungicide, first introduced in 1965 (Van Scoy & Tjeerdema, 2014). A product manufactured under the name BRAVO $^{(R)}$, which has the active ingredient chlorothalonil was used in the mushroom industry in the past (Gandy & Spencer, 1976). However numerous studies detailed the high toxicity levels associated with chlorothalonil. Its approval for use in the EU was fully

withdrawn in 2019 (EC, 2019). Studies have shown that transformation products of chlorothalonil are found in drinking water sources and they are suspected to persist and pose issues for many years despite this ban (Kiefer *et al.*, 2020). The banning of this product once again reduced the number of fungicides products available to mushroom growers in Europe. BRAVO (R) is currently still approved and in use in the USA, Brazil and Canada (with mitigation measures). It has been shown to be extremely toxic to aquatic life and moderately toxic to mammalians which means extreme caution should be taken by growers in these countries to avoid the introduction of chlorothalonil to water systems (Bai *et al.*, 2024).

1.4.2.3 Prochloraz

Prochloraz is an imidazole which belongs to a class of fungicides called demethylation-inhibitors (DMI) fungicides (Leadbeater, 2014). DMI fungicides target fungal sterol biosynthesis to prevent fungal pathogen growth. The C_{14} demethylation step during fungal sterol biosynthesis is inhibited which prevents the conversion of lanosterol to ergosterol (Vinggaard *et al.*, 2006), an important component in fungal cell membranes. The imidazole moiety of prochloraz interacts with the iron atom present in cytochrome P450. This interaction is quite unspecific which makes prochloraz a broad spectrum fungicide (Laignelet *et al.*, 1990). Prochloraz was a popular treatment for mushroom diseases as it was inhibitory towards fungal pathogens, but did not appear to have off-target effects on the *A. bisporus* crops. Prochloraz was also a popular treatment for eyespot disease in wheat (*Oculimacula* spp.) and net blotch in cereals (*Pyrenophora teres) (*Leadbeater*, 2014).* Prochloraz was used in the mushroom industry with the fungicide product marketed as Sporgon(R).

Prochloraz became the fungicide of choice for many growers due to the lack of any alternatives. Unsurprisingly, evidence of tolerance toward prochloraz was reported in the 2000s (Gea *et al.*, 2005, Grogan *et al* 2000). Despite this, the prochloraz fungicide continued to be effectively used and was very popular with mushroom growers. As mentioned, prochloraz is a broad spectrum fungicide product, which means that the chances of impacting non-target organisms within the environment is much higher with prochloraz. Prochloraz was documented as being extremely hazardous to aquatic life and extreme care was recommended when disposing of the product (European parliament, 2009).

1.4.2.4 Metrafenone

The Metrafenone fungicide product (3-bromo-2′,3′,4′,6-tetramethoxy-2,6′-dimethylbenzophenone) was first introduced to control cobweb disease of mushrooms in France in 2014 and has since become approved for use across the EU. Metrafenone is used in the mushroom industry using the product marketed as Vivando(R). Metrafenone belongs to the benzophenone class of fungicides and it was the first commercial product registered from this group in 2006. When introduced, metrafenone was not found to be sensitive to the resistance mechanisms that inhibit the use of other fungicides which suggested it has a potentially novel mode of action (Schmitt *et al.*, 2006). The mode of action of metrafenone is still under investigation, however studies have suggested that it disrupts hyphal morphogenesis and establishment and maintenance of cell polarity (Opalski *et al.*, 2006). Metrafenone was shown to negatively impact several stages of fungal development, including spore germination, appressorial formation, penetration, surface hyphal morphology and sporogenesis (Schmitt *et al.*, 2006). Metrafenone has been used as a treatment for powdery mildew on cereal, grasses, fruit and vegetable crops in Europe since 2006 (Felsenstein *et al.*, 2010; Vielba-Fernández *et al.*, 2020). However resistant isolates against metrafenone have been reported in both *in vitro* and *in vivo* studies (Felsenstein *et al.*, 2010; Kunova *et al.*, 2016). At this current time, metrafenone is the only approved fungicide for cobweb control. If resistance from cobweb isolates became widespread, then approval may be withdrawn.

1.4.3 Fungicide toxicity

There are varied levels of toxicity associated with different chemical fungicides. Despite their effectiveness, we are living in a more environmental and health conscious world. Therefore understanding the toxic effects associated with these fungicides has become extremely important and source of public concern and apprehension. Workers on mushroom farms are most at risk from fungicide exposure. Exposure usually occurs via inhalation but dermal or ocular exposure is also a possibility. Exposure can occur during fungicide mixing, preparations or applications, meaning harvesters are less at risk to fungicides then the person applying them. (Damalas & Eleftherohorinos, 2011). Those who apply fungicides must be trained and certified and must follow health and safety instructions. Powders present respiratory risks more so than liquids.

Formulations have improved over time to reduce or eliminate the use of powders which reduces health risks.

The fungicide chlorothalonil has extremely high soil and sediment absorption rates which risks its exposure to plants and animals (Van Scoy & Tjeerdema, 2014). During murine studies, it was shown that exposure to concentrations greater than 400 mg/kg/day resulted in symptoms of exhaustion, weakness, embryo lethality and reduced foetus survival rates (Farag *et al.*, 2006). It is also toxic to aquatic life. The fresh water mussel, *Lampsilis siliquoidea* in both its adult and juvenile life stage was highly sensitive to chlorothalonil (Bringolf *et al.*, 2007). However, workers who sprayed chlorothalonil, did not show a link between their exposure and increased likelihood of cancer, despite sufficient evidence of its carcinogenic affects in murine studies (International Agency for Research on Cancer, 1999; Mozzachio *et al.*, 2008). Perhaps this suggest that the safety protocols and PPE used by workers is protective against fungicide exposure.

Studies have also shown the fungicide prochloraz to be harmful in murine studies. Pregnant female mice subjects, treated with 30 g/kg prochloraz, experienced increased pregnancy lengths (Vinggaard *et al.*, 2006). Male foetuses born to these female mice, who were exposed to prochloraz from breast milk, experienced a reduction in testosterone levels and increase in progesterone levels, which overall resulted in the feminization of adult male mice (Vinggaard *et al.*, 2006). Similarly, Wilson *et al*., (2004) showed that exposing pregnant female mice to prochloraz at 250 mg/kg increased progesterone and reduced testosterone in male offspring. Another study described how prochloraz can regulate and bind to the efflux transporter gene ABCG2 within the bovine mammary gland (Halwachs *et al*., 2013). It was suggested that the intake of food which contain residues of prochloraz could result in the secretion of harmful compounds in milk (Vinggaard *et al.*, 2006). The use of prochloraz on mushroom farms continued up until 2023, when its approval for use in Europe was revoked. The company failed to provide the toxicology data to support its continued approval (European parliament, 2009).

A risk assessment for metrafenone use was carried out by the European Food Safety Authority (Álvarez *et al.*, 2023). They reported that metrafenone displayed low acute toxicity when administered orally, dermally or inhaled by rats. They also found

metrafenone had moderate-high persistence rates in soil. However they also reported concerns about lack of information and data gaps which were needed to complete their assessment. Further research into potential toxic effects of metrafenone is required, especially as it will be the only option for mushroom growers to use for the foreseeable future. Metrafenone approval is up for renewal (Marchand *et al* 2023a, Marchand *et al* 2023b).

1.4.4 Future of fungicides use

Global population levels are increasing year after year and there is a huge demand on the agriculture sector to produce enough food to feed this growing population. The United Nations estimates that population numbers will rise to 9.7 billion people by 2050 (UN, 2022), therefore it is anticipated that the demand for food crops will increase even more in the future. They also estimated that the highest population increases will be seen in the least developed countries (UN, 2022). This increases pressure even further as many of these countries rely on importing their food produce. Mushroom cultivation has been identified as a potential industry to be expanded in developing countries (Higgins *et al.*, 2017). Despite facing constraints, farmers from developing countries can use locally available materials to build mushroom growing rooms and produce substrate materials for growing mushrooms. Mushrooms can also be grown year round depending on climate and/or availability of air conditioning. This could help supplement profits for farmers growing crops which are limited to seasonal conditions. With help and knowledge sharing from more experienced mushroom growing countries, mushrooms may offer protein rich food sources for developing countries (Predmore *et al.*, 2018).

It is estimated that 20-40% of the world's crops are lost to moulds, pathogens and pests (Oerke, 2006; Savary *et al.*, 2012). The reduction in the number of available fungicides is one of the factors that has the potential to critically disrupt food production. The importance of fungicides cannot be overstated, they will continue to be an important tool in agriculture. However, we do need to make changes to ensure that the fungicides are used in a responsible way to avoid resistance development and damage to the environment. There has been a considerable growth in public concern over the use of chemical fungicides on food crops. There is a call for the use of alternative treatments which are considered to be more natural and safe, both for the environment and

towards human health. Unlike the early days of fungicide use, we now realise that we have to take responsibility for the actions that we take which contribute to the pollution of our planet. There is much more consumer awareness in regard to food safety and environmental impacts on biodiversity. It is for this reason that there has been a huge increase in restrictions imposed around fungicide use. Environmental agencies and governments are more often not renewing approval for fungicide products, which is why many of the fungicide products are no longer available for growers to use. Modern pesticides must meet very strict safety and environmental tests before they are approved.

The mushroom industry currently only has one fungicide, metrafenone which is approved for use. It is expected that pathogen resistance to metrafenone will be difficult to prevent and that this fungicide may also lose its approval for use in the future. One suggestion to avoid this from happening, is that fungicide treatments should be the last resort treatment option for our crops and they should not be used routinely. It is hoped that integrated pest management strategies could be developed and used as the standard crop protection strategy in agriculture. This would reduce our over-reliance on fungicide products.

1.5 Integrated pest management strategies: alternative treatments for mushroom disease

1.5.1 Prevention practices and non-chemical treatments

There is a number of non-chemical methods that can reduce disease occurrence on mushrooms farms but having extremely high hygiene standards on the farm is fundamental to these treatments. Farms should be well equipped with disinfection solutions, hairnets, gloves, foot-dips, door-seals, filters and some method of fly control. As mentioned in section 1.2.7, after a crop is complete, steam cook-out is performed with all remaining spent mushroom compost and also any equipment that was used on the crop. This should be done prior to adding a new crop in the room to ensure there is no disease carried over from the previous crop. It is critical to ensure steam cook out reaches 60-70°C and is held for a minimum of 12 hrs. Other ways to reduce disease include working from new crops to older crops rather than the other

way around and reducing the number of flushes to two rather than three (den Ouden, 2016).

In both diseases, casing material is often identified as a potential source of pathogenic spores which results in disease development. There are specific steps that the growers can take to prevent casing contamination and disease outbreak. Growers are advised to ensure all rooms and equipment which will be in contact with the casing is cleaned and disinfected prior to the casing material arriving to the farm (Grogan & Gaze, 2008). If casing material is stored for any period of time, it should be covered and protected from dust and debris. Personnel applying the casing layer to the beds will need to wear appropriate personal protective equipment including clean overalls, boots and gloves. They should not enter any other active growing rooms prior to or after working with casing (Gaze & Grogan, 2008).

Growers may also rely on rapid detection of potential disease and fast response to limit its negative effect. Once identified, as mentioned in section 1.3.1.4, the area of disease on the bed should be salted. When done correctly, this can be one of the easiest and most effective ways of controlling disease outbreak. The salt will kill the diseased area and prevent spores from spreading from that area, but also acts as a marker for mushroom pickers to actively avoid. Caution while salting is required to avoid unintentionally disrupting the diseased area and releasing spores which will contribute to further disease outbreak. It is also important to extend the salted area a few centimetres beyond the diseased area (Gaze & Grogan, 2008). Ideally salt should be added to cover the infected mushroom which is then left untouched for the remainder of the crop. However in some situations, for example if the infected mushroom is too large to be covered by salt, the mushroom may have to be physically removed. It is important to remove and dispose of infected mushrooms carefully as they can contain spores from the pathogen, which if dislodged will spread around the growing rooms. Salting an area on the mushroom bed will effectively prevent any further mushrooms developing in that area. Furthermore, the mushrooms which develop close to the salted area are also usually aesthetically unpleasing as the salt can cause damage to developing caps, these mushrooms will need to be discarded. Salting can be effective at controlling disease when the levels are low and at an early stage. However, if disease gets out of control, salting disease will have huge impact on healthy yield and therefore

may not be the most economic option for growers. Because of this, many growers rely on the use of preventative chemical fungicides to prevent disease developing on the crops in the first place.

1.5.2 Use of essential oils

Plants and herbs were used in ancient times to treat illness and disease, despite not fully understanding how they were having an effect. We now know that the pharmaceutical properties of these plants was usually due to the essential oils which they contained (Edris, 2007). These essential oils are aromatic oily liquids which can be extracted from plant material and characterised with advancements in gas chromatography and mass spectrometry. Essential oils extracted from plants may be different compound mixtures of terpenes, alcohols, acids, esters, epoxides, aldehydes, ketones, amines and sulphides (Sendra, 2016). Essential oils have also been used historically for their antimicrobial activities. In ancient Egypt, essential oils were used during the embalming rituals to prevent decay (Edris, 2007). With the development of *in vitro* antagonistic tests, we know that various essential oils can prevent the growth of many different bacterial and fungal strains. It is known that essential oils are effective on a broad range of bacterial species, both Gram positive and Gram negative. Tea tree oils have been investigated for use in hospital settings to target multi-drug resistant organisms resistant to antibiotics such as methicillin-resistant *Staphylococcus aureus* (MRSA) (May, 2000). Essential oils are also inhibitory towards fungi and yeast (Kalemba & Kunicka, 2003; Lang & Buchbauer, 2012; Hu *et al.*, 2017). It is believed that the essential oils can disrupt hyphal growth and inhibit the formation of fungal spores (Lang & Buchbauer, 2012). This can be utilized in a clinical setting to treat *Aspergillus* infections, which can lead to asthmatic and allergic reactions. For example essential oils produced by plants *Aegle marmelos* (Indian bael), *Chenopodium ambrosioides* (Mexican tea) and *Ageratum conyzoides* (Billygoat weed) have been investigated for their efficacy against *Aspergillus flavus* (Jardim *et al*., 2008; Singh *et al*., 2009; Nogueira *et al*., 2010). Essential oils have been investigated as an alternative to chemical fungicide use on plant crops. Eugenol can be extracted from *Syzygium aromaticum* (clove) and can be used to inhibit plant pathogens such as *Botrytis cinerea* (grey mould), *Penicillium expansum* (Blue mould) and *Phlyctema vagabunda* (Bull's eye rot disease) (Amiri *et al.*, 2008).

Essential oils have been documented to reduce the growth of pathogens which cause disease to mushroom crops. *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme) were found to produce essential oils which were the most inhibitory towards *Lecanicillium* spp. (dry bubble disease) and *Trichoderma* spp. (green mould disease). It has been reported that carvacrol in the oregano oil and thymol in the thyme oil were the main components which had the strongest activity (Soković & Van Griensven, 2006). Carvacrol and thymol are known to exert their anti-fungal affects by damaging fungal membranes through interactions with sterols (Chavan & Tupe, 2014). Plants belonging to the Lamiaceae family (*Zatariam ultiflora, Satureja hortensis, Mentha piperita*) and *Pelargonium roseum* (rose geranium) produced essential oils which were most inhibitory towards the dry bubble pathogen, *L. fungicola*. The main components of *Zatariam ultiflora* and *Satureja hortensis* were found to be phenolic compounds which include carvacrol and thymol (Mehrparvar *et al.*, 2016). Essential oils from *Cinnamomum verum* (cinnamon) and *Syzygium aromaticum* (clove) both showed high antagonistic potential against *Lecanicillium* and *Cladobotryum* pathogens. *Syzygium aromaticum* essential oil performed better than *Cinnamomum verum* essential oil against both pathogens. The essential oils from *Cinnamomum verum* were shown to be more toxic to *Lecanicillium* than *Cladobotryum* spp. (Lukovic *et al.,* 2018). Essential oils were extracted from *Lavandula intermedia* (Lavender), *Salvia lavandulifolia* (Spanish Sage), *Satureja montana* (Winter savory), *Thymus mastichina* (Spanish Marjoram), and *T. vulgaris* (Thyme) and were analysed by gas chromatography to determine if they could inhibit cobweb disease. Essential oils extracted from *T. vulgaris* and *S. montana* were the most inhibitory against *C. mycophilum*. This work found that applying essential oils from these two species at a high dose was just as effective as fungicide application. Similar to the previous studies mentioned, phenolic compounds like carvacrol and thymol were identified within the *T. vulgaris* and *S. montana* essential oils (Gea *et al.*, 2019).

It is clear that essential oils are useful to growers during the mushroom growth cycle to prevent fungal growth. However, they have also been shown to have applications post-harvest. Essentials oils can improve shelf-life and quality of mushrooms after they have left the growing rooms. Applying essential oils with cinnamaldehyde compounds reduced browning, weight-loss and inoculum levels on *A. bisporus* mushrooms during storage (Gao *et al.*, 2014). Applying a vaporised mixture of essential oils from *Citrus*

bergamia (bergamot orange) and *Citrus×paradisi* (grapefruit) to sliced, packaged, *Agaricus bisporus* mushrooms reduced quality loss compared to an untreated control (López-Gómez *et al.*, 2021).

The natural compounds found in the essential oils of plants can reduce the growth of mushroom disease pathogens, which mean their use as a crop protection strategy in the future is promising. They have a broad spectrum of activity and as a result of their high volatility, they are unlikely to persist and leave toxic residues behind in the mushroom casing/substrate (Alonso-Gato *et al.*, 2021). However, many of the studies listed above looked at *in vitro* interactions between essential oil components and mushroom pathogens. More large-scale crop trial studies investigating these essential oils are required before they are incorporated into disease prevention strategies. Furthermore, the availability of commercial products which utilise essential oils is insufficient. This could be due to the high costs associated with developing and testing such products. Furthermore, any products that claim to control pathogens must be registered and approved as a plant protection product, which may explain why they are not generally available to the professional grower.

1.5.3 Biological control of pathogens

Similar to essential oil compounds, biocontrol treatments are suggested to be an environmentally friendly and more sustainable treatment option for many different agricultural crops, including mushrooms. Biological control or 'Biocontrol' is based on the concept of applying natural enemies to the environment which will directly antagonise the target pest or pathogen and prevent/reduce disease. Biocontrol organisms have included animals, insects and pathogens such as viruses, fungi and bacteria. The term 'biocontrol' appeared first when it was used by Karl von Tubeuf in 1914 (in relation to plant diseases) and Smith in 1919 (in relation to insects) (Hajek & Eilenberg, 2018). However biocontrol techniques were practiced for centuries prior to this, humans have often exploited biocontrol to control pests (Campbell, 1989). It can be said that the earliest forms of biocontrol involved crop manipulation practices such as seasonal crop rotations which would break the cycle of disease in a particular place as well as introduce more diverse microbial populations (Campbell, 1989). One of the earliest reports of biocontrol dates back to China in 900 and 1200 AD. Chinese farmers used *Oecophylla smaragdina*, a large predatory ant to protect their mandarin citrus

trees from predation (Shields *et al.*, 2019). The ants built large nests in the tree and these nests were often transported and sold at markets. In fact predatory ants and coleopteran species such as beetles were usually cited as the antagonist species during these early uses of biocontrol mainly because they could be seen with the naked eye (Shields *et al.*, 2019). It was not until the 1920s, after scientific advances in the field of microscopy and microbiology that there were more studies which explored the use of fungi and bacteria as biocontrol strains. In 1919, Hartley discussed how antagonistic fungi could be used to control 'dampening off', a disease which weakened pine needles (Hartley, 1919). In 1931, Henry investigated the treatment of *Helminthosporium sativum* (wheat foot-rotting fungus) using the microorganisms found within the soil (Henry, 1931). In 1963, a study was published which detailed the treatment of *Heterobasidion annonsus* (conifer rot) with the fungus *Phlebia gigantea* (Rishbeth, 1963). Based off of this work, the first commercially available biocontrol product was released (Campbell, 1989). Since these early works, there has been a continuous expansion in interest and publications in the area of biocontrol.

1.5.3.1 Biocontrol

Generally, biocontrol can be separated intro three strategies; classical biocontrol, conservation and augmentation. These classifications were developed due to the extremely broad and diverse nature of biocontrol studies which are carried out by different scientific disciplines. Biocontrol is studied in the context of controlling populations of either insects, plants or animals and therefore involves entomologists, plant pathologists and zoologists. Often these different areas have different definitions, which has led to a lot confusion in the field (Eilenberg *et al*., 2001; Hajek & Eilenberg, 2018). For simplicity, this thesis is focused mostly on augmentative biocontrol, which involves releasing additional numbers of naturally occurring enemies to reduce the population density of a pest (Van Lenteren, 2012). Augmentative biocontrol is very much a commercial activity as it involves mass rearing the natural enemy to a very large scale so that a product can be distributed and sold. Several different microbial strains are commonly employed for augmentative biocontrol treatments. This study looks at the use of *Bacillus* spp. for the treatment of fungal infections of mushroom crops.

Because of the unique microbial interactions which can occur, species that are pathogenic to some crops, may also be used as biocontrol treatments to protect other crops. For example, *Cladobotryum mycophilum*, which causes cobweb disease to mushrooms, has been shown to antagonise several important crop pathogens such as *Botrytis cinerea*, *Fusarium oxysporum* and *phytophthora* spp (Santos *et al*., 2019). Likewise, *Trichoderma* spp., which cause different green mould diseases in mushrooms are often more studied as a biocontrol agents for several plant crops. *Trichoderma* is the most widely used fungal biocontrol strain (Sood *et al*., 2020).

1.5.3.2 *Bacillus* species in biocontrol treatments

Bacillus are Gram positive, rod shaped, endospore forming, catalase positive bacteria which belong to the Firmicutes phylum. There are several factors which make *Bacillus* species excellent candidates for biocontrol strains. Although some *Bacillus* strains have been identified as pathogenic to humans, for example *B. cereus* (Bottone, 2010), generally *Bacillus* species are regarded as safe and non-harmful. *Bacillus* species are also ubiquitous within the environment, they can be found occupying a wide variety of niches and habitats, including soil, water, air and on the surfaces of plants (Mandic-Mulec *et al.*, 2016). Because of this, *Bacillus* biocontrol species have a wide range of environmental applications.

Competition with the pathogenic organisms for space and nutrition is an important mode of action for biocontrol strains. The rhizosphere provides only a limited amount of nutrients (carbohydrates, nitrogen, iron etc) which can be exploited by the microorganism. It is suspected that *L. fungicola* cannot germinate without an external nutritional source. As *Agaricus* hyphae develop, they will release nutrients. *L. fungicola* growing in close proximity to developing *Agaricus* hyphae can use the nutrients to germinate (Berendsen *et al.*, 2010). Increasing the population numbers of the biocontrol strain will give them a competitive advantage to obtain these nutrients over the pathogen, which in turn reduces pathogen growth. This strategy can be improved by studying the epidemiology and growth patterns of the pathogen to identify the stages where it will be most vulnerable to competition. Competitive assays can be used to identify biocontrol candidates which show the ability to grow rapidly and competitively against the pathogen using systems which mimic the natural environment (Köhl *et al.*, 2019). For example, Di Francesco *et al* (2017) grew fungal

strains of *Aureobasidium pullulans* with the peach pathogen *Monilinia laxa* in peach juice. HPLC analysis identified that asparagine sources were depleted by *A. pullulans* which reduced the growth of *M. laxa*.

Another important feature of the *Bacillus* species which contributes to their success as a biocontrol strain is their ability to produce several secondary metabolites or antimicrobial compounds (Kaspar *et al.*, 2019). These compounds are produced by microorganisms, are released into the environment and can be damaging to other microorganisms within the vicinity. Approximately 4-5% of the *Bacillus subtilis* genome is dedicated to the production of these antimicrobial compounds and has the ability to produce 24 distinct antimicrobial compounds (Stein, 2005). This is why *Bacillus* spp. are often referred to as 'microbial factories'. Cyclic lipopeptides (surfactin, fengycin, iturin) were the first of these compounds to be described and have been extensively studied in *Bacillus* species (Vanittanakom *et al.*, 1986; Ongena & Jacques, 2008; Dias & Nitschke, 2023). These cyclic lipopeptides are known for their surfactant properties. Surfactins are able to tightly bind to extracellular membranes due to their amphiphilic nature which interferes with biological membrane integrity. If the level of surfactants is high enough, there could be complete and irreversible destruction of the membrane (Heerklotz & Seelig, 2007). The iturin and fengycin family of antimicrobials are characterised for their antifungal activity specifically which is a result of cell membrane disruptions and increased permeability due to osmotic stress (Aranda *et al.*, 2005; Deleu *et al.*, 2008). Fengycin is known for its pore-forming abilities. Iturin can also inhibit spore germination and contribute to cell disruption (Wang *et al.*, 2022). The ability of *Bacillus* species to prevent fungal growth within the soil is mostly due to the iturins and fengycins. Surfactins have limited antifungal activity and are more inhibitory toward bacterial growth (Pérez-García *et al.*, 2011). Bacillomycin-D, is a member of the iturin family and similar to the other the lipopeptides discussed can cause morphological changes to the membranes and cell walls (Gu *et al.*, 2017). Many *Bacillus* strains secrete the lipopeptide siderophore bacillibactin, which can chelate ferric iron in the environment and transport it into the *Bacillus* membrane (Chakraborty *et al.*, 2022). This reduces the levels of available iron, which the fungal pathogens require for different biochemical processes like oxygen binding and electron transport (Fukushima *et al.*, 2013).

Biocontrol strains can also produce metabolites such as lytic enzymes which are destructive toward the pathogenic strains and can interfere with growth (Fira *et al.*, 2018). Important lytic enzymes include chitinases, glucanases cellulase, lipases and proteases. Many of these enzymes target and degrade the cell wall of the pathogen. Chitin is one of the most abundant polymers found in nature. Chitinase is a chitin-degrading enzyme which cleaves glucosamine units between the C1 and C4 bonds. Three classes of chitinase enzymes are classified, N-acetylglucosaminidases, endochitinases and exochitinases. N-acetylglucosaminidases cleave glucosamine monomers, endochitinases cleave glucosamine randomly, while exochitinases cleave glucosamine dimers (Viterbo *et al.*, 2002). Glucanases target glucan polysaccharides within fungal cell walls and can be classified as either ß-1,3- or ß-1,6-glucanases (Selitrennikoff, 2001). Proteases can work by degrading the lipids and proteins found in the pathogen cell wall which induces cell lysis (Viterbo *et al.*, 2002).

Bacillus species are well documented for their biofilm formation capabilities (Rabbee *et al.*, 2019; Nie *et al.*, 2022). A biofilm is an aggregation of microbial cells on a solid or liquid surface surrounded by a extracellular polymer matrix (O'Toole *et al.*, 2000). Cells are attracted together after the release of chemical signals. Biofilm formations can be very beneficial to biocontrol strains as producing a biofilm layer on crop tissue can protect that crop from infections by pathogenic strains (Ramey *et al.*, 2004; Pandin *et al.*, 2017). After infection, *Arabidopsis thaliana* has been shown to release malic acid which can attract bacteria including *Bacillus* species and encourage bacteria to produce a biofilm, which in turn protects *A. thaliana* (Rudrappa *et al.*, 2008). *B. velezensis* (FZB42) has been shown to produce biofilm on the fruiting body of *A. bisporus* as a bio-protection strategy (Pandin *et al.*, 2017).

Bacillus species produce spores in unfavourable, dry conditions. This contributes to their ability to survive during long term storage conditions within powdered biocontrol product formulations (Piggot & Hilbert, 2004). The success of *Bacillus*-based biocontrol treatments has been seen for many different agricultural crops including avocado, tomato, melon and strawberries among many others (Jayaraj *et al.*, 2005; Cazorla *et al.*, 2007; Romero *et al.*, 2007; Pertot *et al.*, 2008)

Having multiple modes of action means that resistance developing, although not impossible, is less likely for biocontrol treatments. The pathogen may have to evolve

to overcome the biocontrol strain's effects in different areas rather than a single target. Often these different actions work in unison to have combined maximal anti-fungal effect. For example, *Trichoderma* biocontrol species produce lytic enzymes which increase permeability in fungal cell walls and allow antimicrobials to enter the fungal cell (Karlsson *et al.*, 2017). Despite having several different modes of actions that the biocontrol strain can employ, in most cases, the protection levels that BCAs can provide are not as high as those seen with traditional chemical fungicide use.

1.5.3.3 *Bacillus velezensis*

The biocontrol strains under investigation during this work both belong to the *Bacillus* genus and belong to the species *B. velezensis*. The *B. velezensis* strain was first isolated from the Vélez river, in Spain (Ruiz-García *et al.*, 2005). This newly isolated strain showed a 20% similarity to other *Bacillus* species and therefore was classified as its own distinct species (Ruiz-García *et al.*, 2005). Another *Bacillus* species, *B. amyloliquefaciens* (Priest *et al*., 1987), which is a member of the *'B. subtilis* species complex' was separated into two subspecies, *B. amyloliquefaciens* subsp. *amyloliquefaciens* (strain DSM7) and *B. amyloliquefaciens* subsp. *plantarum* (strain FZB42) (Borriss *et al.*, 2011). *B. amyloliquefaciens* subsp. *plantarum* was often studied for its capabilities as a biocontrol agent in plant studies. With advances in comparative genomics, it was shown that the genome of *B. amyloliquefaciens* subsp. *plantarum* was highly similar to *B. velezensis* as well as *B. methylotrophicus* (Madhaiyan *et al.*, 2010) and *B. oryzicola* (Chung *et al.*, 2015). As *B. velezensis* was published first, *B. amyloliquefaciens* subsp. *plantarum* (strain FZB42), *B. methylotrophicus* and *B. oryzicola* became heterotypic synonyms for *B. velezensis (*Dunlap *et al.,* 2016*)*.

B. velezensis belongs to the *B. amyloliquefaciens* operational group which also contains *B. amyloliquefaciens* subsp. *amyloliquefaciens* and *B. siamensis.* These three clades have a similar phylogenetic relationship and are closely related (Fan *et al.*, 2017). *B. velezensis* strains have been studied for their ability to produce antimicrobial compounds such as surfactin, bacillomycin-D, bacillibactin and fengycin. In fact about 8.5% of the *B. velezensis* genome is dedicated to synthesis of lipopeptide antimicrobials, compared to 5% for *B. subtilis* (Chen *et al*., 2009). These antimicrobial compounds often contribute to the antifungal activities of *B. velezensis*. Surfactin,

bacillomycin-D, and fengycin play a significant role in the inhibition of *Rhizoctonia solani* by *B. velezensis* (FZB42) during bottom rot disease in lettuce crops (Chowdhury *et al.*, 2015). Studies which investigated mutant strains of *B. velezensis* found that double mutants for bacillomycin-D and fengycin genes were severely impaired in their antagonist activities against an important wheat pathogen, *Fusarium graminearum* (Gu *et al*., 2017).

B. velezensis QST 713 is the active bacterial strain in the commercially available product Serenade(R) which has been distributed by Bayer CropScience. The *B. velezensis* QST 713 strain was first isolated in 1995 from a peach tree orchard in California (Anastassiadou *et al.*, 2021). *B. velezensis* QST 713 was often described as *B. subtilis* QST 713 or as *B amyloliquefaciens* subsp. *plantarum* QST 713 (Patel *et al.*, 2011; Lahlali *et al.*, 2013; Matzen *et al.*, 2019). However, after its genome was published in 2018 (Pandin *et al.*, 2018) it was formally assigned as a *B. velezensis* strain based off an in depth comparative phylogenomic studies. It should be noted that the most recent safety data sheet released by Bayer for Serenade(R) lists *B. subtilis* as the active agent. After its development into a biocontrol product, it has been used on a range of different crops in many countries (Lahlali *et al.*, 2013; Punja *et al.*, 2016; Twizeyimana & Hartman, 2019; Ayer *et al.*, 2021). It has also been used in France to protect *A. bisporus* crops, mostly against *Trichoderma aggressivum f. europaeum* infections (green mould disease) (Pandin *et al.*, 2018) .

In 2019, a study was undertaken to identify novel biocontrol strains which may be useful for the treatment of mushroom disease (Kosanovic *et al.*, 2021). Several bacterial strains were isolated from mushroom casing environment for investigation. One unidentified strain which showed the highest antagonistic potential against *T. aggressivum* during *in vitro* test was sequenced and identified as *B. velezensis*. This isolate, which was investigated throughout this thesis, is henceforth referred to as *B. velezensis* Kos. The proteomic response of *T. aggressivum* to *B. velezensis* Kos was investigated by Kosanovic *et al*., (2021). *T. aggressivum* stress related proteins such as oxidoreductase proteins and hydrolases were found to be increased in relative abundance when exposed to *B. velezensis* Kos, while growth proteins such as ribosomal and proteasome subunit proteins were decreased in relative abundance.

Proteomic analysis also showed that *B. velezensis* Kos did not induce any stress responses in *A. bisporus (*Kosanovic *et al.,* 2021*)*.

1.5.4 Crop protection product development

The success in finding new chemical products has been very low in recent years (Marchand *et al* 2023a, Marchand *et al* 2023b). Development of a new fungicide product is a very laborious, costly project which involves many years of testing different chemical groups to find one that can inhibit fungal growth. After that, even more work is required to test the chemical compound and its related compounds for its dosage rates, target range and environmental compatibility. Chemical product development is a very long and expensive process. Historically, a success ratio for registering a new chemical product could have been as low as 1:5000 (Campbell, 1989). In 1995, the success rate for fungicide development was reported 1:52,500 and in 2010-2014 it had risen to 1:159,574 (McDougall, 2016). Some believe that the reason for this drastic reduction of discovery rate, is due to the fact that most of the effective chemical compound groups have already been discovered. The development of a biocontrol product on the other hand is much cheaper to achieve. Generally, as biological products are subjected to the same regulation procedures as chemical products, the time it takes to develop both products is the same. Although growth of the biocontrol or biopesticide market has be increasing significantly in the last few years, it still only represents 10% of the total global pesticide market. However, it is expected to continue to grow and predicted to be worth 15 billion dollars by 2029 (Marrone, 2024).

1.6 Novel methods used to investigate biocontrol activity

Historically, research on the activity of potential biocontrol strains has been limited to classical microbiological interaction experiments between biocontrol and pathogenic strains. These include simple inhibition assays which can tell if the biocontrol strain can reduce the growth of the pathogen, but will not tell how exactly the biocontrol strain accomplishes this. Another question researchers are keen to answer is how the addition of these biocontrol strains may impact the microbiome of the environment. As many of the bacteria and fungi present in the environment cannot be cultured in the lab, this would be impossible to determine with standard microbiological assays and

would require culture-independent approaches. Advances in the field of proteomics and sequencing can be employed to discover more information about how these biocontrol treatments are working, which could result in the improvements to biocontrol product development and application (Chinnasamy, 2006; Massart *et al.*, 2015).

1.6.1 Proteomics as a tool to study microbial interactions

Proteomic analysis is the large scale study of all proteins within complex biological proteomes. The term proteome was first used in 1995 where it was described as ‘‘all proteins expressed by a genome, cell or tissue’ (Wilkins *et al.*, 1996). Early on in the field of proteomics, scientists were limited to identifying and characterising individual or small groups of proteins. However, improvements in knowledge and advances in the technology used in mass spectrometry (MS) and bioinformatics allowed for a more in-depth analysis (Bradshaw & Burlingame, 2005). It became possible to compare the expression of proteomic profiles under different environments/stresses with more reliability, reproducibility and efficiency (Walther & Mann, 2010). Current proteomic techniques allow for a more high-throughput and large-scale analysis which can perform quantitative comparisons of different proteomes (Chandramouli & Qian, 2009). This makes proteomics a suitable technique to determine the response of microbial pathogens to biocontrol species. The response of different fungal pathogens to antifungal agents has been studied extensively using proteomics (Delgado *et al.*, 2015; Tilocca *et al.*, 2019; Álvarez *et al.*, 2021; Álvarez *et al.*, 2022).

Comparative protein expression analysis of different proteomes is one of the key approaches which can be undertaken with proteomics. Gel-based techniques were traditionally used for comparative proteomic analysis. This included the use of 2-dimensional polyacrylamide gel electrophoresis which was commonly employed to separate and identify protein complexes. The main limitations for this gel-based analysis included low reproducibility, difficulty separating ‘extreme proteins’ (such as extremely acidic, basic or hydrophobic proteins) and limitations on the number of proteins which could be run on a single gel (Magdeldin *et al.*, 2014). Significant advancements have been made in quantitative mass spectrometry (MS)-based techniques, which has become the more common technique to employ for comparative expression analysis.

'Top down' and 'bottom up' are the two main approaches which can be taken for MS analysis. Top down proteomics allows for the identification of intact proteins, without the need for prior protein digestion. Proteins are fractionated with the mass spectrometer during the gas phase, and the resulting fragmentation profile can be compared to databases for protein identification (Graham *et al.*, 2007; Toby *et al.*, 2016). Conversely, bottom up proteomic techniques involve digestion of proteins, either chemically or enzymatically into peptides (Gundry *et al.*, 2010; Duong & Lee, 2023). The peptides are also purified prior to loading onto the MS to remove any contaminates which would interfere with the analysis (Gundry *et al.*, 2010). Shot-gun proteomics is commonly used for the bottom up approach. Working with peptides rather than intact proteins comes with a lot less challenges as peptides are easier to fractionate with mass spectrometry compared to whole proteins (Miller & Smith, 2023). The digested, purified peptides are analysed using liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS), collectively known as LC-MS/MS (Meyer, 2021). This technique may be label-based or label-free. Labelling involves the incorporation of chemical isotope labels to the peptides prior to LC-MS/MS analysis. Label-free quantitative proteomics (LFQ) relies on chromatogram peak intensities and spectral counting of peptides for protein quantification (Anand *et al.*, 2017). Label-free methods allow for the characterisation of changes in protein expression levels without focusing on individual proteins which allows for large protein profiles to be analysed whilst limiting bias.

1.6.2 Next generation sequencing as a tool for studying microbial interactions

Another area which has seen significant advances is next generation sequencing (NGS). The development of culture-independent NGS technology has been transformative and revolutionised microbiome studies. The first type of DNA sequencing was developed by Sanger *et al.*, (1977). This technology was highly significant and is seen as the gold standard for DNA sequencing, however early Sanger sequencing was extremely laborious and required reading the DNA film containing the gel separated DNA fragments with the naked eye (Mardis, 2013). Sanger sequencing is still preformed for sequencing small regions of DNA but can be very costly (Metzker, 2010). Over the past few decades, more sequencing platforms have been developed, each with different capabilities and advantages. These newer

technologies have made DNA sequencing faster, cheaper and more reliable (Metzker, 2010). First introduced in 2004, the NGS sequencing platforms were capable of sequencing many strands of DNA simultaneously. The work flow for NGS sequencing generally consists of DNA extraction, DNA fragmentation, library preparation, sequencing, and finally bioinformatic analysis to piece together and interpret the sequencing data. Roche 454, Illumina HiSeq, MiSeq (second generation), PacBio (third generation) and MinION (fourth generation) platforms are all examples of NGS sequencing platforms. Most recently, DNBSEQ sequencing technology was released by MGI Tech Co (Kumar *et al.*, 2019). DNBSEQ utilises DNA Nanoball (DNB) technology to deliver high accuracy sequencing with the lowest error rates reported compared to other short-read sequencing platforms like Illumina (Rao *et al.*, 2020; Jeon *et al.*, 2021; Hu *et al.*, 2024).

Next generation sequencing is one of the best ways to look at the population dynamics of a community within the soil and monitor any changes to that community over time. Prior to this, researchers were depending on culture-dependent and PCR-based approaches which often could not show the full picture of what was happening within the soil. The dynamic nature of soil microbial communities can now be investigated with metagenomic techniques (Garg *et al.*, 2024). Internal transcribed spacer (ITS) ribosomal RNA (rRNA) is the most common amplicon to sequence when investigating fungal DNA (Schoch *et al.*, 2012), while 16S amplicon sequencing is undertaken for bacterial DNA (Weisburg *et al.*, 1991). The 16S amplicon is approximately 1500 bp long and contains nine variable regions. Although 16S sequencing has been in use for a long time and has been very successful, one of the main drawbacks is that it is often limited in its ability to provide species level identifications (Martínez-Porchas *et al.*, 2016; Bailén *et al.*, 2020). The genomes of many important biocontrol strains have now been sequenced using 16S and ITS rRNA sequencing which has improved our knowledge of the mechanisms these strains employ to antagonise pathogens which may improve biocontrol strategies in the future (Hernández-Salmerón *et al.*, 2016; Pandin *et al.*, 2018; Luo *et al.*, 2022; Dong *et al.*, 2023; Yang *et al.*, 2024).

1.7 Overview of thesis objectives

Increasing levels of fungicide resistance and the removal of approved fungicide products due to toxicity issues is a very serious concern for all horticulture sectors. The mushroom industry is especially vulnerable due to limitations in the type of fungicide products it can use. At the time of writing, there is only one approved fungicide for use on mushroom crops in Europe, with no fungicides products being advertised as coming down the pipeline for the future. There is an urgent need to identify potential alternatives for fungicide use in the mushroom industry which is the primary aim of this thesis.

This thesis stems from the discovery of the novel *B. velezensis* Kos isolate in 2019. As mentioned in section 1.5.3.3, this study was the first to note the potential of *B. velezensis* Kos as a biocontrol strain for the mushroom industry. However, during this study only one mushroom disease (green mould disease) had been investigated in relation to *B. velezensis* Kos and no large scale crop trials had been undertaken (Kosanovic *et al.*, 2021). *B. velezensis* QST 713 is another biocontrol strain which had been studied in the mushroom industry in a limited capacity. More research was required in relation to both of these strains to confirm their potential as treatments in the mushroom industry.

The first aim of this project was to confirm the antagonistic potential of *B. velezensis* Kos and QST 713 against the fungal pathogens which cause cobweb disease (*Cladobotryum* spp.) and dry bubble disease (*L. fungicola*) *in vitro*. To achieve this *in vitro* inhibition assays were performed along with fluorescent microscopy to determine if the bacterial strains could reduce the growth of the pathogen and determine if they caused any structural damage to fungal hyphae. In order to gain a better understanding of the response of the fungal pathogens to these biocontrol strains, LFQ proteomics was performed. Alterations to protein expression in the pathogen following exposure to culture filtrate from the biocontrol bacteria were investigated. Efforts to isolate and identify the inhibitory component of *B. velezensis* Kos culture filtrate was also undertaken to try shed light on a potential mode of action for this strain.

The second aim of this project was to determine the effectiveness of biocontrol strain in an *A. bisporus* crop environment. Large scale mushroom disease trials were carried

out in industry-standard growing rooms for both cobweb disease and dry bubble disease. Fungicides common to the mushrooms industry (prochloraz and metrafenone) were included in this work to compare biocontrol with conventional treatment methods. Healthy yield and disease progression was monitored over the course of each crop trial. The resistance profile of *Cladobotryum* and *L. fungicola* against these fungicides was also investigated to determine the effectiveness of fungicides for the future of mushroom disease control.

The third and final aim of this project was to determine if the application of biocontrol agents to the *A. bisporus* crop environment resulted in any alterations to the natural microbial population dynamics within the casing layer. Microbial DNA was extracted from the casing layer at several timepoints over the course of a crop trial. The amplicon sequence variants (ASVs) of biocontrol-treated and control samples were compared after 16S and ITS sequencing.

The results which have been generated in this thesis, which focus on the three aims listed above, will contribute to our knowledge of integrated pest management and biocontrol use in the mushroom industry. Overall this thesis shows that biocontrol treatment does show potential for the treatment of cobweb disease and dry bubble disease on *A. bisporus* crops. However, it also discusses the limitations of biocontrol which may complicate its incorporation into disease control practices within the mushroom industry.

Chapter 2

Analysis of the effect of *Bacillus velezensis* culture filtrate on the growth and proteome of *Cladobotryum mycophilum*

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Author Contributions

in vitro inhibition assays were carried out and analysed by JC

Microscopy was carried out and analysed by JC

Qualitative and quantitative proteomic extractions, LCMS data analysis was carried out and analysed by JC

Manuscript writing was performed by JC and KK

Manuscript editing was performed by DF, HG

Abstract

Cladobotryum mycophilum, the causative agent of cobweb disease on *Agaricus bisporus* results in significant crop losses for mushroom growers worldwide. Cobweb disease is treated through strict hygiene control methods and the application of chemical fungicides but an increase in fungicide resistant *Cladobotryum* strains has resulted in a need to develop alternative biocontrol treatment methods. The aim of the work presented here was to evaluate the response of *C. mycophilum* to a *Bacillus velezensis* isolate to assess its potential as a novel biocontrol agent. Exposure of 48 hr *C. mycophilum* cultures to 25% v/v 96 hr *B. velezensis* culture filtrate resulted in a 57% reduction in biomass ($P < 0.0002$), a disruption in hyphal structure and morphology, and the appearance of aurofusarin, a secondary metabolite which is a known indicator of oxidative stress, in culture medium. Proteomic analysis of *B. velezensis* culture filtrate revealed the presence of peptidase 8 (subtilisin), peptide deformylase and probable cytosol aminopeptidase which are known to induce catalytic activity. Characterisation of the proteomic response of *C. mycophilum* following exposure to *B. velezensis* culture filtrate revealed an increase in the abundance of a variety of proteins associated with stress response (ISWI chromatin-remodelling complex ATPase ISW2 (+24 fold), carboxypeptidase Y precursor (+3 fold) and calmodulin (+2 fold). There was also a decrease in the abundance of proteins associated with transcription (40 S ribosomal protein S30 (-26 fold), 40 S ribosomal protein S21 (-3 fold) and carbohydrate metabolism (L-xylulose reductase (-10 fold). The results presented here indicate that *B. velezensis* culture filtrate is capable of inhibiting the growth of *C. mycophilum* and inducing a stress response, thus indicating its potential to control this important pathogen of mushrooms.

Keywords: *Agaricus bisporus, Cladobotryum mycophilum*, *Bacillus velezensis,* Proteomics

List of abbreviations:

MBC: methylbenzimidazole carbamate

IPM: integrated pest management

SUD: Sustainable Use of Pesticides Directive

GO: gene ontology

BP: biological process

MF: molecular function

CC: cellular component

SSDA: statistically significant differentially abundant

LFQ: Label free quantitative-proteomic

MEA: malt extract agar

NB: nutrient broth

NA: nutrient agar

SDB: Sabouraud dextrose liquid broth

PDA: potato dextrose agar

CF: culture filtrate

ANOVA: analysis of variance

PCA: principal component analysis

GRAS: generally regarded as safe

2.1 Introduction

Mushrooms are susceptible to a wide range of viral, bacterial and fungal pathogens that adversely affect quality and reduce yield. One of the most challenging fungal pathogens of mushrooms, *Cladobotryum mycophilum*, is difficult to control and the recent appearance of fungicide-resistant isolates has compromised treatment of crops (Grogan and Gaze 2000; Grogan 2008). There is an urgent need to identify novel ways to control this increasingly serious pathogen and biocontrol agents may represent a new and effective way to control infection. Other fungal pathogens that affect mushroom cultivation such as wet bubble disease (*Mycogyne perniciosa*), dry bubble disease (*Lecanicillium fungicola*) and green mould disease (*Trichoderma aggressivum*) have also developed resistance to some fungicides and may require biocontrol agents for control in the near future (Fletcher and Gaze, 2008).

Cobweb disease due to *C. mycophilum* can result in significant crop losses, product deformation and consequential revenue loss for mushroom farmers. Although cobweb disease is known to infect various economically important mushroom species (*Pleurotus eryngii, Flammulina velutipes*, and *Ganoderma lucidum*) it is mostly associated with *Agaricus bisporus* (Lange) [Imbach] (Back *et al*., 2010; Gea *et al*., 2011; Kim *et al*., 2012; Zuo *et al*., 2016; Gea *et al*., 2017). *A. bisporus* is an important edible mushroom species, accounting for around 15% of cultivated mushroom production worldwide (Royse *et al*., 2017). The widespread use of white hybrid strains derived from HorstU1 in *A. bisporus* cultivation means that world production is effectively a monoculture, and this has made the species more universally susceptible to attack by the same pathogens due to its lack of genetic diversity (Sonnenberg *et al*., 2017).

Cobweb disease is caused by several members of the *Cladobotryum* genus including *Cladobotryum dendroides, C. mycophilum, Cladobotryum varium, Cladobotryum multiseptatum, Cladobotryum asterophorum, Cladobotryum semicirculare and Cladobotryum protrusum*, occurring naturally in the wild on several different fungal basidiomycete taxa including polypores (Tamm and Poldmaa 2013). Historically, *C. dendroides* (teleomorph: *Hypomyces rosellus*) (Gams and Hoozemans 1970) was most associated with cobweb incidence in commercial *A. bisporus* production. Today it is believed that *C. mycophilum* (teleomorph: *Hypomyces odoratus*) (Gams and

Hoozemans 1970), has overtaken *C. dendroides* as the most common causative agent of cobweb disease (McKay *et al*., 1999; Tamm and Poldmaa 2013). A strain of *Cladobotryum*, resistant to methylbenzimidazole carbamate (MBC) fungicides which had similar characteristics to *C. mycophilum* but lacked the camphor odour distinctive of the species was classified as *C. mycophilum* type II (McKay *et al*., 1999).

In *A. bisporus* cultivation *Cladobotryum* species grow over the casing layer with a white, fluffy mass of mycelium which can spread to, and envelope neighbouring fruit bodies. If left untreated, the fruit body will usually present first with discolouration and eventually rot and become unsellable. If not dealt with immediately, localized outbreaks will appear throughout the mushroom crop. *C. dendroides* spores are very easily dispersed through air conditioning systems or through the action of watering/salting (Adie and Grogan 2000; Adie *et al*., 2006). Conidial dispersion results in the appearance of brown, irregular spots on the mushrooms when the spores land on the *A. bisporus* caps and germinate (Adie and Grogan 2000). The infection then spreads to adjacent mushrooms on the bed, eventually resulting in rotted crops, which are unfit for sale. Conidial dispersion also results in the establishment of secondary points of infections which further reduces crop yield (Adie *et al*., 2006). Cobweb disease is controlled on mushroom farms through strict hygiene control methods and fungicide application. It is important to treat cobweb disease as soon as it becomes apparent on the mushroom farm to prevent conidial dispersion from getting out of control. The recommended treatment method for cobweb disease is to cover the diseased area with a thick layer of damp paper and generously cover with salt (Grogan and Gaze 2008). This is done to prevent the dispersion of conidia from the infected area, however caution is needed as salting without damp paper has been shown to aid conidial dispersion (Adie *et al*., 2006).

Currently, there are only a limited number of chemical fungicides approved for use on mushroom crops. Prochloraz is mostly used in Europe, while chlorothalonil and thiabendazole are popular in North America (Grogan, 2008; Potocnik *et al*., 2015). Reliance upon a select number of fungicides has resulted in various mushroom pathogens becoming resistant to current treatment options (Grogan, 2008). In the 1970s, MBC fungicides such as benomyl and carbendazim were commonly applied to mushroom crops to protect against cobweb disease (Fletcher, 1973). Resistance to

these fungicides emerged within a decade, and were first identified in Great Britain (Gaze, 1995; McKay *et al*., 1998). These resistant strains are also present in Irish isolates, and were responsible for a crisis which reached epidemic proportions in the 1990s (Adie *et al*., 2006). A new fungicide, metrafenone, was approved for use against cobweb disease in several European countries in 2017 (Carrasco *et al*., 2017; Pyck *et al*., 2016) but there are already widespread reports that metrafenone-resistance has developed. Most countries are now planning to reduce the use of fungicides and implement integrated pest management (IPM) strategies. This strategy is promoted under the Sustainable Use of Pesticides Directive (SUD) 2009/128/EC (Anon, 2009). When available, non-chemical biocontrol treatments should be used in place of chemical fungicides, which can be harmful to both human health and the environment.

The application of biocontrol agents offers a potential alternative to fungicide use in mushroom crop cultivation. *Bacillus* species are commonly studied as potential biocontrol agents due to their ability to out compete fungal pathogens in spatial and nutritional competition (Borriss, 2015; Pandin *et al*., 2017). Their use is complicated by the fact that both host, and pathogen are fungal species. SERENADE® (AgraQuest Inc.) is a commercially available biocontrol agent which has been used to treat *T. aggressivum* and *L. fungicola* successfully (Stanojevic *et al*., 2019). Serenade is diluted into water and sprayed onto crops prior to the formation of mushroom pins. It uses *Bacillus velezensis* QST 713 as its active ingredient (Pandin *et al*., 2018). It has not been shown to be effective against cobweb species. Kosanovic *et al*. (2021) isolated an environmental *B. velezensis* strain (strain Kos) which was able to control the growth of *T. aggressivum* without adversely affecting *Agaricus* growth. Here, we aim to investigate the potential of this *B. velezensis* strain as a biocontrol agent against *C. mycophilum in-vitro*.

2.2 Materials and methods

2.2.1 Culture conditions

A carbendazim and metrafenone resistant *C. mycophilum* type II strain (isolate 1546), isolated from an infected mushroom crop and stored in the Teagasc Ashtown culture collection (Dublin, Ireland), was used in these studies. Cultures were grown on malt extract agar medium (MEA) (Oxoid) at 25°C for up to 3 days, in the dark. Liquid cultures of *C. mycophilum* were grown in Sabouraud dextrose liquid broth (SDB), at 25°C, 120 rpm for 48 h. *B. velezensis* (strain Kos) (Kosanovic *et al*., 2021) was obtained from liquid nitrogen stocks at Maynooth University (Kildare, Ireland). *B. velezensis* cultures were maintained on nutrient agar (NA) (Oxoid) and were grown at 25°C, in the dark for 2-3 days.

2.2.2 *B. velezensis* culture filtrate (CF) collection and separation

B. velezensis culture filtrate (CF) was established by adding a loopful of *B. velezensis* from an NA plate culture, into nutrient broth (NB) (Oxoid) (50 ml). Flasks were incubated at 30°C at 120 rpm in an orbital incubator. At 24 h, 48 h, 72 h and 96 h, CF was collected by centrifugation (20 min, x 5000 g), and was passed through 0.45 mm filtropur S filters (Sarstedt Ltd). CF was stored at 20°C until needed. The 96 h CF was filtered through a 0.2 mm filtropur S filters (Sarstedt Ltd) and separated into four fractions; >3 kda polar, > 3 kda non-polar, <3 kda polar and <3 kda non-polar using Vivaspin 20 centrifugal concentrator (satorius) and C18 cartridges (Sep-Pak (R) Vac 3 cc 200 mg). Samples were lyophilised and resuspended in ddH_2O.

2.2.3 The effect of *B. velezensis* culture filtrates on the growth of *C. mycophilum* to identify inhibitory component

A *C. mycophilum* conidial suspension was prepared and its conidia concentration was determined using a haemocytometer. The suspension was then adjusted to x10^5 /ml. A 100 µl aliquot (x10^4 conidia/plate) was spread onto potato dextrose agar (PDA) plates using a sterile spreader, and plates were left to dry for 15 min. Wells (8 mm, diameter) were added to the PDA plates and 50 µl of the four 96 h *B. velezensis* culture filtrate fractions were applied to the wells. Plates were incubated at 25°C, in the dark for three days.

2.2.4 Label free qualitative proteomics of polar, >3 kda fraction from *B. velezensis* 96 h culture filtrate

The plate inhibition assay identified the >3 kda, polar sample as inhibitory against *C. mycophilum*. This sample was acetone precipitated overnight. The protocol for protein extraction and mass spectrometry preparation was as described in Margalit *et al*. (2020). A detailed description can also be found in **Appendix 9.1**. Samples were run on a QExactive (ThermoFisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient from 2% to 40% on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120-min reverse phase gradient at a low rate of 250 nl/min. A full MS scan of range 200-2000 was followed to select the 15 most intense ions prior to MS/MS. Qualitative analysis was carried out using Proteome Discoverer 1.4 and Sequest HT (SEQUEST HT algorithm, Thermo Scientific). Proteins which were identified within the >3 kda *B. velezensis* CF sample were queried against the *B. velezensis* proteome (identifier 492670) (Genome assembly accession: CP026610, proteome ID: UP000425588) downloaded from www.uniprot.org. Proteins with a score of <0 and a peptide number <2 were excluded from the analysis.

2.2.5 The effect of *B. velezensis* culture filtrate on the growth of *C. mycophilum in-vitro*

C. mycophilum (x10^4 conidia/plate) was spread onto PDA plates using a sterile spreader and was left to dry for 15 min. Once dry, wells (8 mm diameter) were cut in the PDA plates and 50 µl of *B. velezensis* culture filtrate at various time points (24-96 h) were added into the wells. *B. velezensis* culture drops (10 µl) were also added directly onto plates. Samples of both CF and cell culture drops were applied in triplicate. All plates were incubated at 25°C for three days. NB was used as a negative control. In order to determine the optimal timepoint of *B. velezensis* CF, samples from 24, 48, 72, 96, 120, 144, 168 and 196 h were applied to PDA plates which contained *C. mycophilum* (x10^4 condida/plate) as described above. Zones of inhibition were calculated by measuring the distance (mm) from the centre of the well to the furthest point of growth disturbance. This radius value was then used to calculate the area of the zone ($A = \pi r^2$).

Cultures of *C. mycophilum* (x10^3 conidia/ml) were grown in 50 mls of SDB for 48 h at 25°C and 120 rpm. Cultures were then supplemented with 96 h *B. velezensis* culture filtrate at a concentration of 25% v/v for treatment 1 and 12% v/v for treatment 2. Control flasks were supplemented with NB to a 25% v/v concentration. Cultures were incubated under the same conditions for a further 24 h. This experiment was carried out with five replicates per treatment. The mycelium wet weight was measured once the culture filtrate had been removed.

One protein present within the *B. velezensis* 96 h CF which was of particular interest was peptidase S8 or subtilisin. Flasks of *C. mycophilum* (x10^3) (30 ml) were grown for 48 h at 25°C and 120 rpm. The flasks were then supplemented with 12.5% v/v purified subtilisin dissolved in PBS (1 mg/ml) (Sigma-Aldrich). Flasks were returned to the same conditions for a further 24 h. Control flasks were supplemented with 12.5% v/v PBS. Each treatment was done in triplicate. After 24 h, the mycelium within the flasks were weighed and the effect of subtilisin on mycelium wet weight per treatment was determined.

2.2.6 Microscopy

Stationary phase *C. mycophilum* (x10^3 conidia/ml) was grown for 48 h and supplemented with either 24 or 96 h *B. velezensis* culture filtrate (25% v/v) for a further 24 h. Fungal cultures supplemented with 25% v/v nutrient broth were used as a control. Hyphae were collected from the cultures and a small sample from each treatment was applied to a glass slide. Hyphae were first washed with PBS (50 µl) three times. Calcofluor white (25 µl, Sigma-Aldrich) was then applied to the hyphae for 5 min at room temperature. The excess stain was washed off with PBS. A cover slip was placed directly on top of the stained hyphae. Slides were visualised on Olympus BX51 fluorescent microscope using visible light (X40 lens).

2.2.7 Label free quantitative proteomics of *C. mycophilum* treated with *B. velezensis* culture filtrates

Proteins were extracted from *C. mycophilum* mycelium which was grown for 48 h before it was supplemented either with 12.5 ml/50 ml *B. velezensis* 96 h CF (treatment 1), 6.25 ml/50 ml *B. velezensis* 96 h CF (treatment 2), or 12.5 ml/50 ml NB (control). Each treatment was performed in replicates of five. The protocol for protein

extractions and mass spectrometry run are described in Margalit *et al*. (2020). A detailed description can also be found in **Appendix 9.1**. For the quantitative run, 0.75 µg of the resuspended *C. mycophilum* digested protein mix was applied to the QExactive rather than 0.50 µg. The steps for proteomic data analysis are also described in Margalit *et al*. (2020). Quantitative analysis was performed using Andromeda search engine in Max-Quant (version 1.6.17 https://www.maxquant.org/). Max-Quant was used to identify the proteins within the sample and to correlate them against the *Trichoderma harzianum* proteome (strain CBS 226.95 (Genome assembly accession: #MBGI01000000, proteome ID: UP000241690) downloaded from www.uniprot.org. There is no *Cladobotryum* database currently available on UniProt, hence why a closely related species, *T. harzianum* was chosen (Xu *et al*., 2020). Perseus (version 1.6.14.0) was employed for data and statistical analysis as well as graphics visualisation (Margalit *et al*., 2020). Gene ontology (GO) mapping was performed in Perseus, the UniProt gene IDs for all identified proteins were queried against a *T. harzianum* annotation file generated from Blast 2 Go tool (https://www.blast2go.com/) and uploaded to Perseus. This provided terms for gene ontology biological process (BP), gene ontology cellular component (CC), gene ontology molecular function (MF) and UniProt name for each protein. ANOVA of significance and t-tests between the treated groups was performed using a p-value of 0.05. Volcano plots were generated by plotting the log2 fold change on the x axis against the log p values on the y axis for each pairwise comparison. Statistically significant and differentially abundant (SSDA) proteins (ANOVA, $p < 0.05$) with a relative fold change greater than ± 0.58 were retained for analysis. A principal component analysis (PCA) was generated with ANOVA significant proteins. (PCA with dataset pre-ANOVA available in **Appendix 9.2**). SSDA proteins were Z-score normalised and then used for hierarchical clustering to produce a heat map. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026467.

2.3 Results

2.3.1 Analysis of the effect of *B. velezensis* culture filtrate on the growth of *C. mycophilum*

B. velezensis CF will contain various substances that have been secreted by the organism during its growth. We wanted to determine whether *B. velezensis* Kos CF had antifungal activity. The effect of *B. velezensis* CF on *C. mycophilum* growth was determined as described and the greatest zone of inhibition was evident when 96 h CF was used (**Figure 2.1**). Clear zones of inhibition could be found surrounding areas where *B. velezensis* CF had been applied on PDA plates containing *C. mycophilum* ($x10^4$ /plate) (**Figure S1A**). CF samples from all collection time points (24, 48, 72 and 96 h) produced distinct zones of clearance. Bacterial cells from each time point also significantly inhibited the growth of *C. mycophilum* ($x10^4$/plate) on PDA plates (**Figure S1B**). The growth within *C. mycophilum* flask cultures which were supplemented with *B. velezensis* 96 h CF was assessed after 24 h. The presence of *B. velezensis* culture filtrate reduced the growth of *C. mycophilum* hyphae within these flask cultures. The average wet weight in control flasks, which contained *C. mycophilum* ($x10^3$/ flask) and NB was 2.8±0.46 g (grams). In flasks which contained either 25% v/v or 12.5% v/v, *B. velezensis* 96 h CF, the growth of *C. mycophilum* was reduced by 57% ($p < 0.0002$). The average wet weight was 1.2 ± 0.24 g and 1.2 ± 0.18 g for treatment 1 and treatment 2 respectively (**Figure 2.2**). It should be noted that there may be nutrients present within the NB applied to the control samples which may account for some increase in wet weight. There was also a physical difference between control and treatment flasks as the medium in the treated flasks was reddish/brown colour (**Figure S2**).

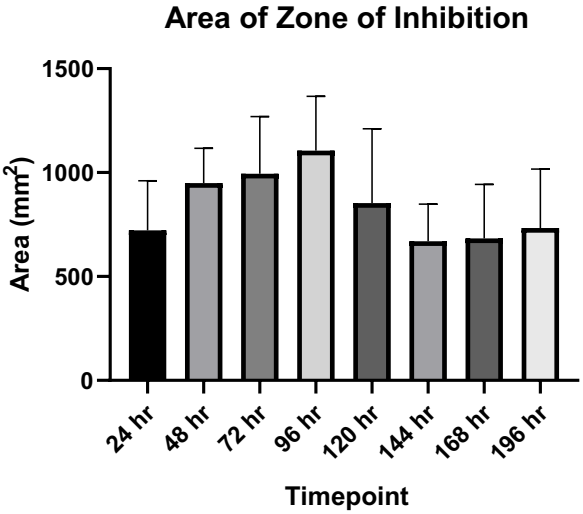


Figure 2.1 The average area ($mm^{2)}$ of the zone of inhibition produced by *B. velezensis* CF of various timepoints (24 hr, 48 hr, 72 hr, 96 hr, 120 hr, 144 hr, 168 hr and 196 hr) when grown on PDA plates with *C. mycophilum* ($x10^4$) for 72 hr at 25°C. (n=3).

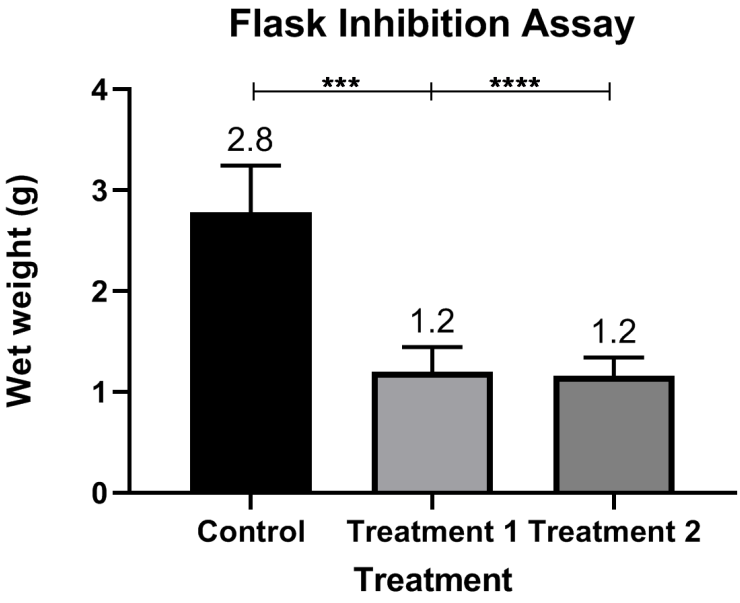


Figure 2.2 Flasks of *Cladobotryum* ($x10^3$) were grown for 48 h and then supplemented with either NB (control), 25% v/v 96 h CF (treatment 1) or 12.5% v/v 96 h CF (treatment 2) for 24 h. Average hyphae wet weight per treatment is graphed above. Error bars represent standard deviation. ***: P value 0.0002, ****: P value < 0.0001.

2.3.2 Microscopic examination of *C. mycophilum* cultures exposed to *B. velezensis* culture filtrate

The images produced of the *C. mycophilum* hyphae by microscopy show that both hyphal growth and development were seriously disrupted when exposed to *B. velezensis* CF. Hyphae taken from control were well defined and linear (**Figure 2.3 A**). Hyphae of *C. mycophilum* treated either with 24 h or 96 h *B. velezensis* CF were clearly disrupted and contained globular structures (**Figure 2.3 B and C**). Fewer hyphae were observed in these treatments, and when present, hyphae appeared short and rounded. The internal organisation of the hyphae also seemed to be adversely affected by *B. velezensis* CF.

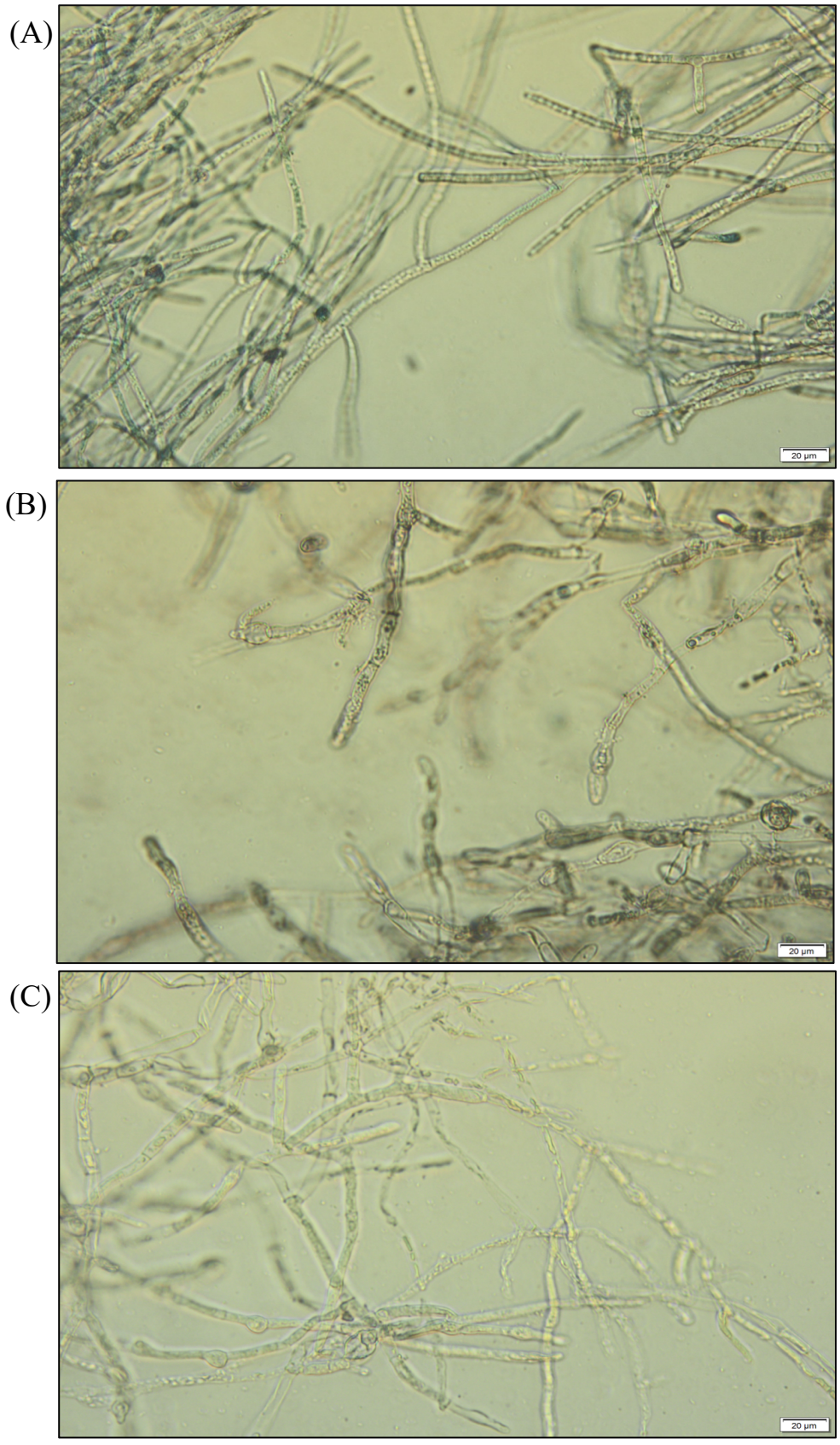


Figure 2.3 Hyphae from treatment and control samples were visualised using an Olympus microscope at magnification X20. All cultures were grown for 48 h in SDB prior to treatment application. **(A)** Control hyphae (supplemented with 25% v/v nutrient broth) appeared to be healthy and well-structured and defined. *Cladobotryum* treated with 25% v/v 24 h CF **(B)** and 25% v/v 96 h CF **(C)** appeared to be damaged and twisted.

2.3.3 The effect of *B. velezensis* culture filtrate fractions on the growth of *C. mycophilum* to identify inhibitory component

B. velezensis CF was fractionated to assist in identifying the agents in it that might be responsible for growth inhibition and distortion to hyphae. A clear zone of inhibition was visualised surrounding the area on the plate where the >3kda, polar samples had been applied. A zone of clearance was also identified around the >3kda, non-polar sample. No inhibition could be seen around the <3 kda (**Figure S3**). As the zone of inhibition was strongest around the >3 kda, polar sample, this was chosen to proceed with for protein extraction and mass spectrometry.

2.3.4 Label free qualitative proteomics of *B. velezensis* >3 kda, polar 96 h culture filtrate

Qualitative proteomic analysis identified a number of proteins which were present within the *B. velezensis* CF > 3kda, polar sample that may be causing inhibition of *C. mycophilum* growth. The majority of proteins identified within this sample were peptidases (peptidase S8 (subtilisin), aminopeptidase YsdC, probable cytosol aminopeptidase) (**Table S1**). There was also a number of other enzymes present including; catalase, citrate synthase, sucrose-6- phosphate hydrolase and peptide deformylase. The effect of purified subtilisin on *C. mycophilum* growth was assessed after 24 h. The average wet weight of *C. mycophilum* exposed to 12.5% v/v subtilisin was 0.49±0.2 g. This represents a percentage decrease of 28% compared to control flasks, which had an average wet weight of 0.68±0.2 g (**Figure S4**).

2.3.5 Label free quantitative proteomics of *C. mycophilum* treated with *B. velezensis* culture filtrates

The whole cell proteomic response of *C. mycophilum* when exposed to *B. velezensis* 96 h culture filtrate (12.5% & 25% v/v) was investigated using label free quantitative (LFQ) proteomics. In total, 1279 proteins were initially identified and 733 remained after various filtration processes which removed proteins only identified by site and potential contaminants. According to analysis carried out in Perseus, there were 81 statistical SSDA proteins in treatment 1 (41 increased and 40 decreased) (analysis of variance [ANOVA], $P < 0.05$). In treatment 2, there were 41 SSDAs (24 increased and 17 decreased) ([ANOVA], $P < 0.05$). Each of these SSDA proteins had a fold change

value of > ± 0.58. All of these proteins were included in further statistical analysis. A total of 26 SSDAs were common to both treatment 1 and treatment 2. Treatment 1 therefore had 55 exclusive SSDAs, while treatment 2 had 15. The fold change of these common SSDAs appear to be higher in treatment 1 compared to treatment 2, meaning that the mutual SSDAs were found at a greater extent in treatment 1. The higher concentration of *B. velezensis* CF in treatment 1 (25% v/v) may be responsible for this larger difference in SSDA's compared to treatment 2 (12.5%v/v).

A PCA performed on filtered proteins confirmed that the two sets of treatment samples, were significantly different from the control (**Figure 2.4 A**). The control samples clustered on their own, while treatment samples clustered close together but remained separate from one another. This indicates a clear difference between proteomes of treated samples and control samples. Hierarchal clustering performed in Perseus, shows that there is a clear difference in protein abundance within the control and treatment sample. This difference is further highlighted in a heat map (**Figure 2.4 B**) which was generated from data gathered during Perseus analysis. In areas where there is decreased relative protein abundance in the treatments, there is increased relative protein abundance compared to the control. This further proved that the presence of the *B. velezensis* CF is influencing the growth of *C. mycophilum*.

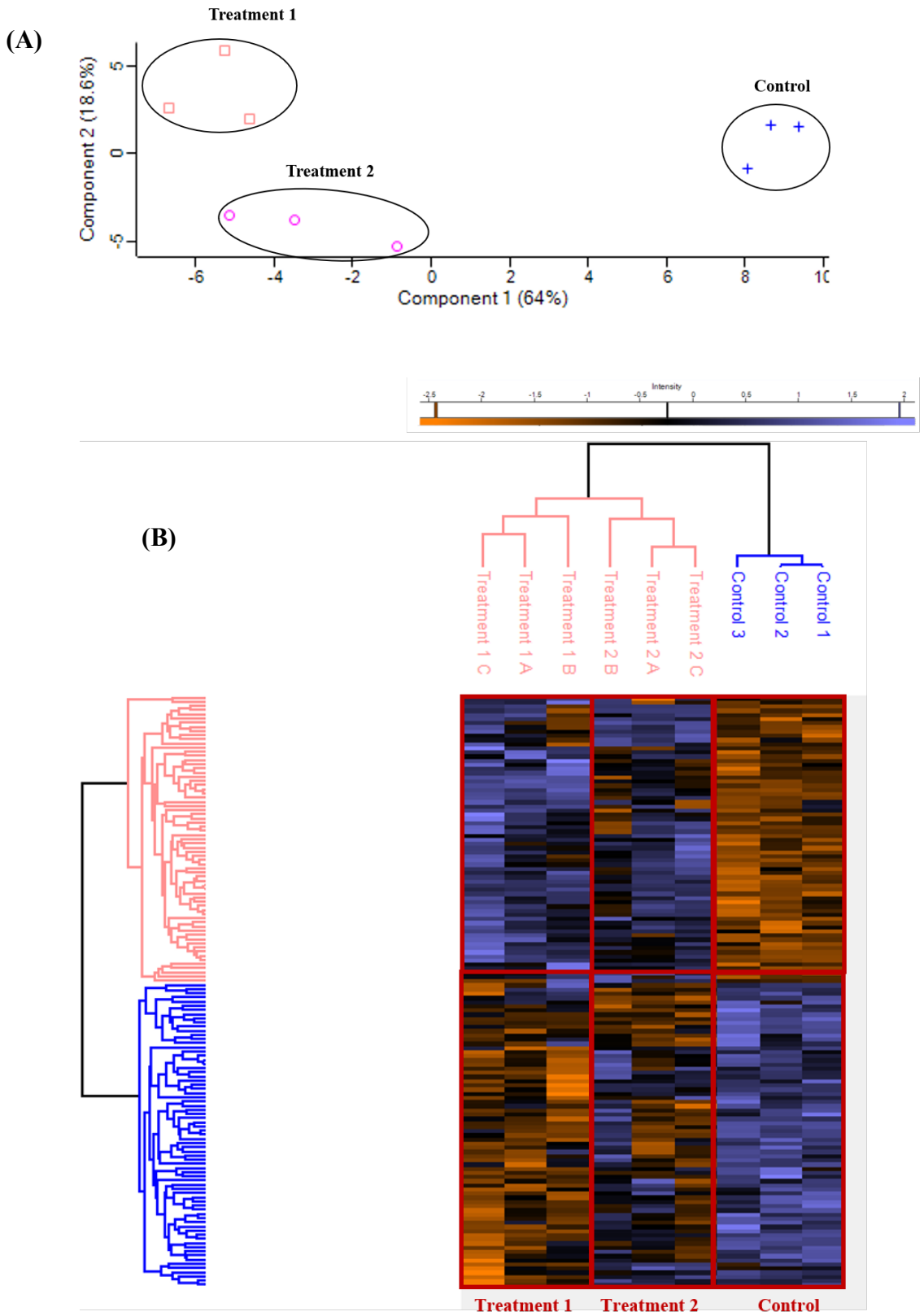
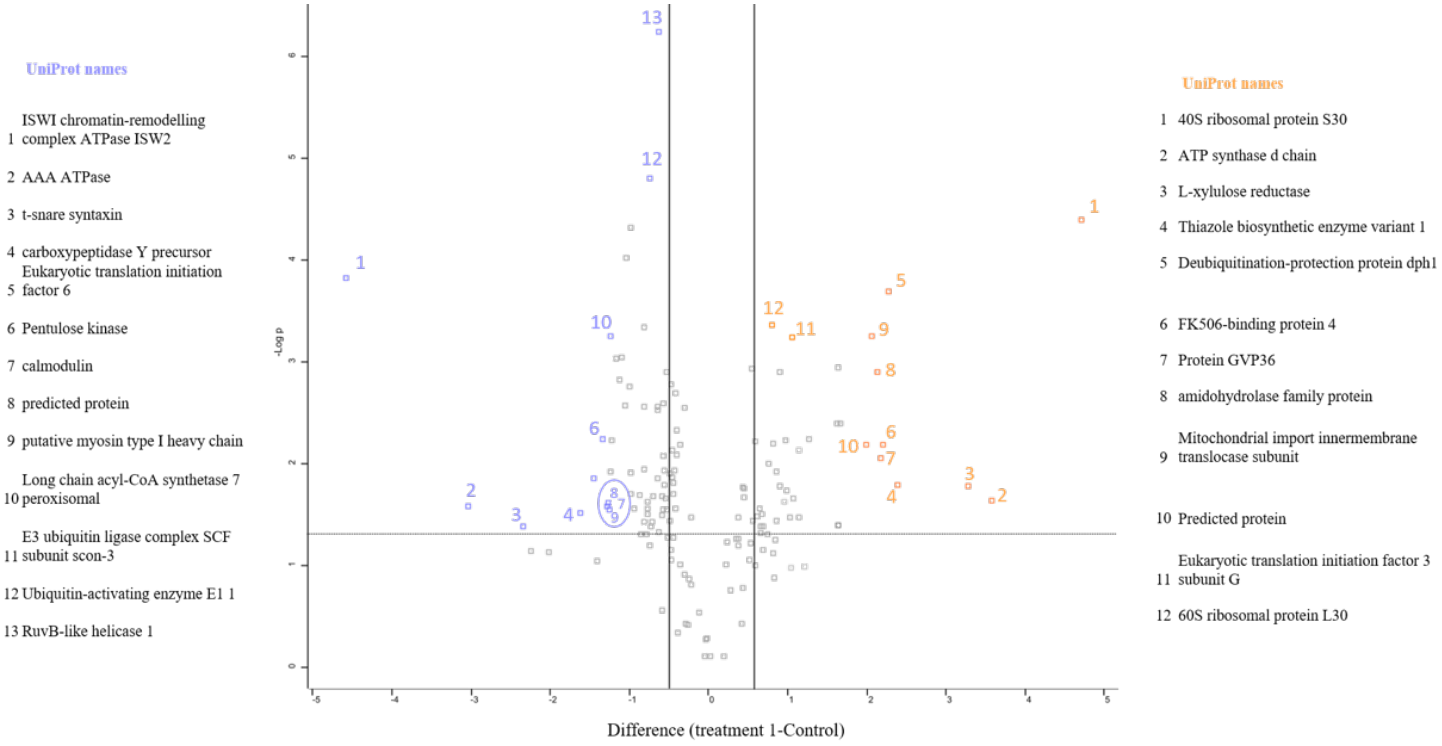


Figure 2.4 A. Principal component analysis generated during Perseus analysis. The PCA groups control samples together and away from treatment 1 and 2 samples. **B.** Heat map depicts the overall pattern of increased (purple) and decreased (orange) protein abundance within the samples Hierarchal clustering is also depicted and groups control samples and treatment samples on separate lineages.

Volcano plots **(Figure 2.5**) show the distribution of SSDA proteins. Proteins which increased in relative abundance in treatment 1 (*C. mycophilum* treated with 25% v/v *B. velezensis* CF) compared to the control include; ISWI chromatin-remodelling complex ATPase (24-fold), AAA ATPase (8-fold), t-snare syntaxin (5-fold) and carboxypeptidase Y precursor (3-fold). Proteins which decreased in treatment 1 compared to the control include; 40 S ribosomal protein S30 (26-fold), ATP synthase d chain (12-fold) and L-xylulose b5 reductase 2 (10-fold) (**Figure 2.5 A**).



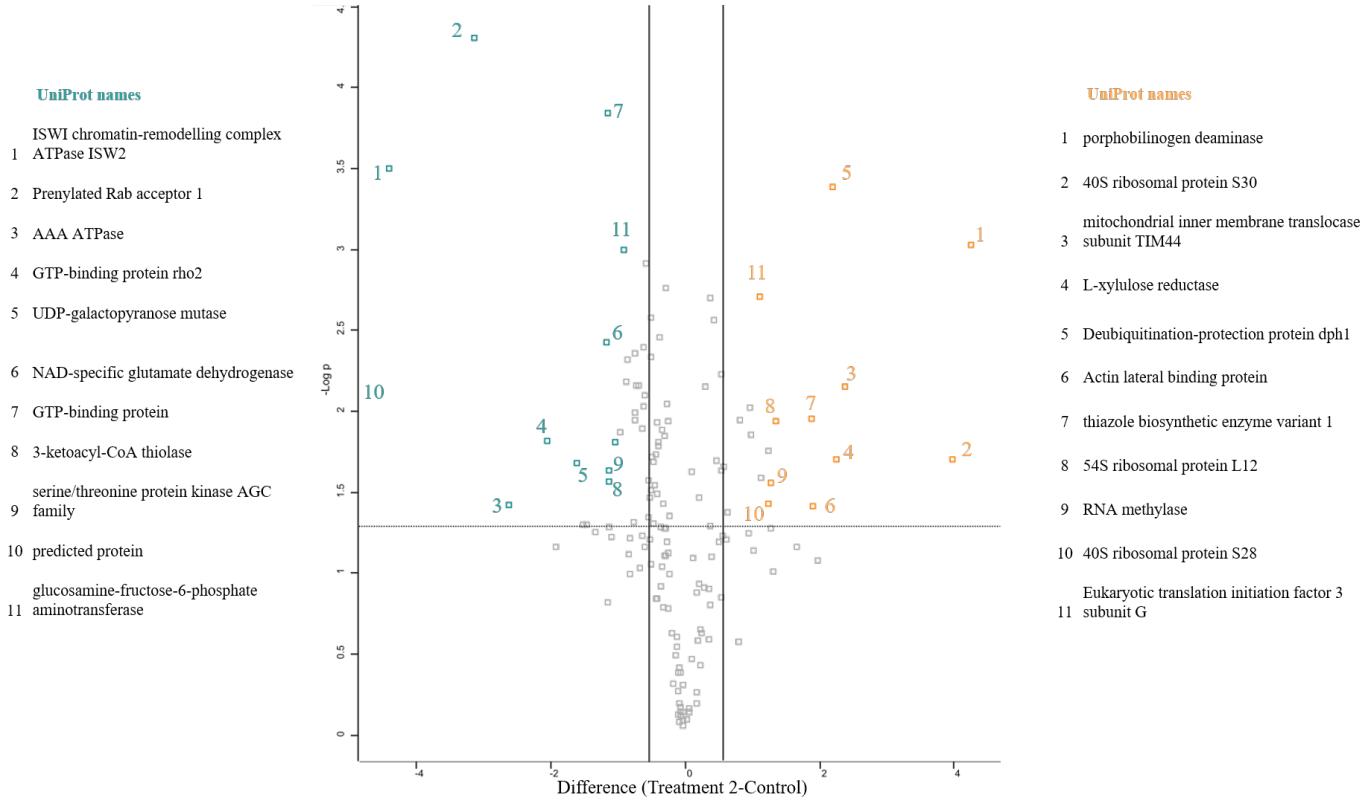


Figure 2.5 Volcano plots display the distribution of statistically significant and differentially abundant (SSDA) proteins which have a -Log (p-value) >1.3 and difference ± 0.58.

Proteins which increased in relative abundance in treatment 2 (*C. mycophilum* treated with 12% v/v *B. velezensis* CF) compared to the control include; Prenylated Rab acceptor 1 (9-fold), GTP binding protein rho2 (4-fold) as well as ISWI chromatin remodelling complex ATPase (21-fold) and AAA ATPase (6-fold), which were also upregulated in treatment 1. Proteins which decreased in treatment 2 included porphobilinogen deaminase (19-fold), mitochondrial inner membrane translocase subunit TIM44 (-5-fold) as well as 40 S ribosomal protein S30 -(16-fold) and L-xylulose reductase (-5-fold) which were also decreased in treatment 1 (**Figure 2.5 B**). The most abundant proteins, either up or downregulated in either group, are listed (**Table S2-5**).

GO mapping from the Blast2Go software tool was also carried out on SSDA proteins from each treatment group. Several GO terms which belong to biological process (BP) were enriched for treatments 1/2 compared to the control including; Proteolysis involved in cellular protein catabolism, cellular catabolic process and DNA repair. BP terms; translation, ribosome biogenesis and nucleotide metabolic process were enriched in control and down in treatments. Molecular functions (MF) such as ATP binding and magnesium ion binding were also higher in treatments compared to the control (**Figure S5**).

2.4 Discussion

The results presented here indicate that the CF of a *B. velezensis* species has a negative effect on the growth of *C. mycophilum*. Both *B. velezensis* culture filtrate and bacterial cells were able to inhibit the growth of *C. mycophilum* on PDA plates. Flask assays showed that the presence of *B. velezensis* culture filtrate in *C. mycophilum* liquid culture, could reduce biomass accumulation by an average of 57%. A review of literature on *Cladobotryum* species suggest the red colour present in *C. mycophilum* cultures exposed to *B. velezensis* culture filtrate was due to the release of aurofusarin (Poldmaa, 2011). This secondary metabolite is released by some *Cladobotryum* species, including *C. mycophilum* around 25 days into their growth. The red pigment is indicative of a cessation in growth and an oxidative stress response (Poldmaa, 2011; Cambaza, 2018). This is another indication that *B. velezensis* CF can halt the growth and development of *C. mycophilum*. Images of hyphae exposed to *B. velezensis* CF also confirm that the growth of *C. mycophilum* cells are stunted in the presence of *B. velezensis* CF. Treated hyphae are clearly being distorted while control hyphae appear healthy. Antimicrobial secondary metabolites such as fengycin are produced by *B. velezensis* species (Chen *et al*., 2009). These metabolites are known to significantly induce damage to fungal cell membranes (Deleu *et al*., 2008). The production of secondary metabolites by *B. velezensis* Kos may be causing the damage to the *C. mycophilum* hyphae.

One of the aims of this research was to identify the specific element within the *B. velezensis* culture filtrate which inhibited *C. mycophilum* growth. The results demonstrated that the inhibitory component was >3 kda. Qualitative proteomic analysis on the >3 kda, polar sample of 96 hr *B. velezensis* CF revealed a number of proteins which were present within this sample including peptidase S8 (subtilisin). Subtilisin was first isolated in *Bacillus subtilis* species, but has since been isolated from a number of *Bacillus* species (Ottesen and Svendsen 1970). This protein has serine-type endopeptidase activity and is known to be highly efficient at protein degradation. Its degradation abilities are so efficient that it is used as a key active ingredient in laundry detergents for the removal of proteinaceous stains (Vojcic *et al*., 2015). Genome mining has shown that *B. velezensis* species contain biosynthetic gene clusters within their genome which are responsible for the production of a number

specialised metabolites, including subtilisin which contribute to their antimicrobial properties (Fazle Rabbee and Baek 2020; Mullins *et al*., 2020). Therefore it is possible that subtilisin released in the *B. velezensis* culture filtrate is responsible for the inhibition of *C. mycophilum* growth. The addition of purified subtilisin to *C. mycophilum* cultures slightly reduced the biomass growth of the fungus. This suggests that although subtilisin may be inhibitory, to see the level of inhibition achieved by the 96 hr *B. velezensis* CF, it may need to work in combination with other metabolites within in the CF. Probable cytosol aminopeptidase and peptide deformylase which are both involved in catalytic activity were also found within the sample (Hernick and Fierke, 2010). Future work, using HPLC fractionation will search to identify the specific proteins, either individually or in combination, which allow *B. velezensis* CF to inhibit the growth of *C. mycophilum*.

Kosanovic *et al*. (2021), examined the response of *T. aggressivum* to the same *B. velezensis* strain investigated in this study and found that, against *T. aggressivum*, zones of inhibition could only be produced when *B. velezensis* cells were applied to plates and no inhibition was recorded around culture filtrate samples. To the best of our knowledge, this is the first time that a *B. velezensis* culture filtrate has caused inhibition against a fungal plate culture. This opens up the possibility of applying the culture filtrate directly onto *A. bisporus* crops as a treatment method, rather than applying *B. velezensis* cells which is done with Serenade.

Proteomic analysis further supports the finding that *B. velezensis* culture filtrate causes significant growth inhibition of *C. mycophilum*. Principal component analysis, hierarchical clustering and a heatmap generated from the proteomic data indicate a clear difference between treatment and control samples. Volcano plots highlighted individual proteins which were SSDA. The majority of proteins which were downregulated in both treatment samples are structural components of the ribosome and were involved in translation. Essential growth processes such as translation appear to be significantly reduced in the presence of *B. velezensis* culture filtrate. Other studies have shown that translation is altered in fungi and yeast in response to stress (Crawford *et al*., 2019; Janapala *et al*., 2019). ATP activity, ubiquitination, proteolysis, DNA repair and oxidation-reduction activity were all upregulated in both treatments, compared to the control samples. This would suggest that the *B. velezensis* culture

filtrate is having a detrimental effect on *C. mycophilum*, as the activities which have increased in response to its presence are associated with cellular stress and growth inhibition.

Overall, activities associated with normal cellular growth (e.g. ribosomal/translation proteins) appeared to be downregulated in the treated samples. Similar proteins, involved in translation were also reduced when this *B. velezensis* culture filtrate was applied to *T. aggressivum* (Kosanovic *et al*., 2021). Proteins associated with cell stress response e.g. oxidative reduction, ATP activity, DNA damage were all increased in the samples exposed to *B. velezensis* culture filtrate compared to the control. Oxidoreductase and DNA repair proteins were also found to be increased in abundance in *T. aggressivum* treated with *B. velezensis* (Kosanovic *et al*., 2021). Blast2go GO analysis further confirmed that these particular activities were altered in treatment/control.

The results presented here indicate that *B. velezensis* culture filtrate is capable of inhibiting the growth of *C. mycophilum in-vitro* and of inducing a stress response. This confirms its potential as a biocontrol agent which could be used for the treatment of cobweb disease on mushroom growing farms and may offer an alternative to chemical fungicides for disease treatment. The number of approved fungicides which have been phased out by government bodies and environmental agencies has increased over the past two decades. Furthermore, the emergence of strains which are resistant to chemical fungicides have greatly limited their use. Continued use of chemical fungicides may exacerbate the problems faced by mushroom growers (Grogan and Gaze, 2000; Grogan, 2006; Potocnik *et al*., 2015). It is important that effective biocontrol methods are developed to replace fungicide use in the future. Serenade (*B. velezensis* QST 713) was approved 21 years ago in the USA and has been established as a biocontrol option for diseases of several cultivated crops, however there are few biocontrol options on the market. The development of resistant strains against Serenade is a worrying possibility (Marrone, 2002). This work will go towards the development of this *B. velezensis* strain as another biocontrol option for growers. *Bacillus* species are found naturally in the mushroom casing and are generally regarded as safe (GRAS). Kosanovic *et al*., (2021) have also shown in their work, that the *B. velezensis* strain used during this research does not negatively affect the growth of *A.*

bisporus. Further experimental work is needed to establish if this strain has potential as a biocontrol agent and we will examine the response of *C. mycophilum*/*A bisporus* to *B. velezensis* culture filtrate in a crop trial in the future.

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2.6 Supplementary material

The following sections contain supplementary figures (2.6.1) and supplementary tables (2.6.2) which accompany Chapter 2 of this thesis.

2.6.1 Supplementary Figures

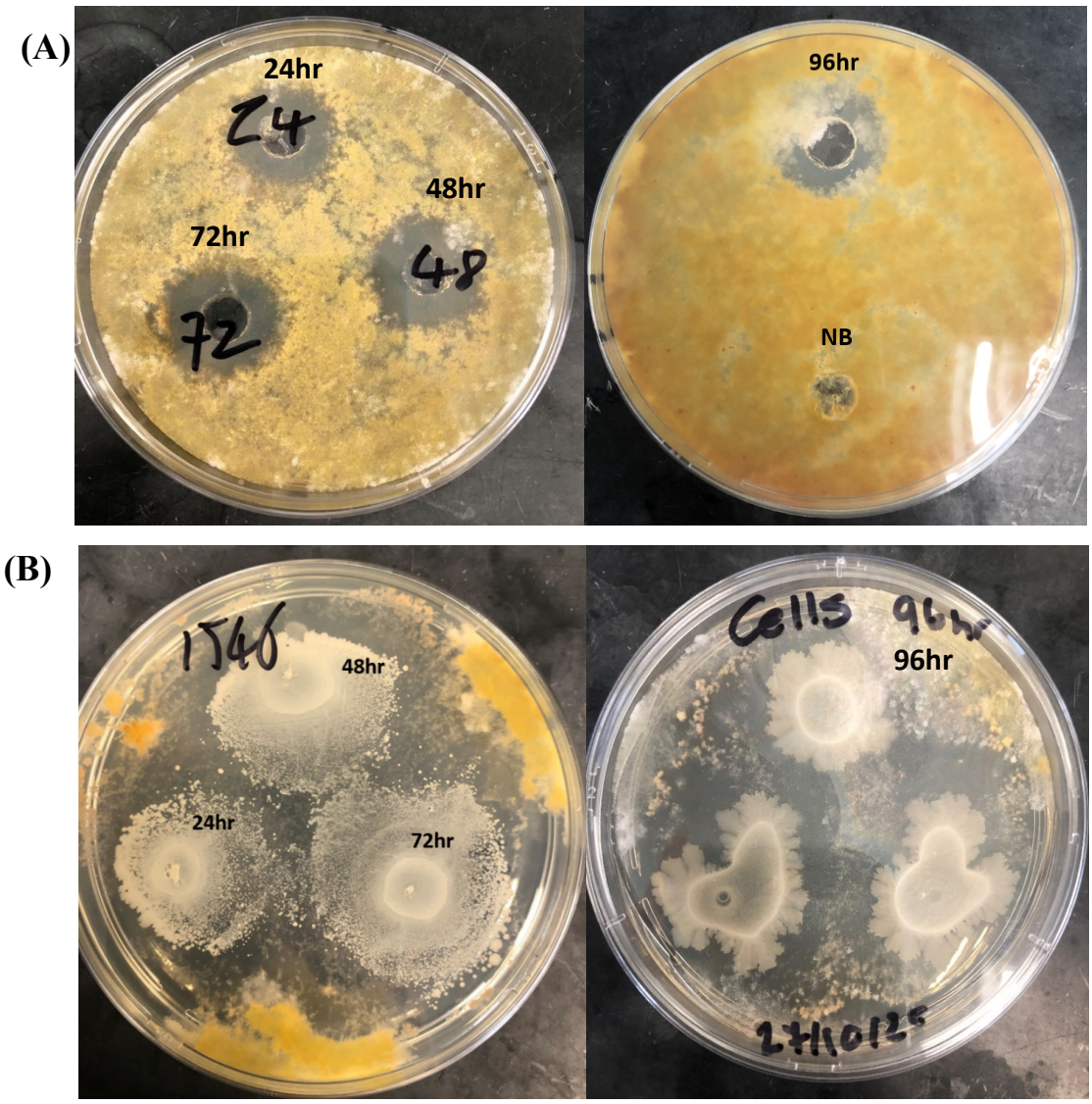

Figure S1: Plates inoculated with *Cladobotryum* (x10^4/plate) showing zones of inhibition when co-incubated with samples of *B. velezensis* culture filtrate (S1A) and culture drops (S1B) at various time points (24, 72, 48 and 96 hr). Nutrient broth (NB) was used as a control.

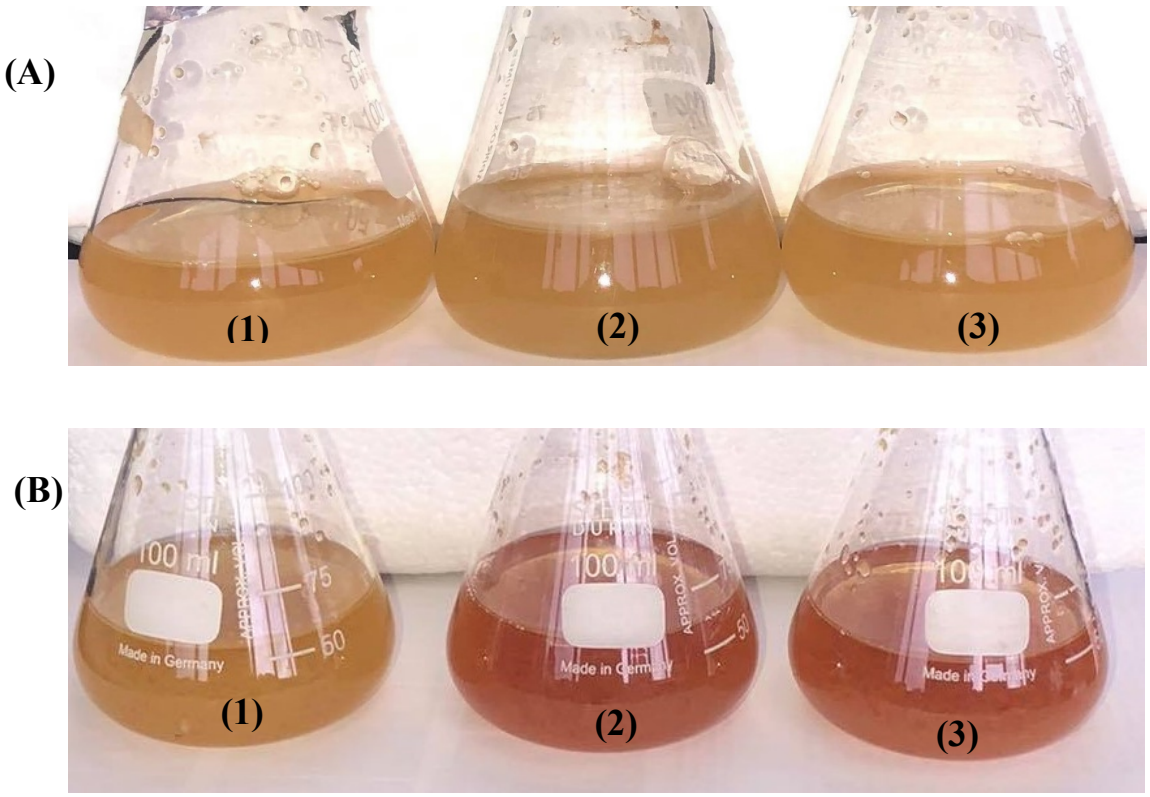

Figure S2: A visible colour difference in the treatment samples appears within 24 hr of inoculation with *B. velezensis* 96 hr CF.

(A) Flasks inoculated with *Cladobotryum* ($x10^3$) and grown for 48hrs.

(B) Same flasks as in (A), 24 hrs after 96 hr *B. velezensis* CF was applied to flask 2 (25% v/v) and 3 (12.5% v/v). NB was applied to flask 1 (25% v/v).

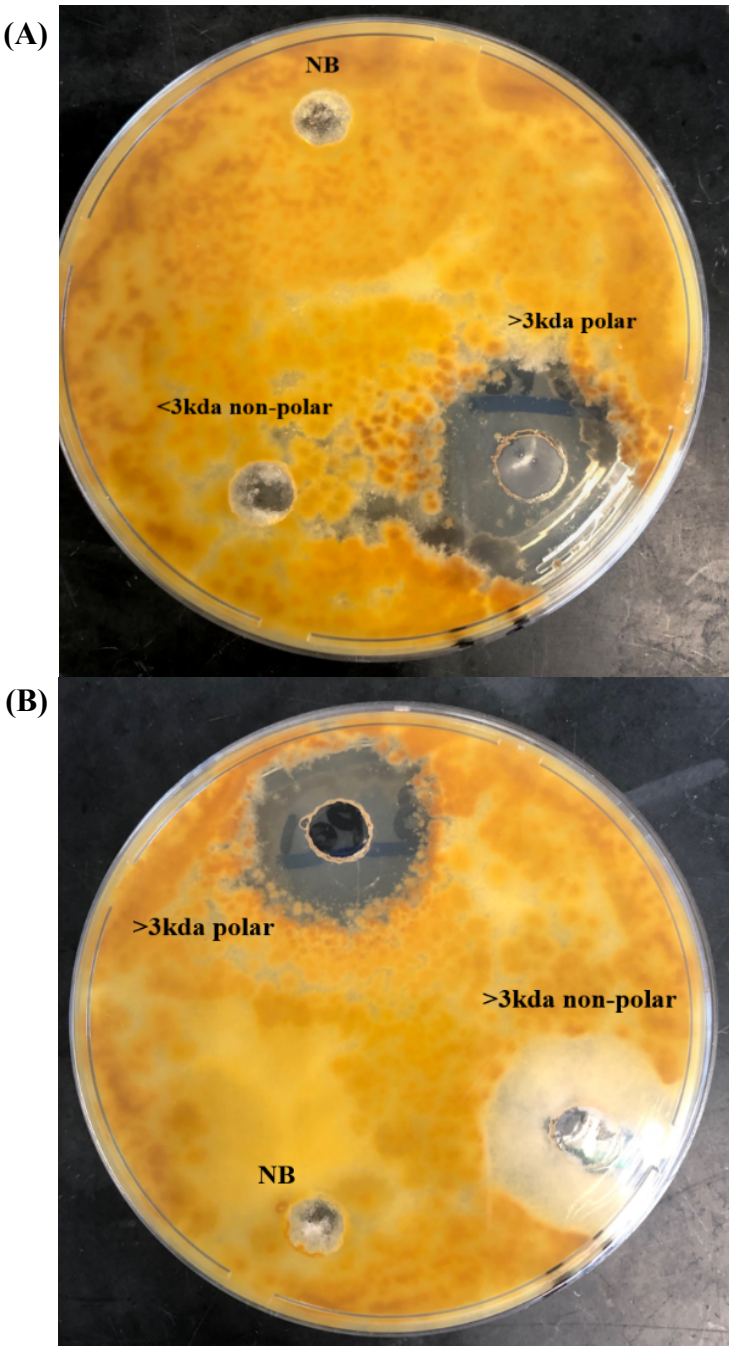

Figure S3: Fractions of 96 hr *B. velezensis* CF (>3kda polar/non polar, <3kda polar/non polar) were applied to *Cladobotryum* (x10^4/plate). Plates were grown for 72 hr at 25 °C. Zones of inhibition can be visualised surrounding >3kda samples only.

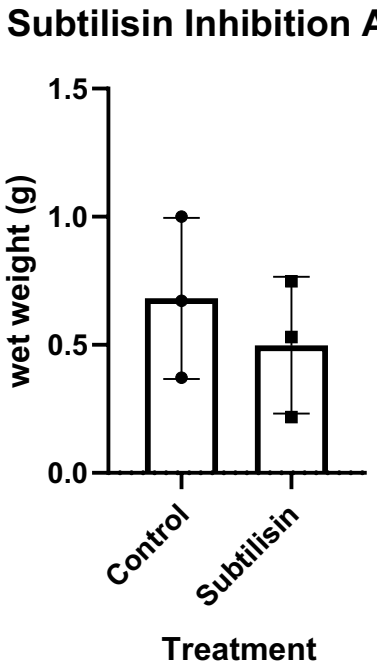

Figure S4: Subtilisin (1 mg/ml dissolved in PBS) added to 48 hr *C. mycophilum* culture (12.5% v/v) results in a 28% reduction in *C. mycophilum* biomass compared to control (12.% v/v PBS). Wet weight measurements of 5 replicates per treatment were recorded. Average wet weight for each treatment is displayed above, Error bars represent standard deviation

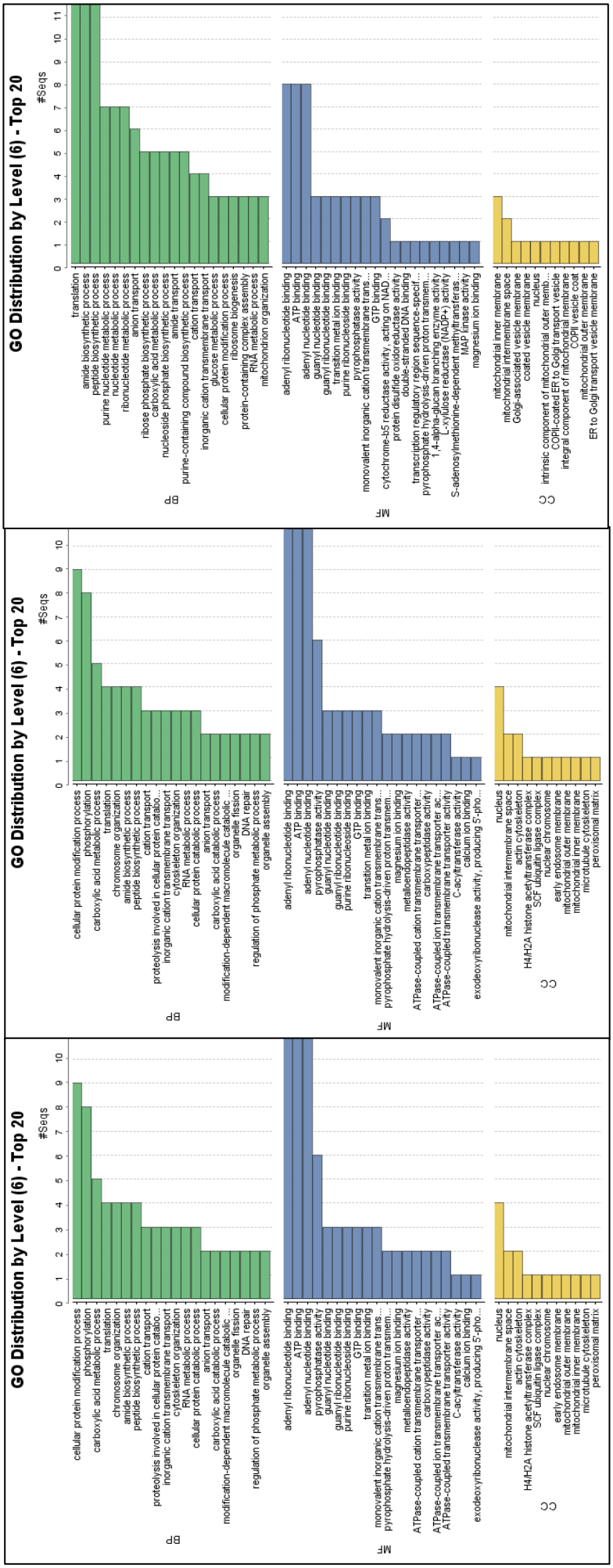

Figure S5: Gene ontology graphs generated in Blast-2-go (level 6). These graphs depict the changes in gene ontology biological process (BP), molecular function (MF) and cellular component (CC) between treatment and control samples.

2.6.2 Supplementary Tables

Table S1: Proteins identified within >3kda, polar 96 hr culture filtrate

Accession	Description	Score	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
A0A6H2V1E4	Alpha-amylase	3.54	8	12	659	72.4	7.02
A0A1D9PMA5	Aminopeptidase YsdC	1.78	8	12	361	39.4	5.97
A0A2G1U0X9	Aminopeptidase YsdC	2.08	8	12	346	38.6	6.2
A0A235BGZ5	Catalase	1.91	8	9	481	54.5	6.95
A0A1D9PQS1	Chitin-binding protein	3.67	6	8	206	22.4	8.27
A0A6I5R679	Citrate synthase	2.24	9	12	372	41.5	5.92
A0A6H2VBZ5	Cupin domain-containing protein	2.13	6	7	386	43.5	5.36
A0A6H2V6B8	Cytochrome c oxidase subunit 1	1.87	4	6	622	68.9	7.36
A0A6H2V7F0	Dihydrolipoyl dehydrogenase	2.43	8	13	473	50.1	5.26
A0A6H2V949	Dipeptidase PepV	5.73	8	8	463	50.9	5.12
A0A6H2VBQ1	DUF1565 domain-containing protein	5.84	11	11	468	50.9	6.06
A0A6H2V5K0	DUF3823 domain-containing protein O	3.39	26	31	1431	154.3	6.44
A0A6I7AXX0	Gamma-glutamyltransferase	1.9	8	8	591	64.3	6.76
A0A1D9PGQ1	Glycine betaine transport system permease protein OpuAB	1.84	3	4	282	30.2	9.64
A0A411A1C0	Inosine-5'-monophosphate dehydrogenase	2.93	9	13	488	52.9	6.55
A0A6I5R6R7	L-threonine 3-dehydrogenase	1.79	3	3	348	37.2	6.65
A0A223CIX6	M20/M25/M40 family metallo-hydrolase	1.77	6	7	371	39.5	5.17
A0A1D9PJR6	Outer spore coat protein CotE	4.34	3	5	181	20.8	4.39
A0A6G9RZ72	Peptidase S8 (Subtilisin)	10.06	6	8	382	39.1	9.29
A0A6H2VD39	Peptidase S8	4.53	15	19	803	85.8	6.84
A0A1D9PJK9	Peptide deformylase	5.39	4	4	184	20.7	5.6
A0A6H2VA46	Probable cytosol aminopeptidase	5.19	11	13	496	53.3	5
A0A235BAD7	Putative NAD(P)H nitroreductase	2.88	4	5	194	22.3	6.57
A0A6I5R982	Sigma-54-dependent transcriptional regulator	1.95	12	13	461	52.3	6.77
A0A6I5QZQ2	Spore coat protein GerQ	3.08	3	5	181	20.4	8.28
A0A6H2V9B4	Sucrose-6-phosphate hydrolase	5.39	11	18	489	56.1	5.63
A0A6H2VDB1	Urocanate hydratase	1.84	9	9	554	60.9	6.18
A0A6I5RC30	YtoQ family protein	1.77	4	4	148	16.8	5.57

Table S1: *B. velezensis* 96 hr S/N was applied to QExactive (ThermoFisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Data analysis was carried out on using Proteome Discoverer 1.4 and Sequest HT (SEQUEST HT algorithm, Thermo Scientific). Proteins which were identified within the sample are listed above. Proteins which scored <0 were discarded from the results. The proteins UniProt accession code, protein score (sum of all peptide Xcorr values above the specified score threshold), coverage, number of peptides, peptide-spectrum match (PSM), amino acids (AA), molecular weight (MW) (kDa) and isoelectric point (calc.Pi) are also included. *B. velezensis* proteome (identifier 492670) (Genome assembly accession: CP026610, proteome ID: UP000425588)- now archived in UniParc.

Table S2: Top Statistically significant and differentially abundant (SSDA) proteins downregulated Treatment 1 (25% v/v *B. velezensis*)

Majority protein IDs	Actual difference	UniProt names	Function
A0A2T4ACS6	26.21	40S ribosomal protein S30	translation
A0A2T4A3H0	11.96	ATP synthase d chain	ATP synthesis coupled proton transport
A0A2T4AJ92	9.73	L-xylulose reductase	glucose metabolic process; mannitol metabolic process; oxidation-reduction process; xylulose metabolic process
A0A2T4A0V4	5.23	thiazole biosynthetic enzyme variant 1	mitochondrial genome maintenance; thiamine biosynthetic process; thiazole biosynthetic process
A0A2T4ADS4	4.86	Deubiquitination-protection protein dph1	Protection of ubiquitin chains from disassembly
A0A2T4AAY8	4.6	FK506-binding protein 4	protein peptidyl-prolyl isomerization
A0A2T4AW62	4.5	Protein GVP36	RNA polymerase I preinitiation complex assembly
A0A2T4ABN7	4.37	Amidohydro_3 domain-containing protein	hydrolase activity acting on carbon-nitrogen (but not peptide) bonds
A0A2T4ADQ1	4.18	Mitochondrial import inner membrane translocase subunit TIM10	protein insertion into mitochondrial inner membrane
A0A2T4A4U2	3.96	Protoglobin domain-containing protein	Heme binding, oxygen binding
A0A2T4AE71	3.08	40S ribosomal protein S21	rRNA processing; translation
A0A2T4AI26	2.87	60S ribosomal protein L37a	translation
A0A2T4AEA0	2.76	50S ribosomal protein L31e	translation
A0A2T4APD5	2.6	40S ribosomal protein S25	translation
A0A2T4ASD0	2.32	40S ribosomal protein S23	translation, small ribosomal subunit
A0A2T4AR00	2.21	54S ribosomal protein L12	translation
A0A2T4APW5	2.2	60S ribosomal protein L27-A	translation
A0A2T4AUW2	2.08	Eukaryotic translation initiation factor 3 subunit G	formation of cytoplasmic translation initiation complex
A0A2T4AJT3	1.88	50S ribosomal protein L26e	translation
A0A2T4AAV1	1.86	50S ribosomal protein L6e	translation
A0A2T3ZWJ7	1.82	ribosomal protein S9	translation
A0A2T4ACK9	1.74	60S ribosomal protein L30	RNA binding
A0A2T4AR37	1.66	Ribosome-interacting GTPase 1	GTP binding; hydrolase activity
A0A2T4A5N4	1.64	40S ribosomal protein S17	translation
A0A2T4AEL1	1.56	60S ribosomal protein L23	translation, large ribosomal subunit rRNA binding
A0A2T4APE1	1.56	40S ribosomal protein S5	rRNA export from nucleus; translation

Trichoderma harzianum proteome (strain CBS 226.95 (Genome assembly accession: #MBGI01000000, proteome ID: UP000241690)

Table S3: Top SSDA upregulated Treatment 1 (25% v/v *B. velezensis*)

Majority protein IDs	Actual difference	UniProt names	Function
A0A2T4AR92	23.96	ISWI chromatin-remodelling complex ATPase ISW2	ATP-dependent chromatin remodelling, ATP binding; helicase activity; nucleic acid binding; nucleosome binding
A0A2T3ZUH7	8.19	AAA ATPase	cell division, ATP binding
A0A2T4AKP8	5.06	t-snare syntaxin	vesicle-mediated transport
A0A2T4API9	3.07	carboxypeptidase Y precursor	proteolysis, serine-type carboxypeptidase activity
A0A2T4A0A3	2.53	Pentulose kinase	carbohydrate phosphorylation; nucleocytoplasmic transport; pentose metabolic process; transmembrane transport
A0A2T4A1Z8	2.42	calmodulin	calcium-mediated signalling; regulation of catalytic activity; regulation of cell cycle; spore germination
A0A2T4ARR8	2.41	predicted protein	regulation of nucleic acid-templated transcription
A0A2T3ZWY4	2.38	putative myosin type I heavy chain	actin binding; ATP binding; motor activity
A0A2T4A0B8	2.36	Long chain acyl-CoA synthetase 7 peroxisomal	ligase activity
A0A2T4AHP5	1.82	E3 ubiquitin ligase complex SCF subunit scon-3	negative regulation of mitotic metaphase/anaphase transition; protein ubiquitination; SCF-dependent proteasomal ubiquitin-dependent protein catabolic process
A0A2T4A9V6	1.68	Ubiquitin-activating enzyme E1 1	DNA metabolic process; nucleic acid phosphodiester bond hydrolysis; protein peptidyl-prolyl isomerization; protein ubiquitination
A0A2T4AIB3	1.54	RuvB-like helicase 1	chromatin organization; DNA duplex unwinding; DNA repair

Trichoderma harzianum proteome (strain CBS 226.95 (Genome assembly accession: #MBGI01000000, proteome ID: UP000241690)

Table S4: Top SSDA proteins downregulated Treatment 2 (12.5% v/v *B. velezensis*)

Majority protein IDs	Actual difference	UniProt names	Function
A0A2T4AMH1	19.1	porphobilinogen deaminase	peptidyl-pyrromethane cofactor linkage; tetrapyrrole biosynthetic process
A0A2T4ACS6	15.74	40S ribosomal protein S30	translation
A0A2T4A174	5.2	mitochondrial inner membrane translocase subunit TIM44	protein transport
A0A2T4AJ92	4.76	L-xylulose reductase	glucose metabolic process; mannitol metabolic process; oxidation-reduction process; xylulose metabolic process
A0A2T4ADS4	4.56	Deubiquitination-protection protein dph1	aspartic-type endopeptidase activity
A0A2T4A5Z9	3.7	Uncharacterized (100% identity to Actin filament-coating protein tropomyosin in Trichoderma guizhouense)	Structural and functional diversification of the actin cytoskeleton
A0A2T4A0V4	3.65	thiazole biosynthetic enzyme variant 1	mitochondrial genome maintenance; thiamine biosynthetic process; thiazole biosynthetic process
A0A2T4AR00	2.54	54S ribosomal protein L12	translation
A0A2T4AK08	2.41	RNA methylase	peptidyl-arginine N-methylation
A0A2T4ATJ1	2.36	40S ribosomal protein S28	translation
A0A2T4AUW2	2.16	Eukaryotic translation initiation factor 3 subunit G	formation of cytoplasmic translation initiation complex
A0A2T4APZ3	1.54	40S ribosomal protein S7	translation
A0A2T4AR61	1.53	Eukaryotic translation initiation factor 3 subunit A	formation of cytoplasmic translation initiation complex
A0A2T4AG75	1.54	ubiquitin-40S ribosomal protein S27a	translation

Trichoderma harzianum proteome (strain CBS 226.95 (Genome assembly accession: #MBGI01000000, proteome ID: UP000241690)

Table S5: Top SSDA proteins upregulated Treatment 2 (12.5% v/v *B. velezensis)*

Majority protein IDs	Actual difference	UniProt names	Function
A0A2T4AR92	21.29	ISWI chromatin-remodelling complex ATPase ISW2	ATP-dependent chromatin remodelling
A0A2T4APQ6	8.78	Prenylated Rab acceptor 1	endoplasmic reticulum to Golgi vesicle-mediated transport, ATP binding; helicase activity
A0A2T3ZUH7	6.12	AAA ATPase	cell division, ATP binding
A0A2T4AIX6	4.13	GTP-binding protein rho2	establishment or maintenance of actin cytoskeleton polarity
A0A2T4APL6	3.07	UDP-galactopyranose mutase	oxidation-reduction process
A0A2T3ZWJ1	2.24	NAD-specific glutamate dehydrogenase	glutamate catabolic process to 2-oxoglutarate; oxidation-reduction process
A0A2T4AKL6	2.23	GTP-binding protein	GTP binding; hydrolase activity
A0A2T4AAK3	2.19	3-ketoacyl-CoA thiolase	fatty acid beta-oxidation; acetyl-CoA C-acyltransferase activity; mRNA binding
A0A2T4A4W3	2.19	serine/threonine protein kinase AGC family	gluconeogenesis; glycolytic process; protein phosphorylation; ATP binding
A0A2T4AKC9	1.88	glucosamine-fructose-6-phosphate aminotransferase	carbohydrate derivative metabolic process
A0A2T4A9K7	1.82	acyl-CoA dehydrogenase	oxidation-reduction process
A0A2T4ALS1	1.56	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit	Cellular bud neck septin ring organization ;DNA repair; positive regulation of autophagosome assembly

Trichoderma harzianum proteome (strain CBS 226.95 (Genome assembly accession: #MBGI01000000, proteome ID: UP000241690)

Chapter 3

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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Author Contributions

Crop trial experimental design, set up and maintenance was performed by JC, BMG and HG.

in vitro fungicide resistance experiments were carried out by BMG. Data analysis was carried out by JC.

Disease and yield assessments were carried out by JC

Data analysis and manuscript writing was performed by JC and HG.

Manuscript editing was performed by BMG, KK, DF

Abbreviations

EC: European Commission (EC)

IPM: integrated pest management (IPM)

SUD: Sustainable Use of Pesticides Directive (SUD)

BCAs: biological control agents (BCAs)

AI: Active ingredients (AI)

CFUs: colony forming units (CFUs)

CF: Culture filtrate (CF)

NB: nutrient broth (NB)

MEA: malt extract agar (MEA)

PBS: phosphate buffered saline (PBS)

DI: Disease Incidence (DI)

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.

Keywords: Cobweb disease, Biocontrol, *Bacillus velezensis,* fungicide resistance

3.1 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 (*Cladobotryu*m 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 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.

3.2 Materials and methods

3.2.1 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 3.1**). All were isolated from cobweb-infected mushroom crops between 1995 and 2019 and are stored in liquid nitrogen at −80 °C in the Teagasc Ashtown culture collection (Dublin, Ireland). Four *C. mycophilum* isolates (1545, 1546, 1583 and 1588) were obtained in 2019 from independent 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 independent mushroom farms with cobweb disease in the UK in 1995 (Grogan and Gaze, 2000), and one (618) had been isolated from an independent 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.

Table 3.1 *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*.

3.2.2 Fungicides and biological control agents (BCAs)

The chemical fungicides prochloraz (Sporgon® 50WP) (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).

3.2.3 *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 (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).

3.2.4 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 the 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.

3.2.5 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.

3.2.6 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}. This inoculation rate was selected as it is commonly used to test efficacy of fungicide treatments in crop trial studies. In crop trial 2, inoculum was prepared for isolate 1546 only. This isolate was focused on as it was most relevant for the mushroom industry. 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 $A = \pi r^2$ where π = 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.

3.2.7 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 3.2**. Eight treatments were repeated in both trials.

Table 3.2 Details of treatments in crop trials 1 and 2.

Crop trial 1		
Treatment	**Fungicide /BCA treatment**	**Inoculum treatment**
1. Control uninoculated	None	None
2. Control 1546	None	Isolate 1546
3. Control 618	None	Isolate 618
4. Prochloraz uninoculated	Prochloraz	None
5. Prochloraz 1546	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	QST 713 (*B. velezensis*)	None
11. QST 713 1546	QST 713 (*B. velezensis*)	Isolate 1546
12. Kos uninoculated	Kos (*B. velezensis*)	None
13. Kos 1546	Kos (*B. velezensis*)	Isolate 1546
Crop trial 2		
Treatment	**Fungicide /BCA treatment**	**Inoculum treatment**
1. Control uninoculated	None	None
2. Control 1546 $1x10^6$	None	Isolate 1546
3. Control 1546 $5x10^5$	None	Isolate 1546
4. Prochloraz uninoculated	Prochloraz	None
5. Prochloraz 1546 $1x10^6$	Prochloraz	Isolate 1546
6. Prochloraz 1546 $5x10^5$	Prochloraz	Isolate 1546
7. QST 713 uninoculated	QST 713 (*B. velezensis*)	None
8. QST 713 1546 $1x10^6$	QST 713 (*B. velezensis*)	Isolate 1546
9. QST 713 1546 $5x10^5$	QST 713 (*B. velezensis*)	Isolate 1546
10. Kos uninoculated	Kos (*B. velezensis*)	None
11. Kos 1546 $1x10^6$	Kos (*B. velezensis*)	Isolate 1546
12. Kos 1546 $5x10^5$	Kos (*B. velezensis*)	Isolate 1546

3.2.8 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 **Tables S1–5**.

3.3 Results

3.3.1 *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 **Figure 3.1** and **Tables S1A and S1B**. 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 (**Tables S1A and S1B**) and had ED50 values of between 0.1 and 1 mg kg^{-1} (**Figure 3.1**). 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.

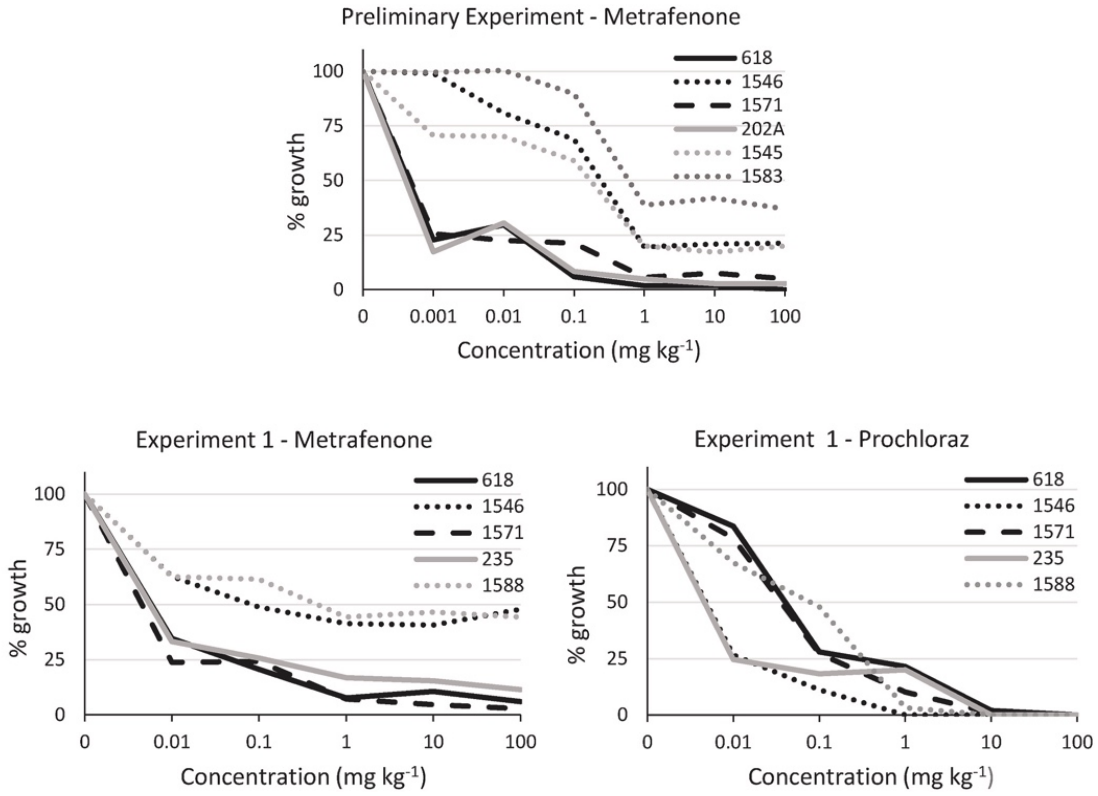

Figure 3.1 *in vitro* response of six *Cladobotryum* isolates to metrafenone (Preliminary Experiment, n = 3) and five *Cladobotryum* isolates to metrafenone and prochloraz (Experiment 1, n = 5). Values are mean % growth at each concentration. ANOVA data in Tables S1A and S1B

3.3.2 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 **Figure 3.2**. 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 **Table S2**.

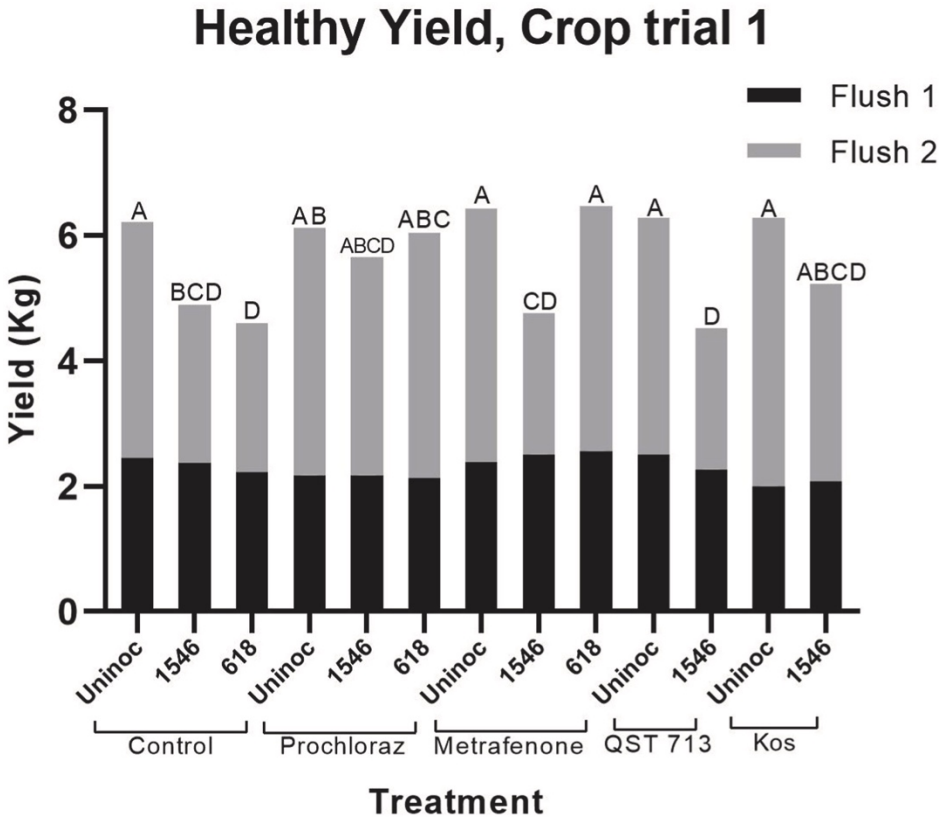

Figure 3.2 Yield of healthy mushrooms over two flushes following treatment with the fungicides prochloraz or metrafenone or the BCAs QST 713 (cell culture) or Kos (culture filtrate) (all 1L m^{-2} application rate), followed by inoculation with one of two different C. mycophilum isolates, 1546 or 618. 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 (Table S2).

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), (**Figure S1**). An assessment of cobweb growth was taken at the end of flush 1 (**Figure S2**). All uninoculated control plots remained free of cobweb with just a few small patches developing by the end of the second flush, with average disease incidence levels of <3% (**Figure 3.3**).

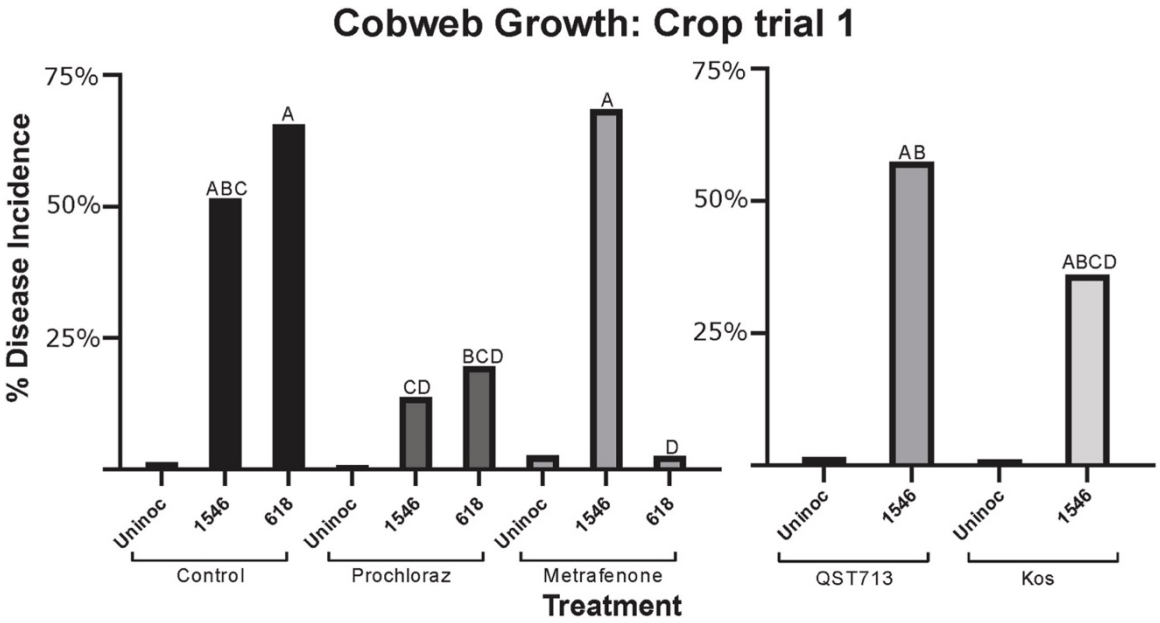

Figure 3.3 Cobweb disease incidence (%) developing after two flushes following treatment with the fungicides prochloraz or metrafenone or the BCAs QST 713 (cell culture) or Kos, (culture filtrate) (all 1L m^{-2} application rate) followed by inoculation with one of two different *C. mycophilum* isolates, 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 (Table S3).

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 (**Figure 3.3**). 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% (**Figure 3.3, Table S3**). 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 (**Figure 3.3**). 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 (**Figure 3.3, Table S3**). Images of representative plots at the end of flush 2 are presented in **Figure 3.4**.

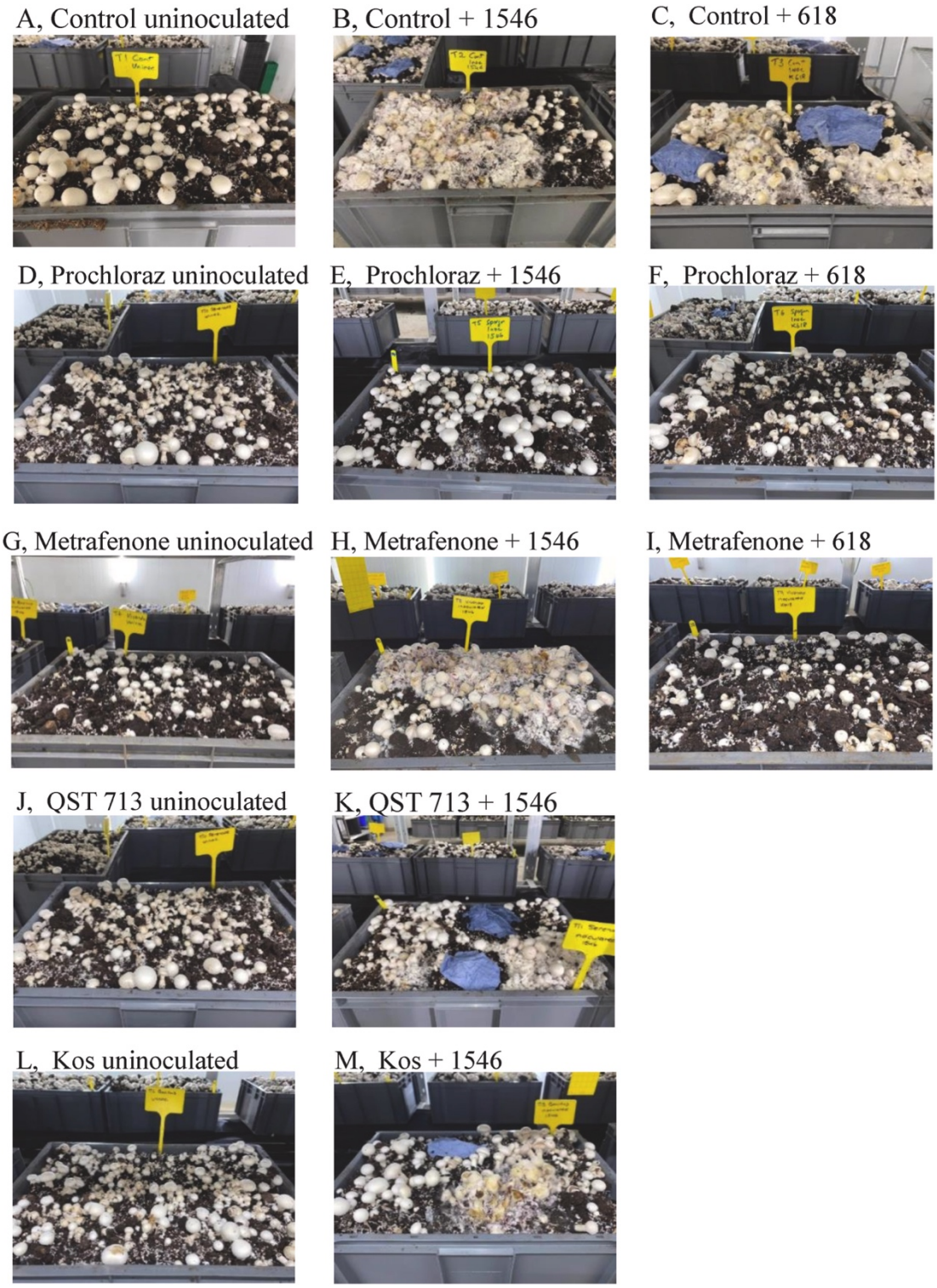

Figure 3.4 A representative plot from each fungicide/BCA/inoculum treatment showing cobweb growth at the end of flush 2 in crop trial 1.

3.3.3 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 **Figure 3.5** 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 **Table S4**.

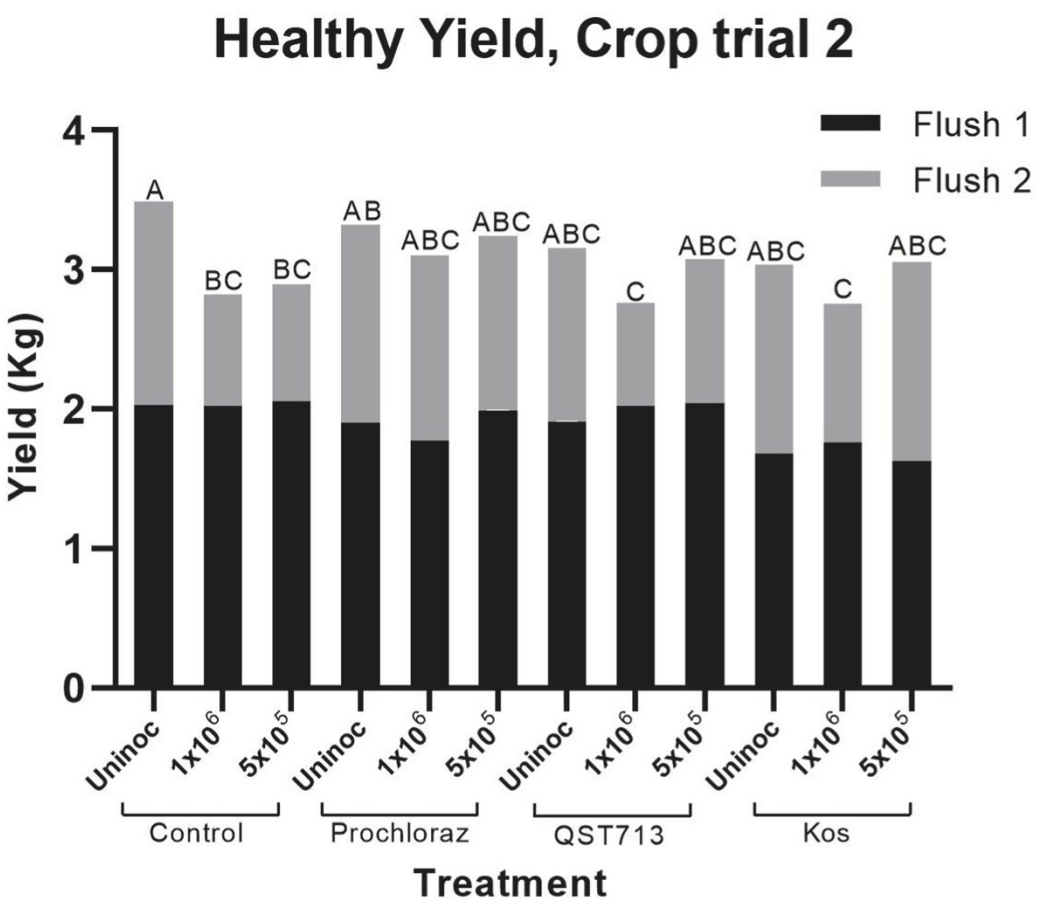

Figure 3.5 Yield of healthy mushrooms over two flushes following treatment with the fungicide prochloraz or the BCAs QST 713 or Kos, followed by inoculation *with C. mycophilum* isolate 1546 at either 1×10^6 or 5×10^5 spores m^{-2}. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukey's pairwise comparisons test. (**Table S4**).

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* QST 713 had the highest average number of spotted mushrooms (**Figure S3**). 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 (**Figure S4**) (**Table S5**).

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 (**Figure 3.6**). 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 (**Figure 3.6**). 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 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 ($P < 0.05$) (**Figure 3.6**, **Table S5**). 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 **Figure S5**.

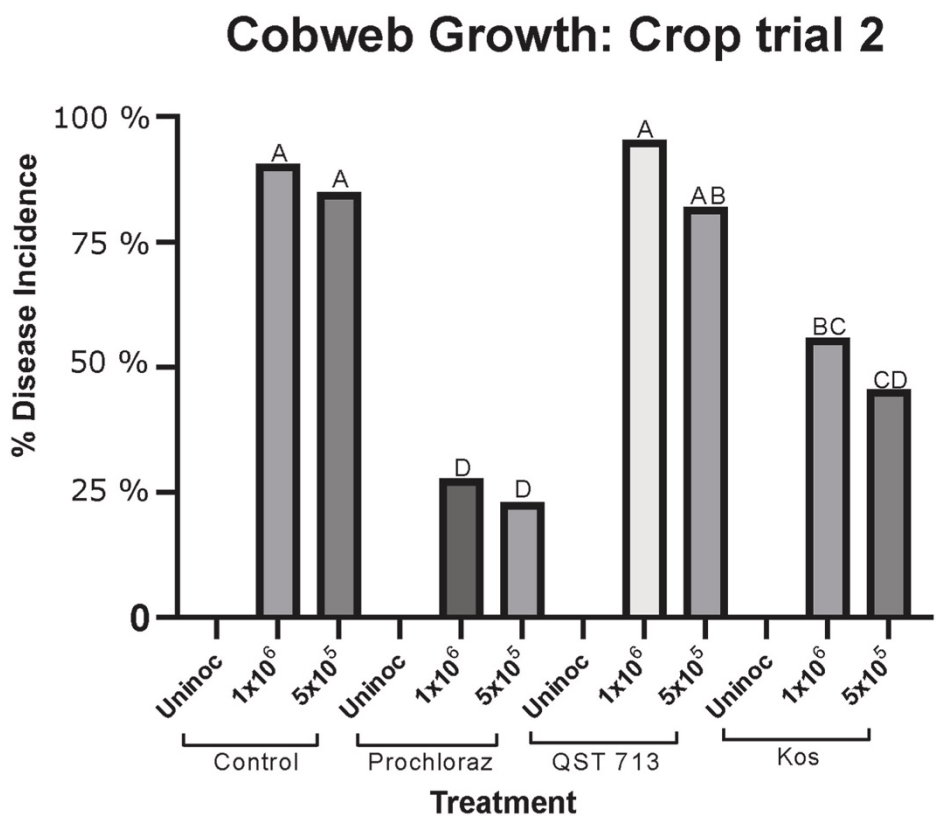

Figure 3.6 Cobweb disease incidence (%) developing after two flushes following treatment with the fungicide prochloraz or the BCAs QST 713 or Kos, followed by inoculation with *C. mycophilum* isolate 1546 at either 1×10^6 or 5×10^5 spores m^{-2}. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukey's pairwise comparisons test (Table S5).

3.4 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 (**Figure 3.1**). 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.

3.4.1 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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3.6 Supplementary material

The following sections contain supplementary figures (3.6.1) and supplementary tables (3.6.2) which accompany Chapter 3 of this thesis.

3.6.1 Supplementary Figures

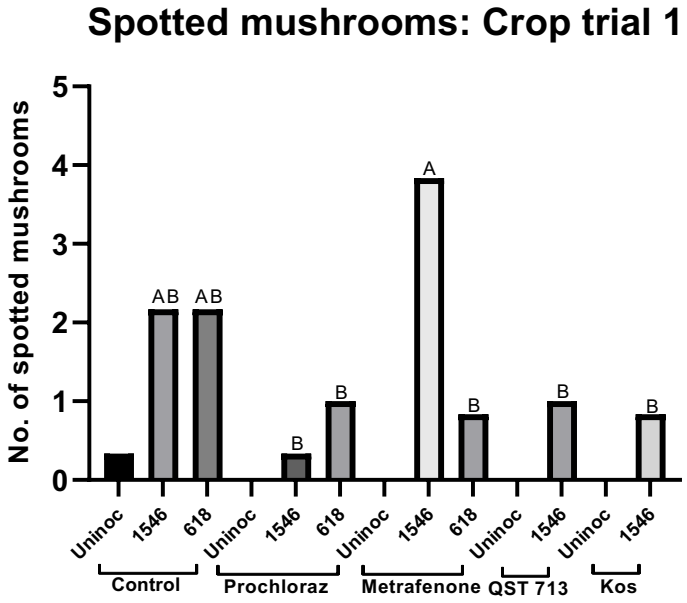

Figure S1: Crop trial 1, Average number of spotted mushrooms, flush 1, following treatment with the fungicides prochloraz or metrafenone or the BCAs QST 713 (cell culture) or Kos, (culture filtrate) (all 1L m^{-2} application rate) followed by inoculation with one of two different *C. mycophilum* isolates, 1546 or 618. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys test. Uninoculated treatments not included in ANOVA.

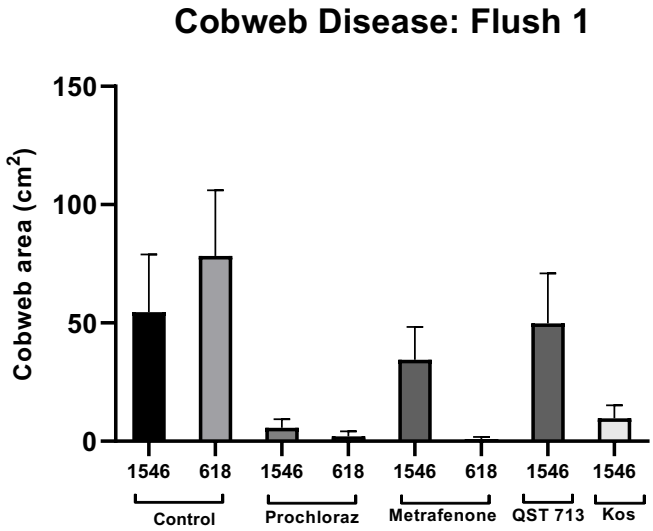

Figure S2: Crop trial 1, Average area of cobweb disease, flush 1, following treatment with the fungicides prochloraz or metrafenone or the BCAs QST 713 (cell culture) or Kos, (culture filtrate) (all 1L m^{-2} application rate) followed by inoculation with one of two different *C. mycophilum* isolates, 1546 or 618. Error bars represent SEM. Maximum coverage area is 2000 cm^2.

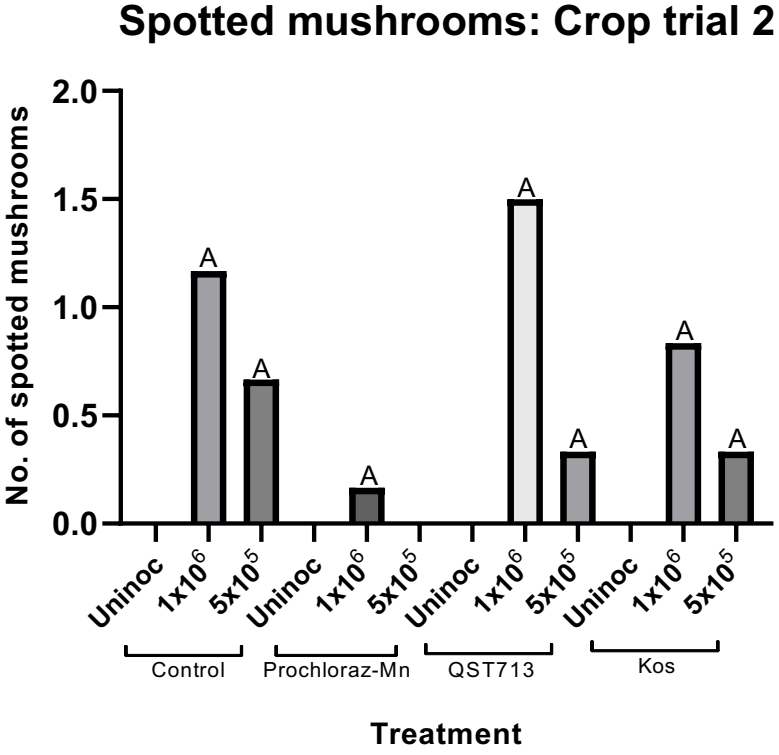

Figure S3: Crop trial 2, Average number of spotted mushrooms, flush 1 (n=6). following treatment with the fungicides prochloraz or metrafenone or the BCAs QST 713 (cell culture) or Kos (culture filtrate) (all 1L m^{-2} application rate) followed by inoculation with *C. mycophilum* isolate 1546 at a rate of either $1x10^6$ or $5x10^5$ cfu/m^2. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukeys test. Uninoculated treatments not included in ANOVA.

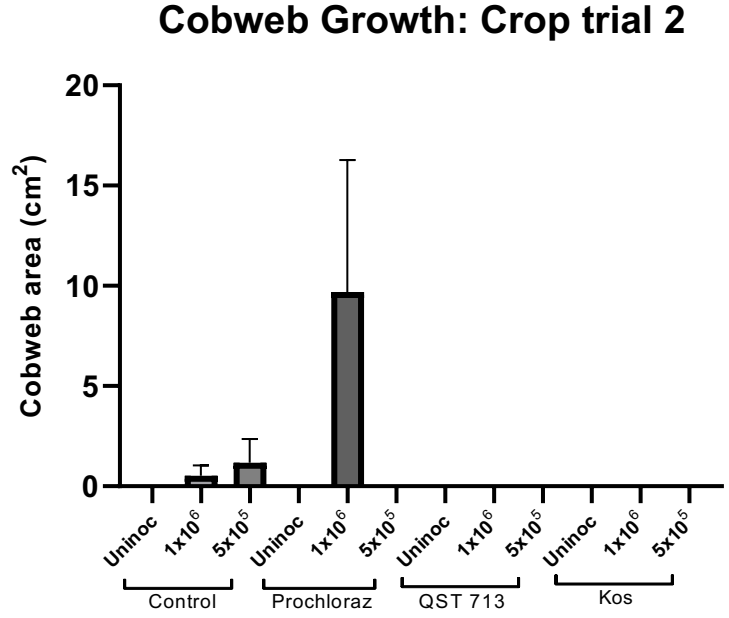

Figure S4: Crop trial 2, Average area of cobweb disease, flush 1(n=6), following treatment with the fungicides prochloraz or metrafenone or the BCAs QST 713 (cell culture) or Kos (culture filtrate) (all 1L m^{-2} application rate) followed by inoculation with *C. mycophilum* isolate 1546 at a rate of either $1x10^6$ or $5x10^5$ cfu/m^2. Error bars represent SEM. Maximum coverage area is 2000 cm^2.

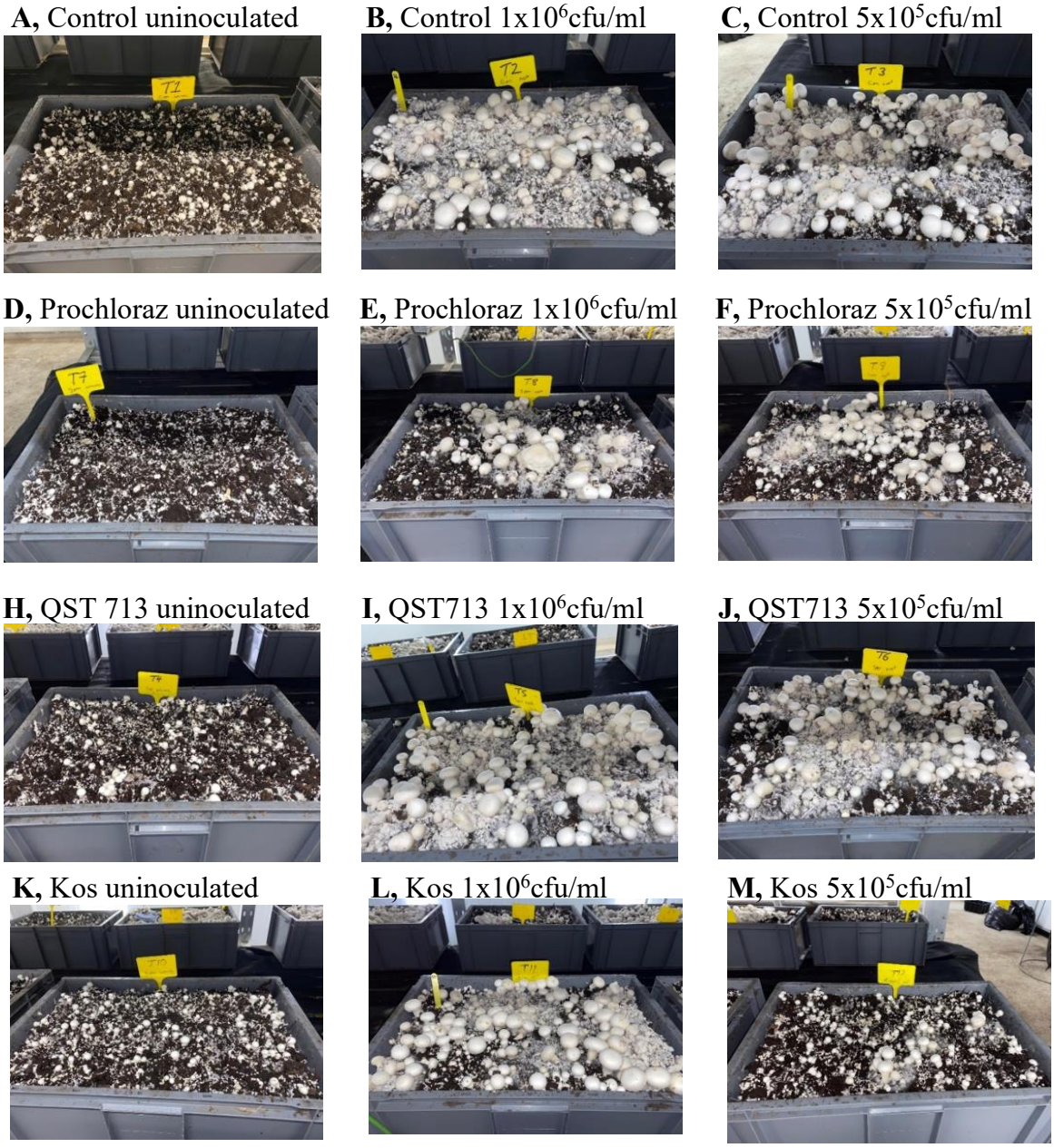

Figure S5: A representative plot from each fungicide/BCA/inoculum treatment showing cobweb growth at the end of flush 2 in crop trial 2

3.6.2 Supplementary Tables

Table S1A: Average data and ANOVA analysis of radial growth (mm) of *Cladobotryum isolates* at various concentrations of metrafenone from the preliminary experiment

Metrafenone							
Isolate	**0 mg kg^{-1}**	**0.001 mg kg^{-1}**	**0.01 mg kg^{-1}**	**0.1 mg kg^{-1}**	**1 mg kg^{-1}**	**10 mg kg^{-1}**	**100 mg kg^{-1}**
1545	71.7	50.7^{B}	50.3^{B}	42.3^{B}	14.33^{B}	12.3^{B}	14.3^{B}
1546	75	74.3^{A}	60.7^{AB}	51.7^{B}	14.67^{B}	15.7^{B}	16^{B}
1571	53.3	13.7^{CD}	12^{C}	11.3^{C}	3^{C}	4^{C}	2.7^{C}
1583	74	73.7^{A}	74.3^{A}	66.3^{A}	28.7^{A}	31^{A}	27.3^{A}
202A	48	8.3^{D}	14.7^{C}	4^{C}	2.3^{C}	1.3^{C}	1.3^{C}
618	73	16.7^{C}	21.7^{C}	4.3^{C}	1.3^{C}	1.3^{C}	0^{C}
ANOVA F-value		($F_{5,12}$) = 524.87, p<0.05)	($F_{5,12}$) = 63.16, p<0.05)	($F_{5,12}$) = 168.02, p<0.05)	($F_{5,12}$) = 59.06, p<0.05)	($F_{5,12}$) = 90.15, p<0.05)	($F_{5,12}$) = 46.35, p<0.05)

Data sets which were normally distributed were analysed by ANOVA, n = 5. Means sharing the same letter are not significantly different at P <0.05 by Tukeys test.

Table S1B: Average growth (mm) of *Cladobotryum isolates* at various concentrations of prochloraz and metrafenone

Prochloraz						
Isolate	**0 mg kg^{-1}**	**0.01 mg kg^{-1}**	**0.1 mg kg^{-1}**	**1 mg kg^{-1}**	**10 mg kg^{-1}**	**100 mg kg^{-1}**
1546	61	16.2 ^{B}	6.8 ^{C}	0 ^{C}	0	0
618	70	58.6 ^{A}	19.6 ^{B}	15 ^{A}	1.4	0
1588	70	47.2 ^{A}	33.6 ^{A}	2.2 ^{BC}	0	0
1571	64.6	50.6 ^{A}	17.6 ^{B}	6.6 ^{B}	0.6	0
235	70	17.2 ^{B}	12.8 ^{BC}	14 ^{A}	0	0
ANOVA F-value		($F_{4,20}$) = 15.43, p < 0.05)	($F_{4,20}$) = 29.20, p > 0.05)	($F_{4,20}$) = 34.78, p > 0.05)		
Metrafenone						
Isolate	**0 mg kg^{-1}**	**0.01 mg kg^{-1}**	**0.1 mg kg^{-1}**	**1 mg kg^{-1}**	**10 mg kg^{-1}**	**100 mg kg^{-1}**
1546	61	38.2 ^{AB}	29.8 ^{B}	25.2 ^{A}	24.8 ^{B}	29.2 ^{A}
618	70	24. 2^{BC}	14.4 ^{C}	5.4 ^{C}	7.4 ^{C}	4.2 ^{BC}
1588	70	43.8^{A}	43 ^{A}	31 ^{A}	32.6 ^{A}	31 ^{A}
1571	64.6	15.4^{C}	15.6 ^{C}	4.6 ^{C}	3 ^{D}	1.8 ^{C}
235	70	23.2^{BC}	18 ^{C}	11.8 ^{B}	10.8 ^{C}	8 ^{B}
ANOVA F-value		($F_{4,20}$) = 8.88, p < 0.05)	($F_{4,20}$) = 27.00, p < 0.05)	($F_{4,20}$) = 67.08, p < 0.05)	($F_{4,20}$) = 176.88, p < 0.05)	($F_{4,20}$) = 194.68, p < 0.05)

Data sets which were normally distributed were analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys test.

Table S2: Average yield of healthy mushrooms, crop trial 1

Treatment	Average weight of healthy mushrooms Flush 1	Average weight of healthy mushrooms Flush 2	Average weight of healthy mushrooms End of trial
Control uninoculated	2.45 kg ^{A}	3. 77 kg ^{AB}	6.2 kg ^{A}
Control (inoculated 1546)	2.37 kg ^{A}	2.53 kg ^{BC}	4.90 kg ^{BCD}
Control (inoculated 618)	2.23 kg ^{A}	2.38 kg ^{C}	4.60 kg ^{D}
Prochloraz uninoculated	2.18 kg ^{A}	3.95 kg ^{A}	6.13 kg ^{AB}
Prochloraz (inoculated 1546)	2.17 kg ^{A}	3.48 kg ^{ABCD}	5.66 kg ^{ABCD}
Prochloraz (inoculated 618)	2.13 kg ^{A}	3.91 kg ^{A}	6.04 kg ^{ABC}
Metrafenone uninoculated	2.39 kg ^{A}	4.04 kg ^{A}	6.43 kg ^{A}
Metrafenone (inoculated 1546)	2.51 kg ^{A}	2.25 kg ^{C}	4.76 kg ^{CD}
Metrafenone (inoculated 618)	2.56 kg ^{A}	3.90 kg ^{A}	6.47 kg ^{A}
QST 713 uninoculated	2.50 kg ^{A}	3.78 kg ^{A}	6.29 kg ^{A}
QST 713 (inoculated 1546)	2.26 kg ^{A}	2.25 kg ^{C}	4.52 kg ^{D}
Kos (uninoculated)	2.00 kg ^{A}	4.28 kg ^{A}	6.28 kg ^{A}
Kos (inoculated 1546)	2.07 kg ^{A}	3.15 kg ^{ABCD}	5.22 kg ^{ABCD}
ANOVA F- value	($F_{12,65}$ = 1.44, p > 0.05)	($F_{12,65}$ = 8.49, p < 0.05)	($F_{12,65}$= 7.83, p < 0.05)

Data which were normally distributed were analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys test.

Table S3: Cobweb disease, crop trial 1

	Flush 1			Flush 2		
Treatment	**Average area of cobweb (cm^2)**	**% Disease Incidence**	**Treatment Efficacy**	**Average area of cobweb (cm^2)**	**% Disease Incidence**	**Treatment Efficacy**
Control uninoculated	0	0		27.36	1.60%	
Control (inoculated 1546)	54.6	2.73		1032.365 ^{ABC}	51.62%	
Control (inoculated 618)	78.31	3.92		1312.97 ^{A}	65.65%	
Prochloraz-Mn uninoculated	0	0		15.445	0.77%	
Prochloraz-Mn (inoculated 1546)	5.66	0.28	89.74%	275.28 ^{CD}	13.76%	73.34%
Prochloraz-Mn (inoculated 618)	2.095	0.1	97.44%	393.09 ^{BCD}	19.65%	70.10%
Metrafenone uninoculated	0	0		52.1	2.60%	
Metrafenone (inoculated 1546)	34.43	1.72	36%	1370.32 ^{A}	68.52%	
Metrafenone (inoculated 618)	0.885	0.04	98.90%	50.40 ^{D}	2.52%	96.16%
QST 713 uninoculated	0	0		31.28	1.56%	
QST 713 (inoculated 1546)	49.8	2.5	8.42%	1148.93 ^{AB}	57.45%	
Kos uninoculated	0			21.995	1.01%	
Kos (inoculated 1546)	9.72	0.49	82.05%	720.6 ^{ABCD}	36.03%	30.20%
ANOVA F- value				($F_{7,40}$ = 8.49, p < 0.05)		

Data which were normally distributed were analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys test.

% Disease Incidence = [(Average area of disease in cm^2/total area of plot (2000 cm^2)) x 100]. Treatment efficacy given as % efficacy = [(Ic -It)/Ic] x 100, where Ic = disease incidence in the inoculated control; It = disease incidence in treated samples

Table S4: Average yield of healthy mushrooms, crop trial 2

Treatment	Average weight of healthy mushrooms Flush 1	Average weight of healthy mushrooms Flush 2	Average weight of healthy mushrooms End of trial
Control uninoculated	2.03 kg ^{AB}	1.46 kg ^{A}	3.49 kg ^{A}
Control (inoculated $1x10^6$)	2.02 kg ^{AB}	0.80 kg ^{C}	2.82 kg ^{BC}
Control (inoculated $5x10^5$)	2.06 kg ^{A}	0.84 kg ^{C}	2.90 kg ^{BC}
Prochloraz-Mn uninoculated	1.90 kg ^{ABC}	1.42 kg ^{A}	3.32 kg ^{AB}
Prochloraz-Mn (inoculated $1x10^6$)	1.78 kg ^{ABC}	1.33 kg ^{AB}	3.10 kg ^{ABC}
Prochloraz-Mn (inoculated $5x10^5$)	1.99 kg ^{AB}	1.26 kg ^{AB}	3.25 kg ^{ABC}
QST 713 uninoculated	1.91 kg ^{ABC}	1.24 kg ^{AB}	3.16 kg ^{ABC}
QST 713 (inoculated $1x10^6$)	2.02 kg ^{AB}	0.74 kg ^{C}	2.76 kg ^{C}
QST 713 (inoculated $5x10^5$)	2.05 kg ^{A}	1.03 kg ^{BC}	3.08 kg ^{ABC}
Kos uninoculated	1.68 kg ^{BC}	1.36 kg ^{AB}	3.04 kg ^{ABC}
Kos (inoculated $1x10^6$)	1.76 kg ^{ABC}	1.00 kg ^{BC}	2.76 kg ^{C}
Kos (inoculated $5x10^5$)	1.63 kg ^{C}	1.43 kg ^{A}	3.06 kg ^{ABC}
ANOVA F-value	$(F_{11,60} = 4.18, p < 0.05)$	$(F_{11,60} = 12.42, p < 0.05)$	$(F_{11,60} = 4.06, p < 0.05)$

Data which were normally distributed were analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys test.

Table S5: Cobweb disease, crop trial 2

	Flush 1			Flush 2		
Treatment	**Average area of cobweb (cm^2)**	**% Disease Incidence**	**Treatment Efficacy**	**Average area of cobweb (cm^2)**	**% Disease Incidence**	**Treatment Efficacy**
Control uninoculated	0	0		0	0%	
Control (inoculated $1x10^6$)	0.524	0.03%		1813.33 ^{A}	90.66%	
Control (inoculated $5x10^5$)	1.178	0.06%		1700.00 ^{A}	85%	
Prochloraz-Mn uninoculated	0	0	100%	0	0%	100%
Prochloraz-Mn (inoculated $1x10^6$)	0	0	100%	557.00 ^{CD}	27.85%	69.28%
Prochloraz-Mn (inoculated $5x10^5$)	0	0	100%	460.54 ^{D}	23.03%	72.91%
QST 713 uninoculated	0	0	100%	0	0%	100%
QST 713 (inoculated $1x10^6$)	9.685	0.48%		1906.67 ^{A}	95.30%	-5.12%
QST 713 (inoculated $5x10^5$)	0	0	100%	1640.00 ^{AB}	82%	3.53%
Kos uninoculated	0	0	100%	0	0%	100%
Kos (inoculated $1x10^6$)	0	0	100%	1116.67 ^{BC}	55.80%	38.45%
Kos (inoculated $5x10^5$)	0	0	100%	913.18 ^{CD}	45.66%	46.28%
ANOVA F-value				($F_{7,40}$ = 20.85, p < 0.05)		

Data which were normally distributed were analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys test.

Chapter 4

Characterising the proteomic response of mushroom pathogen *Lecanicillium fungicola* to *Bacillus velezensis* QST 713 and Kos biocontrol agent

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Author Contributions

in vitro inhibition assays were carried out and analysed by JC

SEM microscopy was carried out and analysed by JC, with help from technician Orla Fenelon (Maynooth University)

Qualitative and quantitative proteomic extractions, LCMS data analysis was carried out and analysed by JC

Manuscript writing was performed by JC and KK

Manuscript editing was performed by DF, HG

Abstract

The fungal pathogen *Lecanicillium fungicola* causes dry bubble disease in *Agaricus bisporus* cultivation and affected mushrooms significantly reduce the yield and revenue for mushroom growers. Biocontrol agents may represent an alternative and more environmentally friendly treatment option to help control dry bubble on mushroom farms. Serenade® is a commercially available biocontrol product used for disease treatment in plant crops. In this work, the *in vitro* response of *L. fungicola* to the bacterial strain active in Serenade, *Bacillus velezensis* (QST 713) and a newly isolated *B. velezensis* strain (Kos) was assessed. *B. velezensis* (QST713 and Kos) both produced zones of inhibition on plate cultures of *L. fungicola*, reduced the mycelium growth in liquid cultures and damaged the morphology and structure of *L. fungicola* hyphae. The proteomic response of the pathogen against these biocontrol strains was also investigated. Proteins involved in growth and translation such as 60S ribosomal protein L21-A (−32- fold) and 40S ribosomal protein S30 (−17-fold) were reduced in abundance in *B. velezensis* QST 713 treated samples, while proteins involved in a stress response were increased (norsolorinic acid reductase B (47-fold), isocitrate lyase (11-fold) and isovaleryl-CoA dehydrogenase (8-fold). *L. fungicola* was found to have a similar proteomic response when exposed to *B. velezensis* (Kos). This work provides information on the response of *L. fungicola* to *B. velezensis* (QST 713) and indicates the potential of *B. velezensis* (Kos) as a novel biocontrol agent.

Keywords: *Agaricus bisporus*, Biocontrol, *Lecanicillium fungicola, Bacillus velezensis*, Proteomics, Dry bubble disease

List of abbreviations:

IPM: Integrated pest management

SUD: Sustainable Use of Pesticides Directive PDA Potato dextrose agar

SDB: Sabouraud dextrose liquid broth

NA: Nutrient agar

NB: Nutrient broth

CF: Culture filtrate

PMSF: Phenylmethylsulfonyl fluoride

DTT: Dithiothreitol

IAA: Iodoacetamide

PCA: Principal component analysis

GO: Gene ontology

BP: Biological process

MF: Molecular function

SSDA: Statistically significant differentially abundant

LFQ: Label free quantitative-proteomic

ANOVA: Analysis of variance

4.1 Introduction

Lecanicillium fungicola (Preuss), Zare and Gams [synonyms: *Verticillium fungicola* (Preuss), Hassebrauk] is a pathogenic fungus which causes dry bubble disease during white button mushroom cultivation (*Agaricus bisporus*) (Lange) Imbach. *A. bisporus* is one of five main commercially grown species, which together account for 85% of the world's mushroom supply. *Lentinula* accounts for 22% of world mushroom production while *A. bisporus* currently contributes 15% (Royse *et al*., 2017). *L. fungicola* infection results in severely deformed crops which greatly reduce the yield of marketable mushrooms (Berendsen *et al*., 2010). An increase in fungicide resistant *L. fungicola* strains has meant that this pathogen is currently one of the biggest problems in commercial mushroom production. The application of biocontrol agents is being investigated as a potential alternative to fungicide use. Other pathogens which are responsible for mushroom disease and potentially may require biocontrol treatment in the future include *Trichoderma aggressivum* (Green Mould Disease), *Cladobotryum* species (Cobweb Disease) and *Mycogyne perniciosa* (Wet Bubble Disease) (Fletcher & Gaze, 2008; Largeteau & Savoie, 2010).

L. fungicola was previously referred to as *Verticillium fungicola* (Gams & Van Zaayen, 1982; Hassebrauk, 1936). In 2008, Zare and Gams confirmed that *V. fungicola* was more closely related to a plant pathogenic genus *Lecanicillium*, thus *Verticillium* was renamed to *Lecanicillium* (Zare & Gams, 2008). The variety *var. fungicola* is mostly associated with disease incidence in Europe, while *var. aleophilum* occurs mostly in the USA and Canada (Largeteau *et al*., 2004). While *L. fungicola* has been isolated from a number of other cultivated mushroom species (e.g. *Pleurotus ostreatus, Pleurotus sapidus, Coltricha perennis*) (Marlowe & Romaine, 1982), it is rarely identified growing on wild mushrooms (Berendsen *et al*., 2012).

Mushroom casing, which is a mixture of peat and a neutralising agent such as sugar-beet lime or ground limestone, is easily contaminated during preparation and therefore it is commonly considered as a primary source of infection of *L. fungicola* on mushroom farms (Berendsen *et al*., 2012; Carrasco *et al*., 2019; Cross & Jacobs, 1969). Centralised casing production has vastly improved in Europe and casing is less likely to be contaminated at source, however prepared casing is still open to contamination. Possible primary sources of infection may include flies which act as vectors and carry

the spores of *L. fungicola* from infected mushrooms (Ware, 1933; Tibbles *et al*., 2005; Shamshad *et al*., 2010). The spores of *L. fungicola* are covered in a sticky mucilage which allows them to adhere to pests such as *Lycoriella ingenua* and *Bradysia ocellaris* (Shamshad *et al*., 2010). Contaminated equipment and dust/debris from the growing rooms and surrounding areas have also been shown to aid the spread of *L. fungicola* spores around mushroom farms (Grogan, 2001; Largeteau *et al*., 2004). The spores of *L. fungicola* will not survive at temperatures higher than 40 °C, therefore the compost in which *A. bisporus* mycelium grow and develop can be ruled out as a primary source of infection due to the high temperatures it is exposed to.

A primary infection of *L. fungicola* results in small undifferentiated masses or 'bubbles' of *A. bisporus*. A primary infection occurs when the mushroom pins are infected, and if left untreated they will develop into large, undifferentiated masses of mushroom tissue. Spores from these primary infections are produced and may be dispersed by water splash and/or flies. If they land on other mushrooms on the beds it results in the development of brown spots. Stipe blow out results in a splitting of the stalk tissue and is common in heavily diseased crops and results in grossly deformed mushrooms (Berendsen *et al*., 2010; North & Wuest, 1993). The germination of *L. fungicola* spores is inhibited by the presence of microbiota within the soil, this is referred to as soil fungistasis (Lockwood & Filonow, 1981). This inhibition is annulled by *A. bisporus* mycelium which provides nutrients such as carbon for *L. fungicola* spore germination (Carrasco *et al*., 2019).

Mushroom growers can limit the presence of *L. fungicola* on their crops through strict hygiene control methods and the use of chemical fungicides. For the past few decades, fungicides have provided good protection against this pathogen. However, complete control of this disease has proven difficult due to fungicide resistance and the increasing number of fungicides which are being phased out by various governmental and environmental agencies. Integrated pest management (IPM) strategies are promoted under the Sustainable Use of Pesticides Directive (SUD) 2009/128/EC (Anon, 2009) which states that the use of chemical fungicides should be avoided where possible as they can be harmful to both human and environmental health. Many strains of *L. fungicola* have also developed resistance or tolerance to fungicides over the years, such as the benzimidazoles and prochloraz-manganese which are commonly used to

prevent their growth (Gea *et al*., 2005; Gea *et al*., 2021; Grogan, 2008). There is an urgent need to identify alternative treatment options to control mushroom diseases. SERENADE® (AgraQuest Inc.) (*B. velezensis* strain QST 713) and Serifel (*Bacillus amyloliquefaciens* strain MBI 600) are commercially available biocontrol agents which have shown potential to control *L. fungicola* growth (Stanojević *et al*., 2019). *B. velezensis* QST 713 has been shown to produce biofilms and antimicrobial compounds as bio-protection strategy against *T. aggressivum* (Marrone, 2002; Pandin *et al*., 2019). However, due the low number of biocontrol options commercially available, there is a need to identify more species which may be used as biocontrol agents in the future.

B. velezensis (strain Kos) is a newly identified bacterial species which was isolated from mushroom casing by Kosanovic *et al*., (2021) and was shown to be inhibitory towards *Trichoderma aggressivum* which causes green mould disease (Kosanovic *et al*., 2021) and *Cladobotryum mycophilum* which causes cobweb disease (Clarke *et al*., 2022). It was also shown that this strain does not negatively impact *Agaricus bisporus*. This strain was named *B. velezensis* R8.3 during initial studies by Kosanovic *et al*., (2021) but was then renamed to *B. velezensis* (Kos) for this work. The aim of this work is to characterise the impact of two strains of *B. velezensis* (QST 713 & Kos) on the growth and proteomic response of *L. fungicola in vitro*.

4.2 Materials and methods

4.2.1 Culture conditions

L. fungicola (Teagasc isolate: 1722) was isolated from an infected mushroom crop and was stored in a culture collection located at Teagasc Research Centre, Ashtown (Dublin, Ireland). The *L. fungicola* cultures were grown and maintained on potato dextrose agar (PDA) (Biokar diagnostics) at 25 °C for 5 days, in the dark. Liquid cultures of *L. fungicola* were grown in Sabouraud dextrose liquid broth (SDB) (Oxoid) for 48 h at 25 °C and 120 rpm. *B. velezensis* (strain Kos) was originally isolated from mushroom compost by Kosanovic *et al*., (2021). The strain was obtained from liquid nitrogen stocks at Maynooth University (Kildare, Ireland) and was grown on nutrient agar (NA) plates (Oxoid) in the dark at 25 °C for 3 days.

B. velezensis QST 713, the active strain in the commercial product SERENADE® was used during this work. *B. velezensis* QST 713 was grown and maintained on NA at 25°C for 3 days.

4.2.2 Collection of bacterial culture filtrate

Liquid cultures of *B. velezensis*, strain QST 713 and Kos were grown in 50 ml nutrient broth (NB) (Oxoid) at 30 °C, 120 rpm, in the dark in an orbital incubator. At various time points (24, 48, 72 and 96 h), flasks were removed from the incubator and the culture filtrate (CF) from the bacterial cultures were collected through centrifugation (1000 x g, 20 min). CF was filtered through a 0.45 µm filtropur S filters (Sarstedt Ltd). CF stocks from the bacterial strains were kept frozen at −20°C until required.

4.2.3 The effect of *B. velezensis* culture filtrate and cells on the growth of *L. fungicola in vitro*

A conidial suspension of *L. fungicola* was prepared and adjusted to $\times 10^5$/ml using a haemocytometer. Aliquots (100 µl) of *L. fungicola* were spread onto PDA plates ($\times 10^4$/plate) using a sterile spreader. The plates were left to dry for 15 min before adding wells (8 mm diameter) to the PDA. Culture filtrates (50 µl) of *B. velezensis*, strain QST 713 and Kos isolated at 24, 48, 72 and 96 h were added to individual wells in triplicate. Plates were incubated at 25 °C, in the dark.

Cell culture drops (10 µl) of *B. velezensis* strain QST 713 and Kos from 24, 48, 72 and 96 h cultures were applied directly onto PDA which contained *L. fungicola* ($\times 10^4$/plate). Plates were incubated at 25 °C, in the dark.

The density of a spore suspension of *L. fungicola* (1×10^6/ml) was ascertained using a haemocytometer and 1 ml of this was added to SDB (50 ml) to give a final spore density of 2×10^4/ml. Liquid cultures of *L. fungicola* were grown for 48 h at 25 °C and 120 rpm. *L. fungicola* cultures were supplemented with either NB (control) (25% v/v), 96 h *B. velezensis* (Kos) CF (25% v/v) or 96 h *B. velezensis* (QST 713) CF (25% v/v). Five replicates were used per treatment. *B. velezensis* (Kos) 96 h CF was chosen to proceed with for further experiments as previous work has shown that this time point results in the largest zone of inhibition (Clarke *et al*., 2022). Cultures were grown under the same conditions for a further 24 h. The mycelia within the flasks were separated from the liquid supernatant using Miracloth (Merck). The wet weight (g) of the mycelium in each treatment was then determined.

4.2.4 Microscopy

A small sample of *L. fungicola* hyphae from each of the liquid culture treatments (*L. fungicola* treated with either NB (A), 96 h *B. velezensis* (QST 713) (B) or 96 h *B. velezensis* (Kos) CF (C)) was collected and applied to the centre of a glass microscopic slide. The hyphae were washed three times with PBS (50 µl). Calcofluor white (25 µl, Sigma-Aldrich) was applied for 5 min to stain the hyphae and the stain was removed by washing with PBS once more. A glass cover slip was then placed on top of the samples and the hyphae were imaged using an Olympus BX51 fluorescent microscope (X40 lens) (bright-field).

The hyphae were visualised using a scanning electron microscope (SEM). A droplet of the PBS washed hyphae from each treatment was placed onto a sterilised microscopic cover slip. Samples were fixed to the slide with 5% (v/w) glutaraldehyde (Sigma-Aldrich) for 2 h, unadhered cells were removed by gentle washing with pre-warmed PBS. Slides were subjected to sequential washing with increasing ethanol concentrations (35, 50, 70, 80, 90 and 100%) to facilitate dehydration. The samples were treated with hexamethyldisilazane (Sigma-Aldrich) and air-dried overnight.

Samples were sputtered with gold (6–12 nm) prior to imaging. Hyphae were imaged using a HITACHI S-3200 N Scanning electron microscope (X500 lens).

4.2.5 Label free quantitative proteomics of *L. fungicola* treated with *B. velezensis* culture filtrates

Proteins were extracted from *L. fungicola* mycelium that had been treated with either 96 h *B. velezensis* (QST 713) CF or 96 h *B. velezensis* (Kos) CF. *L. fungicola* cultures were grown for 48 h before being supplemented with either treatment (25% v/v). *L. fungicola* cultures supplemented with NB (25% v/v) were used as a control. Fungal hyphae were crushed in liquid nitrogen using a pestle and mortar. Cell lysis buffer (8 M urea, 2 M thiourea, and 0.1 M Tris-HCl (pH 8.0) dissolved in 25 ml ddH_2O) which had been supplemented with various protease inhibitors (leupeptin, pepstatin A and Phenylmethylsulfonyl fluoride (PMSF) (10 µg/ml)) was applied to the crushed hyphae to collect the cell lysate. The protocol for protein extraction and mass spectrometry sample preparation is described in Margalit *et al*., (2020) and can also be found in **Appendix 9.1**.

Samples were analysed on a QExactive (ThermoFisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptide mix (0.75 µg) was applied to the QExactive. Peptides were separated by an increasing acetonitrile gradient from 2%–40% on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120-min reverse phase gradient at a low rate of 250 nl/min. A full MS scan of range 200–2000 was followed to select the 15 most intense ions prior to MS/MS.

Quantitative analysis of the data generated from the QExactive run was preformed using Andromeda search engine in Max-Quant (version 1.6.17 https://www.maxquant.org/). Max-Quant was used to identify the proteins within the sample and then correlate them against a *Trichoderma reesei* proteome fasta file (Proteome ID: UP000024376, Genome accession: #JABP01000000) downloaded from www.uniprot.org. There is no *L. fungicola* proteome database available on UniProt therefore a closely related species, *T. reesei* was chosen to analyse the data.

Perseus (version 1.6.14.0) was used for data analysis and graphical generation (Margalit *et al*., 2020). The LFQ intensities were log2-transformed and filtered to

remove proteins with non-existent values which suggest absence or low abundance within the sample. A principal component analysis (PCA) was used to group the data sets based on their similarities. Gene ontology (GO) mapping was carried out using a Blast 2 Go tool (https://www.blast2go.com/). The UniProt gene IDs from all of the proteins within the Perseus dataset were run against a *Trichoderma reesei* fasta file. The GO file which resulted was then uploaded to Perseus to provide terms for gene ontology biological process (BP), gene ontology cellular component (CC), gene ontology molecular function (MF) and UniProt name for each protein identified. Multiple sample t-tests and ANOVA significance tests were used to identify the statistically significant and differentially abundant (SSDA) proteins. The SSDA proteins with a relative fold change greater than ± 0.58 were retained for analysis. These proteins were then used to make volcano plots by plotting the log2 fold change on the x axis against the log p values on the y axis for each pairwise comparison. SSDA proteins were also Z- score normalised and used for the generation of a heatmap and hierarchical clustering.

The SSDAs for both *B. velezensis* (QST 713) and *B. velezensis* (Kos) samples were run through a Omicsbox software tool (v. 2.0.10) to preform GO mapping. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD028506.

4.3 Results

4.3.1 The effect of *B. velezensis* (QST 713) and *B. velezensis* (Kos) culture filtrate on the growth of *L. fungicola in vitro*

L. fungicola plate cultures treated with *B. velezensis* (QST 713 and Kos) culture filtrate from various timepoints were analysed after 72 h and there were no zones of inhibition detected for either treatment. This would suggest that the culture filtrates from *B. velezensis* (Kos) and *B. velezensis* (QST 713) are not capable of inhibiting the growth of *L. fungicola* on plate cultures (**Figure S1A**). However, zones of clearance were identified around the areas on the plate where *B. velezensis* (QST 713) and *B. velezensis* (Kos) cell culture drops were applied indicating that both bacterial cell cultures could inhibit the growth of *L. fungicola* (**Figure S1B**).

Liquid cultures of *L. fungicola* treated with either NB (control), 25% v/v *B. velezensis* (Kos) 96 h CF or 25% v/v *B. velezensis* (QST 713) 96 h CF were analysed after 24 h. The wet weight of the mycelium within each flask was recorded. The control flasks had an average wet weight of 2 ± 0.22 g. The mycelium within the *B. velezensis* (QST 713) treated flasks weighed an average of 0.74 ± 0.16 g and *B. velezensis* (Kos) treated flasks weighed 1.1 ± 0.14 g. This represents a percentage biomass decrease of 63% for *B. velezensis* (QST 713) ($P < 0.0001$) and 45% for *B. velezensis* (Kos) treatment (($P < 0.0002$) (**Figure 4.1**). It should be noted that there may be nutrients present within the NB applied to the control samples which may account for some increase in wet weight.

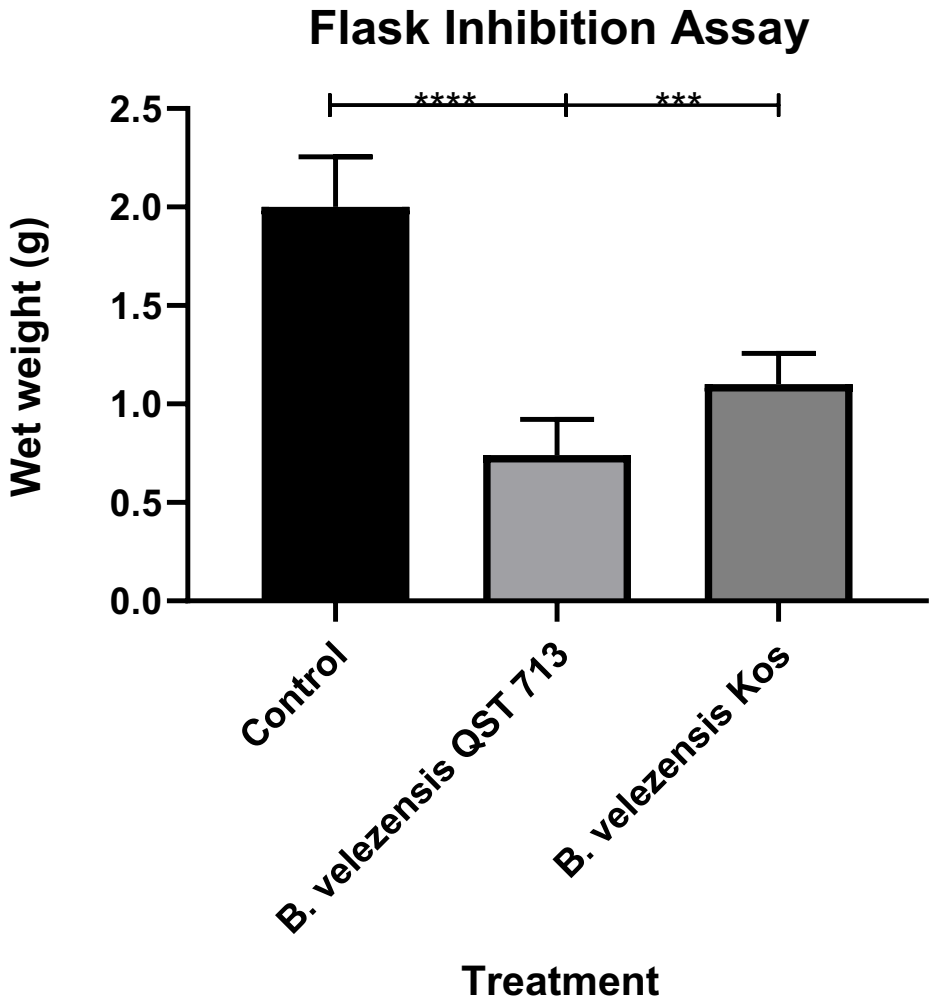


Figure 4.1 *Lecanicillium fungicola* liquid cultures (x10^4/ml) were grown in SDB and were supplemented with either 12.5% v/v NB (control), 12.5% v/v 96 h *Bacillus velezensis* (QST 713) CF or 12.5% v/v 96 h *B. velezensis* (Kos) CF. Wet weight measurements of 5 replicates per treatment were recorded. Average wet weight for each treatment is displayed above, Error bars represent standard deviation. **** = <0.0001 *** = 0.0002

4.3.2 Microscopy

The effect of *B. velezensis* (QST 713) and *B. velezensis* (Kos) 96 h CF (25% v/v) on *L. fungicola* hyphae was visualised after 24 h. The SEM images show a clear difference between the hyphae taken from control flasks, from those taken from *B. velezensis* (QST 713) and *B. velezensis* (Kos) treated flasks. The hyphae from the control (treated with NB only) appear to be cylindrical and regular in shape **(Figure 4.2 A**). This is in contrast to the *B. velezensis* (QST 713) and *B. velezensis* (Kos) CF treated hyphae which appear irregular, rounded, and deformed (**Figure 4.2 B, C**). This indicates that *L. fungicola* hyphae are disrupted when either *B. velezensis* (QST 713) or *B. velezensis* (Kos) CF is present. The images taken on the Olympus BX51 fluorescent microscope using visible light also show similar damage to *B. velezensis* (QST 713) and *B. velezensis* (Kos) treated hyphae (**Figure S2**). The *Bacillus*-treated hyphae appear to be irregular and damaged compared to the control hyphae.

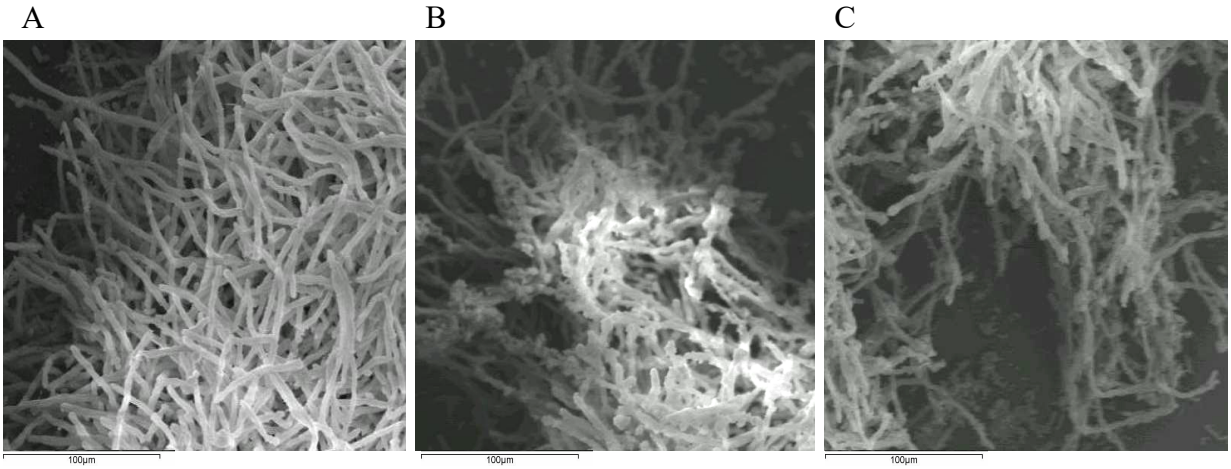


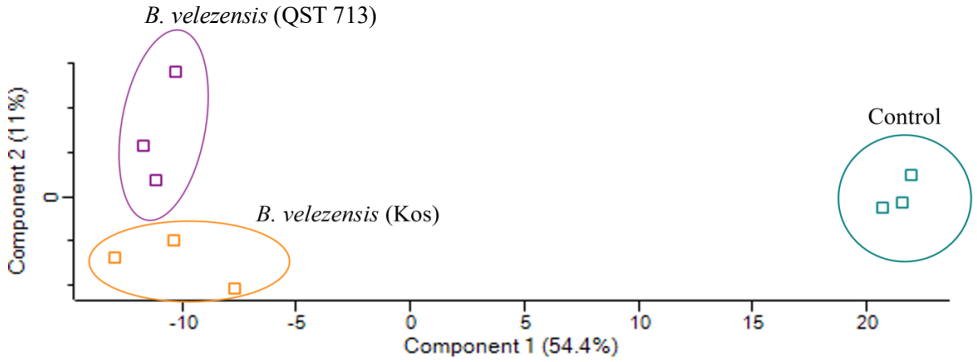
Figure 4.2 *Lecanicillium fungicola* cultures (x10^4/ml) grown for 48 h at 25 °C and then grown for a further 24 h when supplemented with either; A: 25% v/v NB. B: 25% v/v 96 h *Bacillus velezensis (*QST 713) CF or C: 25% v/v 96 h *B. velezensis* (Kos) CF. Hyphae from each treatment were collected and imaged on an HITACHI S-3200 N Scanning electron microscope at magnification X500.

4.3.3 Label free quantitative proteomics of *L. fungicola* treated with *B. velezensis* culture filtrates

The whole cell proteomic response of *L. fungicola* when exposed to *B. velezensis* (Kos) 96 h CF (25% v/v) and *B. velezensis* (QST 713) 96 h CF (25% v/v) was investigated using label free quantitative (LFQ) proteomics. A total of 1962 proteins were initially identified using Perseus (v 1.6.14.0). This number was reduced to 866 after various filtration steps. A PCA was generated with the resulting data set. Samples with similar proteomes will cluster together on a PCA. The PCA groups the control samples together and distances them away from the two sets of treatment samples. It grouped the samples treated with *B. velezensis* (QST 713) and *B. velezensis* (Kos) CF close together but still remaining separate from one another (**Figure 4.3A**).

Hierarchal clustering places the control samples on a separate lineage to either *B. velezensis* (QST 713) or *B. velezensis* (Kos) treatment samples. The pattern on the heat map shows that in areas indicating an increase in protein abundance in the treatment samples, there was a corresponding decreased relative protein abundance for the control samples (**Figure 4.3B**).

A:



B:

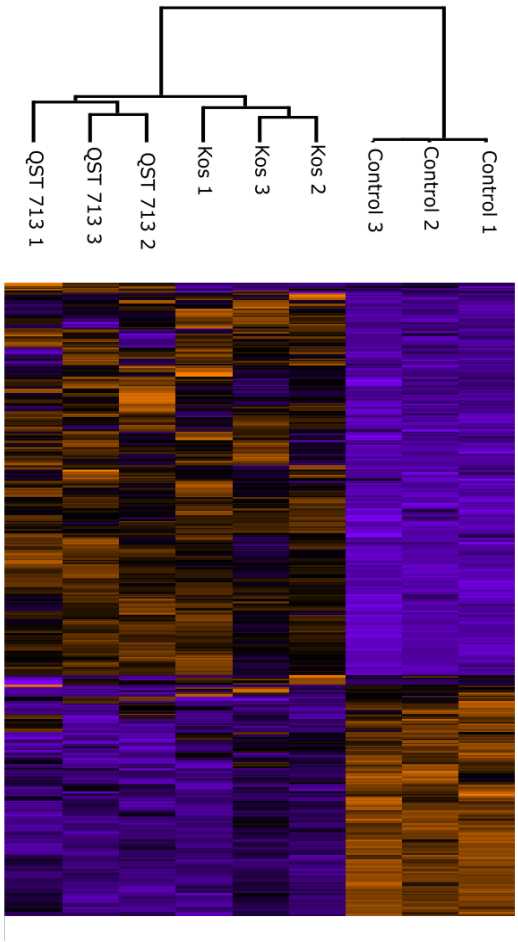
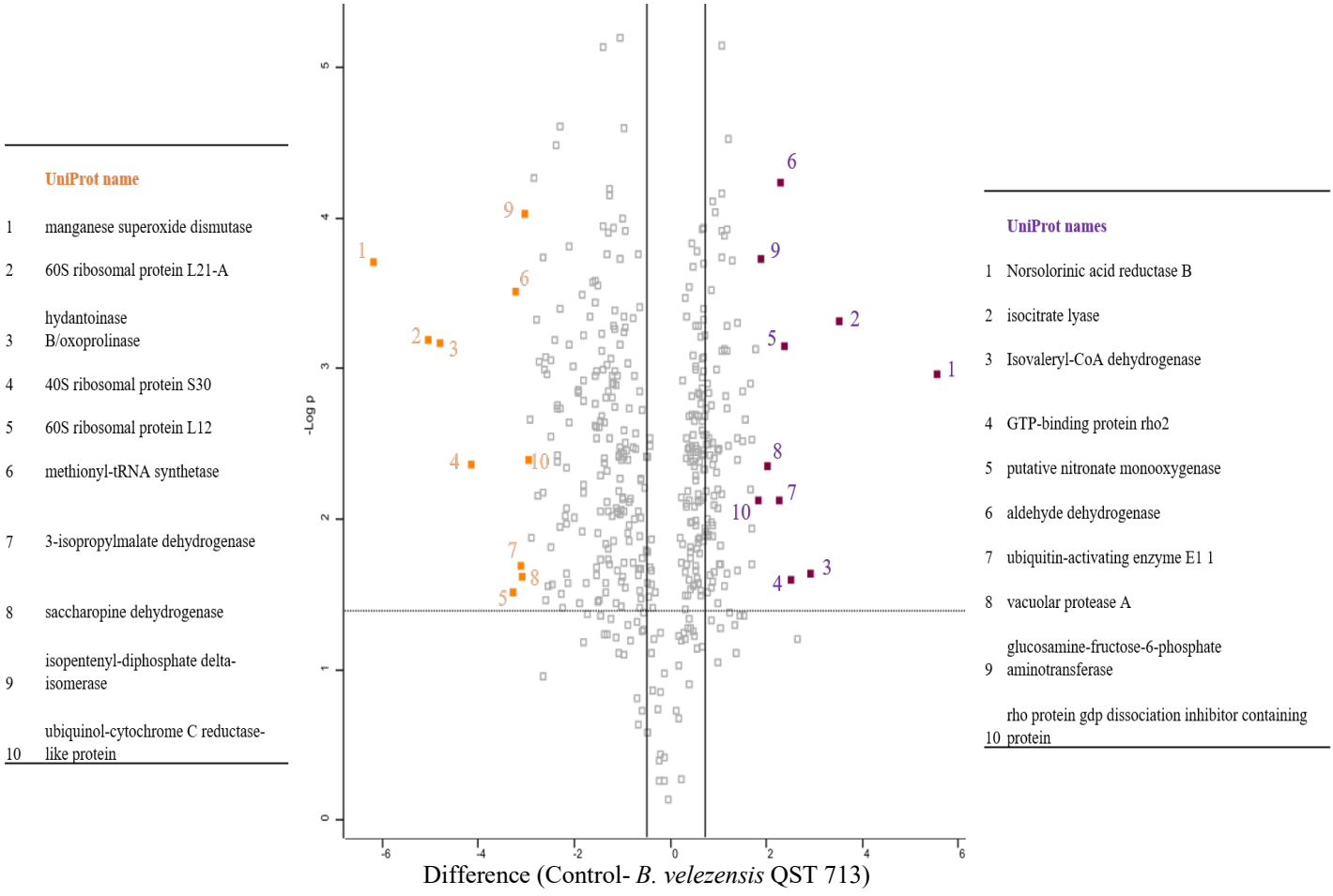


Figure 4.3 A: PCA grouping the samples based on similarities within their proteome. Control samples (blue) clustered together and were distanced away from the *Bacillus velezensis* (QST 713) (purple) and *B. velezensis* (Kos) (orange) samples. *B. velezensis* (QST 713) and *B. velezensis* (Kos) samples clustered close together but remained separate from one another. **B:** Hierarchical clustering separates the control samples from the *B. velezensis* (QST 713) and *B. velezensis* (Kos) samples on separate lineages. The heat map pattern also indicates that in areas of increased protein abundance in control samples (purple) there is decreased protein abundance in *B. velezensis* (QST 713) and *B. velezensis* (Kos) (orange).

Volcano plots show the distribution of SSDA proteins in *B. velezensis* (QST 713) and *B. velezensis* (Kos) treated samples compared to the control (**Figure 4.4**). A total of 328 SSDA proteins were identified in *B. velezensis* (QST 713) treated samples (129 increased and 199 decreased in abundance (**Figure 4.4 A**). SSDA proteins which had increased in abundance with the highest fold change included norsolorinic acid reductase B (47-fold), isocitrate lyase (11-fold) and isovaleryl-CoA dehydrogenase (8-fold). The proteins which were decreased at the highest fold change included; manganese superoxide dismutase (−73-fold), 60S ribosomal protein L21-A (−32-fold) and 40S ribosomal protein S30 (−17-fold) (**Table S1–2**).



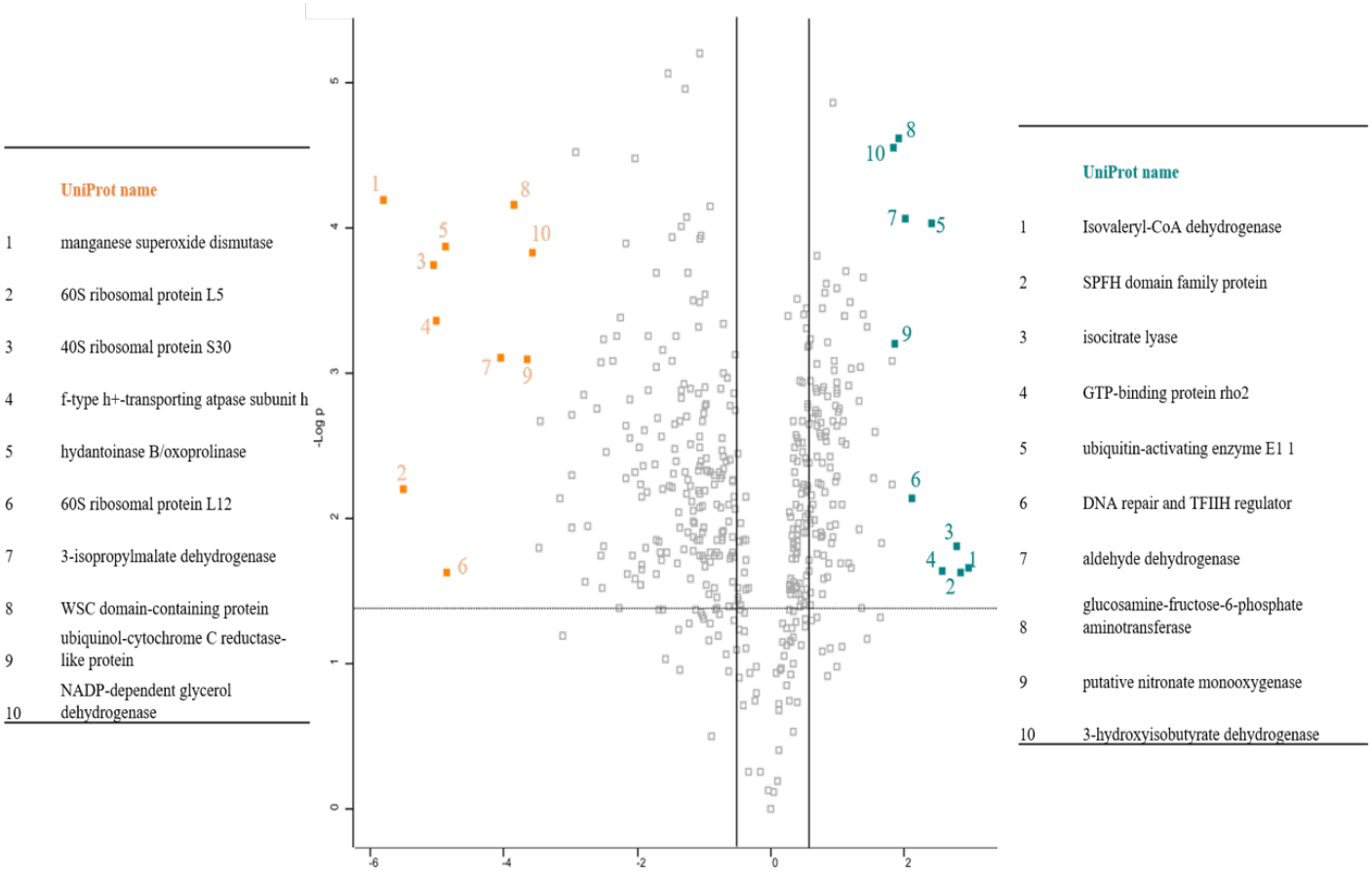


Figure 4.4 Volcano plots display the distribution of statistically significant and differentially abundant (SSDA) proteins which have a -log p fold change >1.3 and difference > ± 0.58 within either **A:** Control/*Bacillus velezensis* (QST 713) or **B:** Control/*B. velezensis* (Kos) treatment groups

A total of 288 SSDA proteins were identified in *B. velezensis* (Kos) treated samples (103 increased and 85 decreased in abundance (**Figure 4.4B**). Many of the SSDAs identified were the same proteins identified as significant for the *B. velezensis* (QST 713) treated samples. Isovaleryl-CoA dehydrogenase (8-fold) and isocitrate lyase (7-fold) were also increased in *B. velezensis* (Kos) treated samples as well as SPFH domain family protein (7-fold) and MMS19 nucleotide excision repair protein (4-fold). Manganese superoxide dismutase (−56-fold), 60S ribosomal protein L5 (−45- fold) and 40S ribosomal protein S30 (−33-fold) were also reduced in abundance in *B. velezensis* (Kos) treated samples as well as NADP-dependent glycerol dehydrogenase (−12fold) (**Table S3–4**). There was a total of 263 common SSDA proteins shared

between the *B. velezensis* (QST 713), and *B. velezensis* (Kos) treatments, meaning there were 65 SSDA proteins exclusive to *B. velezensis* (QST 713) and 25 proteins exclusive for *B. velezensis* (Kos) treatment. The SSDAs with the highest fold change in either *B. velezensis* (Kos) or *B. velezensis* (QST 713) treated *L. fungicola* samples are listed in **Table S1–4**.

Omicsbox GO mapping shows that biological processes like translation and peptide biosynthetic process are reduced in *B. velezensis* (Kos) and *B. velezensis* (QST 713) treated samples. It also indicates that biological process like glutamine family amino acid metabolic process and molecular functions like isocitrate lyase activity are increased in abundance in *B. velezensis* (QST 713) and *B. velezensis* (Kos) samples. Proteasomal protein catabolism and proteolysis are listed as increased in *B. velezensis* (QST 713) treated samples, while D-threoaldose-1-dehydrogenase (oxidoreductase) activity is increased in *B. velezensis* (Kos) treated samples (**Figure S3A and S3B**).

4.4 Discussion

This work highlights the *in vitro* growth inhibition and proteomic response of *L. fungicola* to *B. velezensis* (QST 713), present in the commercially available biocontrol product, Serenade ®, and to a newly isolated *B. velezensis* (Kos) strain. The aim was to establish how similar these two strains were to each other and whether they were inhibiting the growth of *L. fungicola* in a similar manner.

The results of the plate inhibition assay showed that cell culture drops from *B. velezensis* (QST 713) and *B. velezensis* (Kos) cells produced zones of inhibition on *L. fungicola* plate cultures. The CF of *B. velezensis* (QST 713) and *B. velezensis* (Kos) were both able to inhibit *L. fungicola* biomass accumulation in liquid cultures. The bacterial CF was able to inhibit growth of *L. fungicola* in liquid cultures, but not in plate cultures. Kosanovic *et al*., (2021) found a similar result when testing *B. velezensis* (Kos) against *T. aggressivum*. In previous work, it was demonstrated that this CF is capable of inhibiting the growth of *C. mycophilum* on plates and this appears to be the only case of plate inhibition from this *B. velezensis* (Kos) strain (Clarke *et al*., 2022). It is possible that the CF was unable to inhibit the growth of *L. fungicola* in the plate inhibition assay due to the physical difference between PDA and SDB. In a liquid broth, the *L. fungicola* cells are emerged and surrounded entirely by the CF which may make them more vulnerable to inhibition. On a plate culture, it is possible that the CF may dissolve partially into the agar under the surface of the *L. fungicola* and may not interact fully with the pathogen. SEM imaging of hyphae treated with the bacterial CF also confirmed that *B. velezensis* (QST 713) and B. *velezensis* (Kos) CF disrupted the *L. fungicola* hyphae. The growth of *L. fungicola* is stunted in the presence of *B. velezensis* and this results in irregular, shorted hyphae which contrast with the healthy control hyphae.

Proteomic analysis further supports the finding that *B. velezensis* (QST 713) 96 h CF causes significant growth inhibition and stress to *L. fungicola*. The proteomics results also suggest that *B. velezensis* (Kos) is inhibiting *L. fungicola* in a similar way to the *B. velezensis* (QST 713) strain. The PCA, hierarchical clustering and heatmap all point to differences between the proteome of *L. fungicola* samples treated with either *B. velezensis* (QST 713) or *B. velezensis* (Kos) 96 h CF compared to the control samples. Clearly, *B. velezensis* is inducing an abnormal *L. fungicola* proteomic response. The

high degree of separation between treatment and control samples in the PCA indicates that the proteome of *B. velezensis* (QST 713) and *B. velezensis* (Kos) treated samples differ to the control samples. The PCA groups the *B. velezensis* (QST 713) and *B. velezensis* (Kos) samples close together, indicating that they have a similar impact on the proteome of *L. fungicola* suggesting that they are working in a similar way. This finding is reflected in the separation between control and treatment samples, and similarities between the two treatment samples highlighted in hierarchical clustering and the heat map.

The proteomic response of *L. fungicola* against *B. velezensis* (QST 713) and *B. velezensis* (Kos) is similar as there was a high proportion of shared SSDA proteins between them. This suggests that similar activities are being triggered or reduced in response to both treatments. *B. velezensis* (QST 713) had a higher number of SSDA and had more SSDAs which were exclusive. This may indicate that *B. velezensis* (QST 713) is inducing a greater proteomic response from *L. fungicola*. This would support the results from the flask inhibition assay in which *B. velezensis* (QST 713) resulted in a larger biomass reduction compared to *B. velezensis* (Kos). This indicates that although both strains are working in a similar manner, *B. velezensis* (QST 713) appears to be better at inhibiting the growth of *L. fungicola in vitro*. The SSDAs which statistically increased in abundance for both treatments were involved in processes such as oxidoreductase activity, ubiquitination and DNA repair which are all associated with an oxidative stress response. Isocitrate lysase was also increased in abundance in both treatments. This enzyme is involved in the glyoxylate cycle, which acts as a variant of the tricarboxylic acid cycle (Dunn *et al*., 2009); Lorenz & Fink, 2001). The glyoxylate cycle has been shown to be required for fungal virulence and stress response (Lorenz & Fink, 2001). The majority of SSDAs which were statistically significantly decreased in abundance for both treatments were involved in growth process and translation. *L. fungicola* may be reducing processes like growth to conserve energy to maintain a stress response against the *B. velezensis* strains. Manganese superoxide dismutase had the largest protein fold decrease in both *B. velezensis* (QST 713) (−73-fold) and *B. velezensis* (Kos) (−55-fold) treated samples compared to the control. Manganese superoxide dismutase has been shown to play a key role in protection in fungal species against oxidative stress (Holley *et al*., 2011). Its reduced abundance means that *L. fungicola* from these samples has a reduced

antioxidant ability to protect itself from the stress initiated by the *B. velezensis* strains. Omicsbox GO mapping also confirms that activities associated with growth are reduced in *B. velezensis* treated samples, while activities associated with stress are increased.

The continuous application and reliance on chemical fungicides to treat mushroom diseases has resulted in reduced sensitivity and resistant strains (Bollen & Zaayen, 1975; Gea *et al*., 1996; Gea *et al*., 2021). Developing non-chemical treatment methods is listed in the eight principals of IPM as outlined in EU Directive 2009/128/EC (Anon, 2009). Unfortunately, in the field the level of protection achieved by biocontrol agents is often inferior compared to fungicides and there is an understandable hesitation to adopt this approach. Biocontrol treatment alone may not be an adequate replacement for fungicides. However, combining biocontrol treatments with other strategies of IPM (Prevention and suppression, monitoring and decision making) would reduce our reliance on chemical fungicides (Barzman *et al*., 2015). As *Bacillus* species are found naturally in mushroom casing and are generally regarded as safe (GRAS) (Borriss, 2015), it would also be a more environmentally friendly option.

Both the *in vitro* growth assays and proteomic analysis have demonstrated that *B. velezensis* (QST 713) can reduce the growth of *L. fungicola* and induce a stress response in the pathogen. To the best of our knowledge, this is the first time the proteomic response of *L. fungicola* to this important biocontrol strain has been published. We have also shown that the newly identified *B. velezensis* (Kos) is working in a similar manner to *B. velezensis* (QST 713), which is already approved for use on many crops. Previous work has also demonstrated that *B. velezensis* (Kos) can inhibit both cobweb disease (*C. mycophilum*) and green mould disease (*T. aggressivum*) *in vitro* (Clarke *et al*., 2022; Kosanovic *et al*., 2021). The results show that *B. velezensis* (QST 713) can achieve a high level of inhibition against *L. fungicola in vitro*. The results also indicate the potential of *B. velezensis* (Kos) as a new biocontrol treatment for mushroom disease control in the future. Future *in vivo* trials are planned to characterise the full impact of these biocontrol strains on *L. fungicola* and dry bubble disease.

4.5 References

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4.6 Supplementary material

The following sections contain supplementary figures (4.6.1) and supplementary tables (4.6.2) which accompany Chapter 4 of this thesis.

4.6.1 Supplementary Figures

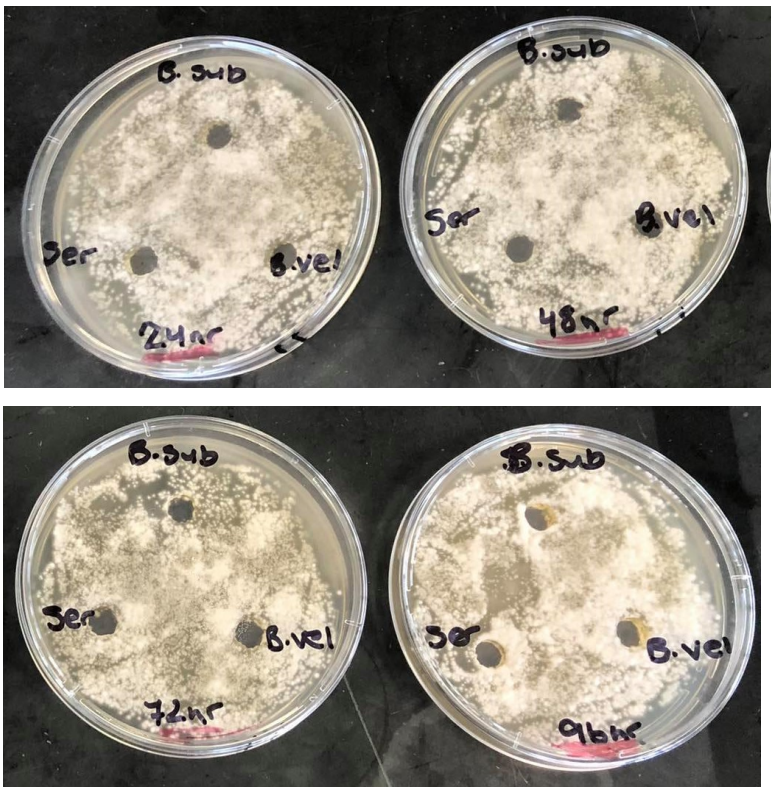


Figure S1A: No zones of inhibition present when *B. velezensis* (Kos) and *B. velezensis* (QST 713) culture filtrate (24, 48, 72 and 96 hr) was grown with *L. fungicola* (x10^4) grown on PDA plates for 72 hr at 25°C. (Note: *B. subtilis* depicted in this image was not included in manuscript data).

(A)

(B)

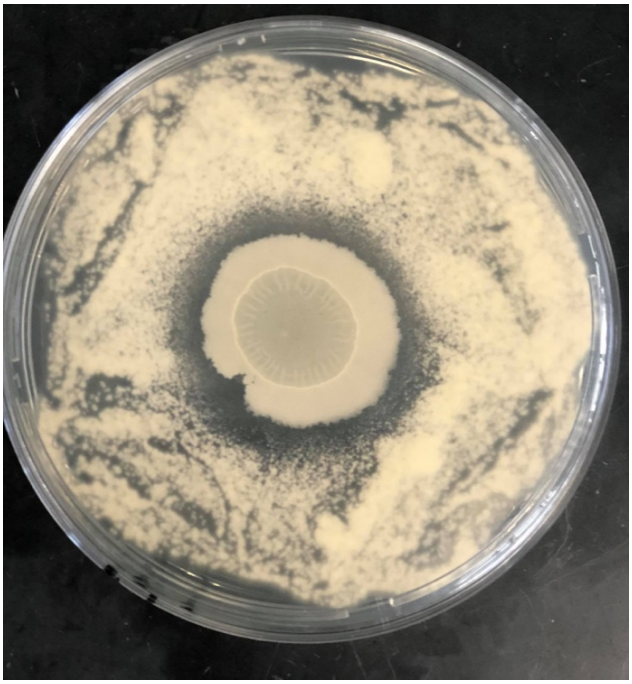
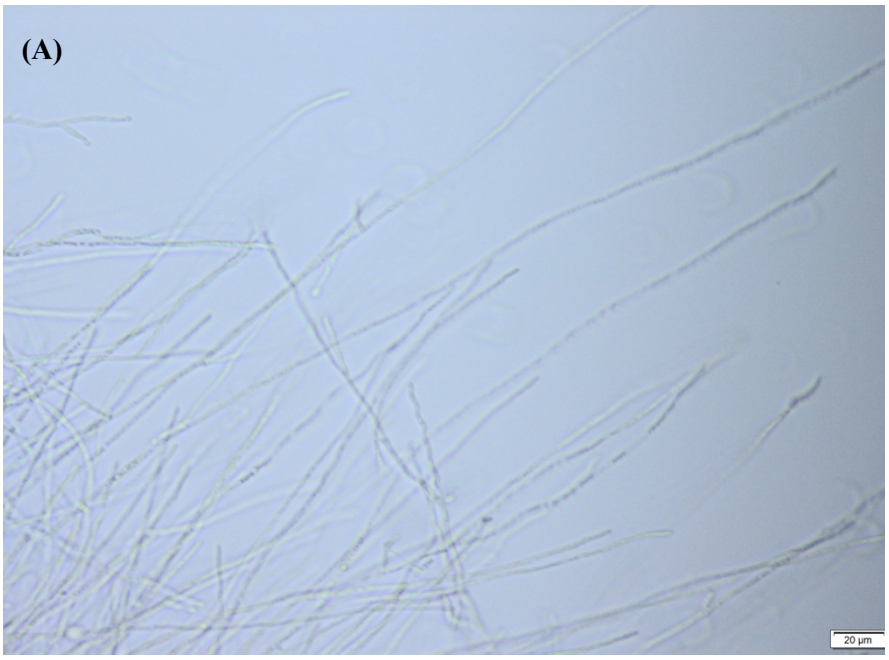
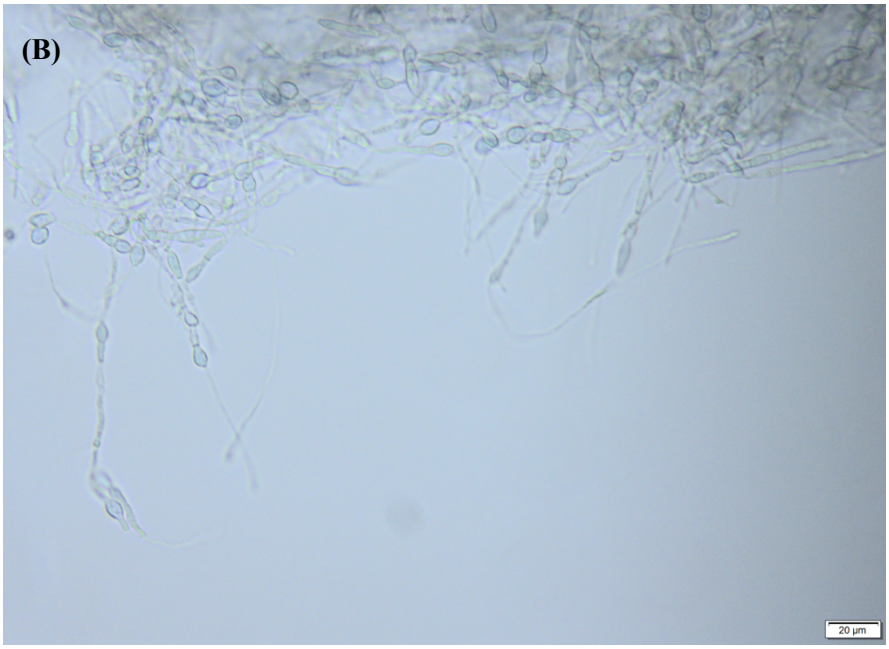


Figure S1B: Zones of inhibition produced by *B. velezensis* (Kos) (A) and *B. velezensis* (QST 713) (B) cells against *L. fungicola* (x10^4) when grown together on PDA plates for 72 hr at 25°C .


(A)
20 µm


(B)
20 µm

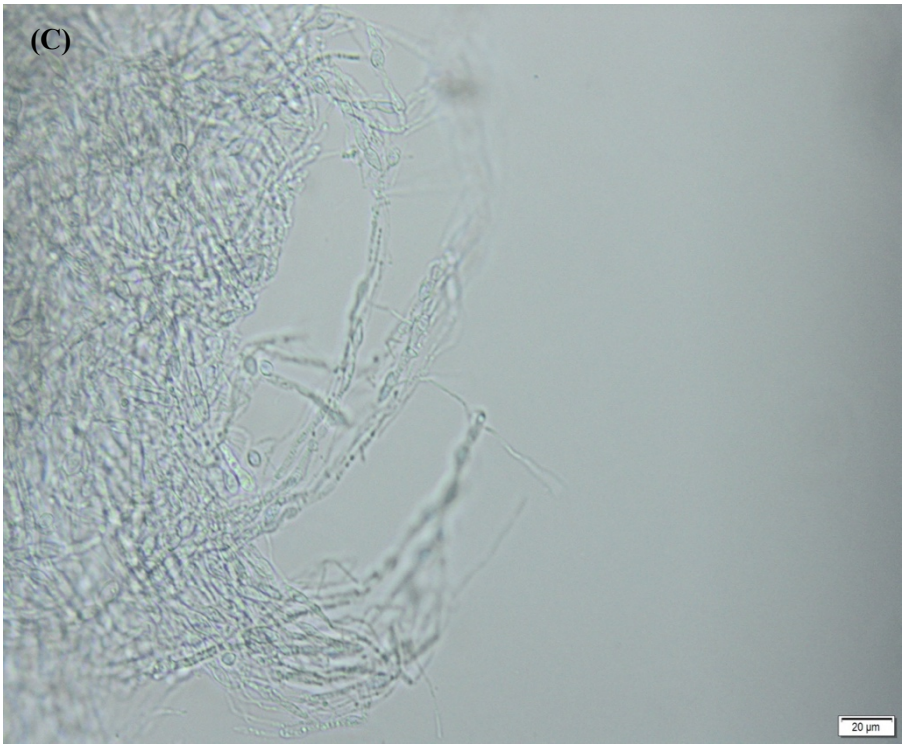
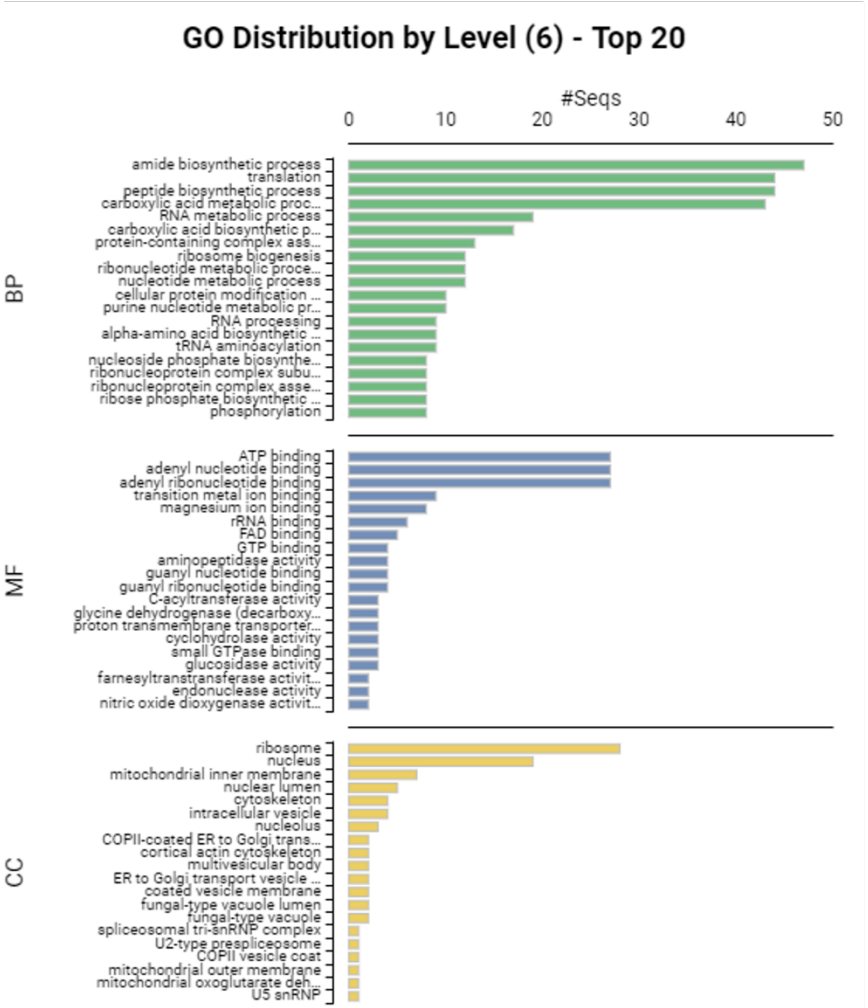
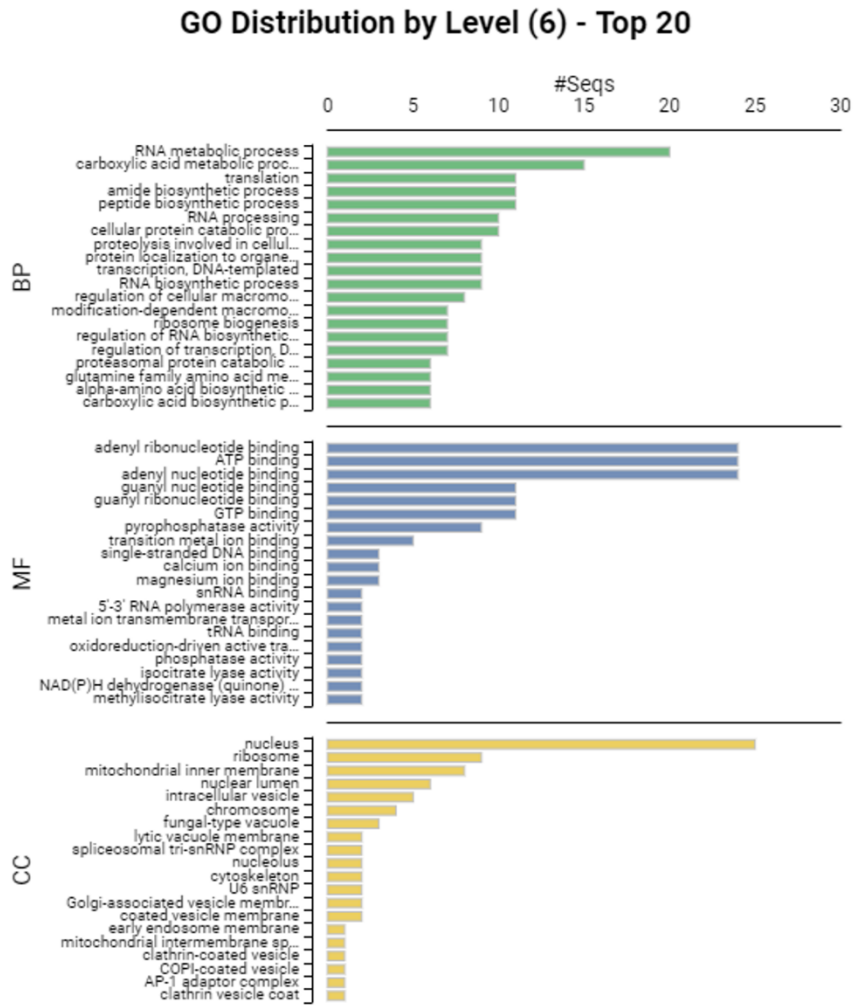


Figure S2: Images taken on an Olympus BX51 fluorescent microscope (X40 lens) of *L. fungicola* hyphae treated with either NB (A), *B. velezensis* QST 713 96 hr CF (B) or *B. velezensis* Kos 96 hr CF (C).

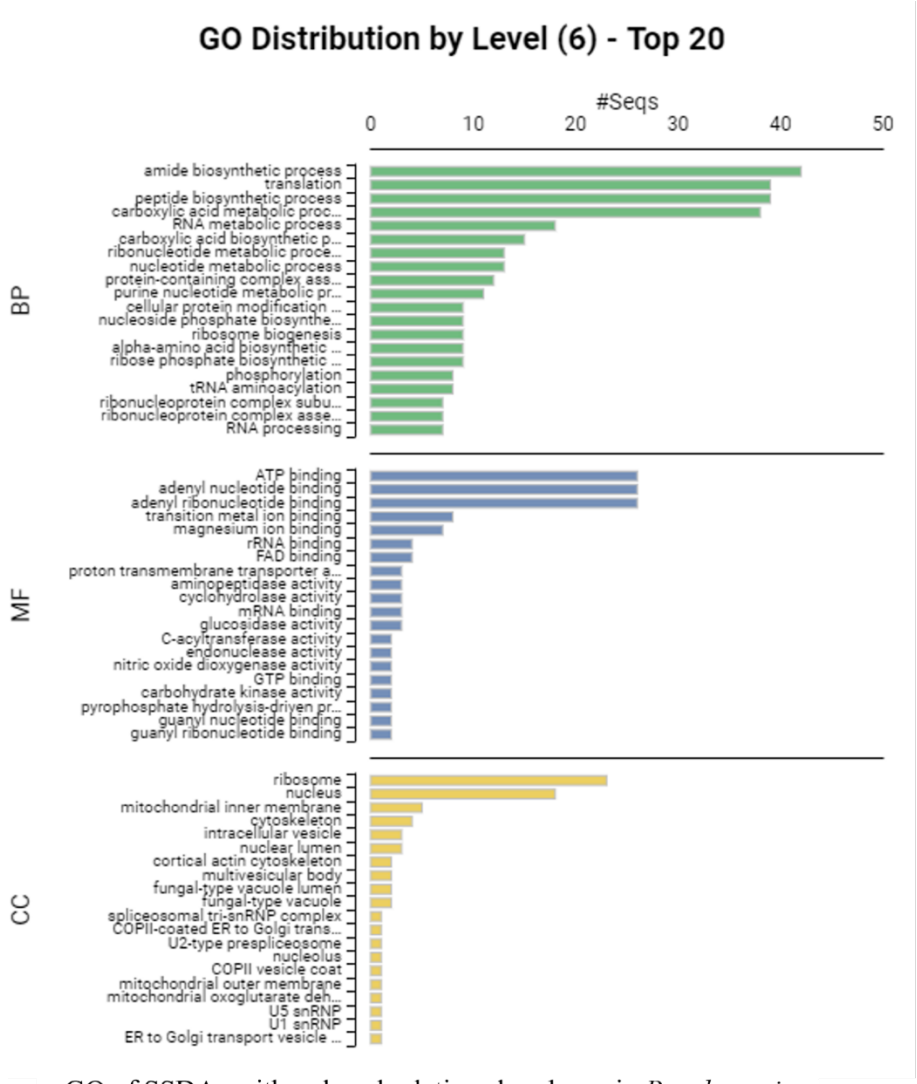


GO of SSDAs with reduced relative abundance in Serenade samples

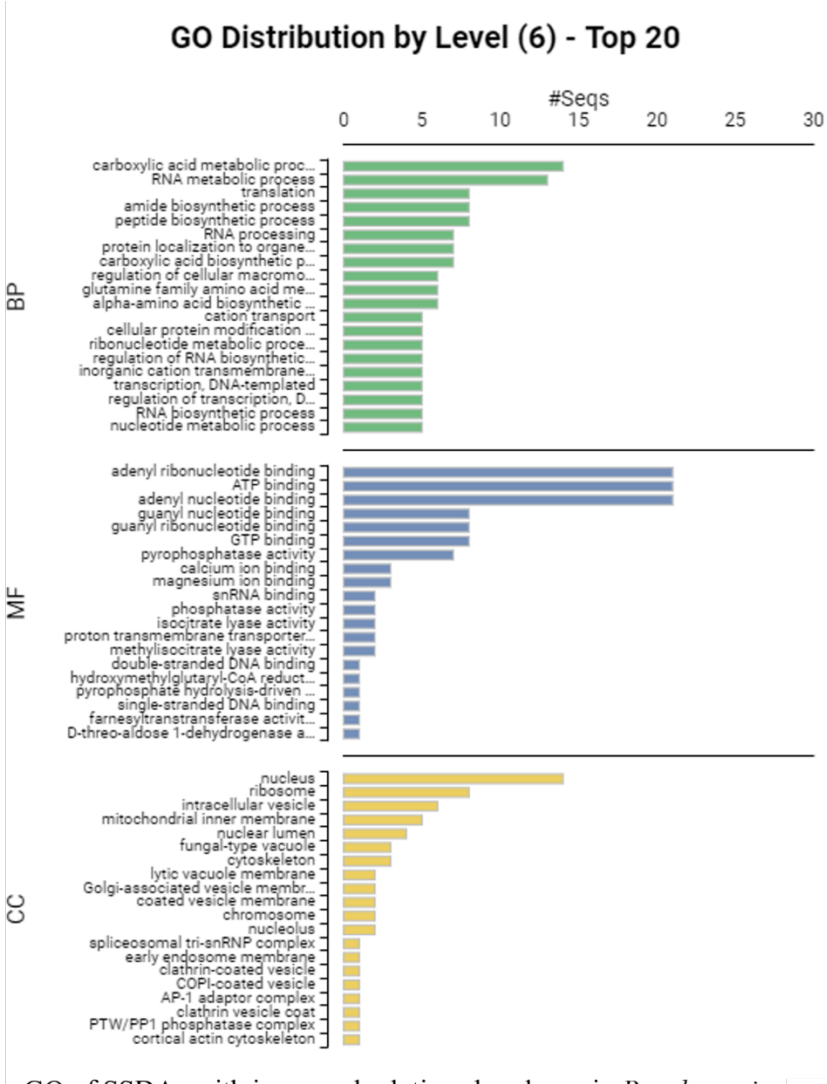


GO of SSDAs with increased relative abundance in Serenade samples

Figure S3A: Omicsbox gene ontology (GO) mapping of Serenade statistically significant and differentially abundant (SSDA) proteins. Graphs depict biological process (BP), molecular function (MF) and cellular component (CC).



GO of SSDAs with reduced relative abundance in *B. velezensis* (Kos) samples



GO of SSDAs with increased relative abundance in *B. velezensis* (Kos) samples

Figure S3B: Omicsbox GO mapping of *B. velezensis* (Kos) SSDA proteins. Graphs depict biological process (BP), molecular function (MF) and cellular component (CC).

4.6.2 Supplementary Tables

Table S1: Top SSDA upregulated in *B. velezensis* QST 713 treated *L. fungicola*

Protein IDs	Actual Difference	Description	Function
G0R973	47.31	Norsolorinic acid reductase B	Oxidoreductase activity, involved in mycotoxicosis (toxic response of fungi)
G0RE21	11.37	Isocitrate lyase	Carboxylic acid metabolic process
G0RMM8	7.56	Isovaleryl-CoA dehydrogenase	Acyl-CoA dehydrogenase activity
G0R8P2	5.73	GTP-binding protein rho2	GTPase activity, GTP binding
G0RU04	5.37	3-ketoacyl-CoA thiolase-like protein	Transferase activity
G0RAZ7	5.17	Putative nitronate monooxygenase	Nitronate monooxygenase activity
G0RKZ7	4.91	Aldehyde dehydrogenase	Oxidoreductase activity
G0RBS0	4.78	Ubiquitin-activating enzyme E1 1	ATP binding, glutathione transferase activity, antioxidant defence
G0RIW3	4.07	Vacuolar protease A	Proteolysis
G0RM90	3.68	Glucosamine-fructose-6-phosphate aminotransferase	Glutamine metabolic process (essential amino acid in states of stress)

Table S2: Top SSDA downregulated in *B. velezensis* QST 713 treated *L. fungicola*

Protein IDs	Actual Difference	Description	Function
G0RQS7	73.07	Manganese superoxide dismutase	Removal of superoxide radicals
G0RGJ8	32.84	60S ribosomal protein L21-A	Translation
G0RTN4	27.68	Hydantoinase B/oxoprolinase	Hydrolase activity
G0RTH1	17.67	40S ribosomal protein S30	Translation
G0RR73	9.76	60S ribosomal protein L12	Translation
G0RQ41	9.34	Methionyl-tRNA synthetase	Nucleic acid-binding protein
G0RLM5	8.57	3-isopropylmalate dehydrogenase	Magnesium ion binding, NAD binding
G0RTR0	8.46	Saccharopine dehydrogenase	Lysine metabolism
G0RAZ0	8.19	Isopentenyl-diphosphate delta-isomerase	Hydrolase activity
G0RWQ8	7.68	Ubiquinol-cytochrome C reductase-like protein	Mitochondrial respiratory chain complex III assembly

Table S3: Top SSDA upregulated in *B. velezensis* Kos treated *L. fungicola*

Protein IDs	Actual difference	Description	Function
G0RKC4	7.83	FKBP-type peptidyl-prolyl cis-trans isomerase	Peptidyl-prolyl cis-trans isomerase activity (stress response)
G0RMM8	7.77	Isovaleryl-CoA dehydrogenase	Acyl-CoA dehydrogenase activity
G0RJG3	7.13	SPFH domain family protein	Plasma membrane component
G0RE21	6.86	Isocitrate lyase	Carboxylic acid metabolic process
G0RDJ7	6.20	Glycosyltransferase family 66 protein	Protein glycosylation
G0R8P2	5.94	GTP-binding protein rho2	GTPase activity, GTP binding
G0RBS0	5.35	Ubiquitin-activating enzyme E1 1	Protein Ubiquitination
G0RQC8	4.33	MMS19 nucleotide excision repair protein	Iron-sulfur cluster assembly, DNA repair
G0RKZ7	4.02	Aldehyde dehydrogenase	Oxidoreductase activity
G0RJG5	3.85	Vacuolar ATP synthase subunit D	Vacuolar transport, proton transmembrane transport

Table S4: Top SSDA downregulated in *B. velezensis* Kos treated *L. fungicola*

Protein IDs	Actual difference	Description	Function
G0RQS7	55.53	Manganese superoxide dismutase	Removal of superoxide radicals, superoxide dismutase activity, metal ion binding
G0RSJ8	45.04	60S ribosomal protein L5	Translation
G0RTH1	32.97	40S ribosomal protein S30	Translation
G0R734	32.05	f-type h+-transporting atpase subunit h	ATP synthesis coupled proton transport
G0RTN4	29.12	Hydantoinase B/oxoprolinase	Hydrolase activity
G0RR73	28.98	60S ribosomal protein L12	Translation
G0RLM5	16.59	3-isopropylmalate dehydrogenase	Leucine biosynthetic process, branched-chain amino acid biosynthetic process
G0R900	14.33	WSC domain-containing protein	Cellular oxidant detoxification, cellular response to DNA damage stimulus
G0RWQ8	12.59	Ubiquinol-cytochrome C reductase-like protein	Aerobic respiration
G0RFB4	11.94	NADP-dependent glycerol dehydrogenase	Carbohydrate metabolic process

Chapter 5

The control of mushroom pathogen *Lecanicillium fungicola* with fungicides and *Bacillus* based biocontrol treatments during crop trial studies

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Author Contributions

in vitro fungicide resistance experiments were carried out and analysed by JC.

Crop trial experimental design, set up and maintenance was performed by JC and HG. With assistance from Brian McGuinness (Teagasc).

Disease assessments were carried out by JC.

Statistical analysis was carried out by JC.

Manuscript writing was performed by JC and HG.

Manuscript editing was performed by KK, DF.

Abbreviations

A.I.S: active ingredients

BCA: Biological control agent

EC European Commission

EU: European Union

IPM: integrated pest management

SUD: sustainable use of pesticides directive

PDA: potato dextrose agar

SDB: Sabouraud dextrose broth

CF: culture filtrate

NB: nutrient broth

CFUs: colony forming units

DI: disease incidence

ANOVA: analysis of variance

Abstract

Lecanicillium fungicola is a fungal pathogen of the white button mushroom (*Agaricus bisporus*) and is the causative agent of dry bubble disease. Infected mushrooms reduce healthy yield resulting in significant financial loss for growers. Dry bubble disease is traditionally managed through the application of chemical fungicides. However, due to the recent removal of approval for the most common fungicide prochloraz, only one approved fungicide, metrafenone can be used on mushroom crops in Europe. There is an urgent need to find alternatives to fungicides due to their potential effects on the environment and human health, and the emergence of resistant strains. Biocontrol uses antagonist bacteria and is considered an organic and sustainable treatment option. *B. velezensis* (QST 713) is the active agent in a commercially available biocontrol product, while *B. velezensis* (Kos) is a novel strain and both have shown antagonistic activity against *L. fungicola in vitro*. The aim of this work was to evaluate the management of dry bubble disease during large scale crop trials using both fungicide and biocontrol treatments, and using a range of inoculation levels to establish a level which best reflects on-farm conditions. An *L. fungicola* inoculation rate of $1x10^4 cfu/m^2$ was determined to reflect disease conditions most closely. At this inoculation rate, both the fungicide and biocontrol treatments were able to significantly reduce disease development ($p<0.05$). It was also shown that applying salt to diseased areas on the beds significantly prevented disease outbreak and is a technique that growers should continue to employ. This work provides important information to the mushroom sector on the treatment of dry bubble disease and provides suggestions to researchers when considering inoculation levels to include for testing biocontrol treatments at a crop level.

Key words: *Agaricus bisporus*, dry bubble disease, biocontrol, , *Bacillus velezensis*, prochloraz, metrafenone, salting

5.1 Introduction

Dry bubble disease is a serious concern for growers of the white button mushroom (*Agaricus bisporus* (Lange) [Imbach]). *A. bisporus* is one of the few mushroom species which can be grown commercially and on an industrial scale (Royse *et al.*, 2017). Of the 43 million tonnes of cultivated mushrooms produced worldwide between 2018-2019, around 11% (4.7 million tonnes) were button mushrooms (Singh *et al.*, 2020). Globally *A. bisporus* production ranks fourth behind *Pleurotus ostreatus* (oyster) (16%), *Auricularia auricular* (wood ear) (21%) and *Lentinula edodes* (shiitake) (26%) due to the popularity of these species in the Asian commercial market, but *A. bisporus* is still the most popular and commercially grown mushroom species in Europe, Australia and the United States (Singh *et al.*, 2020; Li & Xu, 2022).

A. bisporus cultivation can be negatively impacted by several diseases which can be caused by either fungal, bacterial, or viral pathogens (Largeteau & Savoie, 2010; Gea & Navarro, 2017; Gea *et al.*, 2021). Disease will have a direct effect on reducing yield for growers and consequently result in significant revenue losses. The four main fungal diseases that affect *A. bisporus* include dry bubble disease (*Lecanicillium fungicola*), wet bubble disease (*Mycogone perniciosa*), green mould disease (*Trichoderma aggressivum*) and cobweb disease (*Cladobotryum* spp.) (Fletcher & Gaze, 2008). Dry bubble disease is caused by the fungal pathogen *Lecanicillium fungicola* (Preuss) (Zare & Gams, 2008) (previously known as *Verticillium fungicola (*Hassebrauk, 1936; Gams & Van Zaayen, 1982*)*).

The severity of the symptoms of dry bubble disease depends on the timing of infection with more serious disease symptoms occurring later in the crop cycle, after mature fruiting bodies have developed (Holmes, 1971; Berendsen *et al.*, 2012). A primary infection occurs when the mushroom pins are infected with the pathogen. The mushroom which emerges will be severely deformed and made up of a large undifferentiated mass of mushroom tissue, this symptom is described as bubble (**Figure 5.1 A-B**). Spores of the pathogen are produced on the infected bubble mushrooms, which are characterised as being easily transferable due to a sticky mucilage covering. The spores are dispersed by water splash, during crop watering events. Dispersal of the sticky spores is further aided through their attachment to insect vectors, dust, equipment, pickers' hands/clothes and many other surfaces (Shamshad

et al., 2010; McGuinness *et al.*, 2021). Spores which land on the cap of developing mushrooms result in the development of spotting symptoms (**Figure 5.1C**). Another symptom reported for dry bubble disease is stipe blow out, this is generally seen in heavily diseased crops and is characterised by the splitting of stalk tissue (Berendsen *et al.*, 2010).

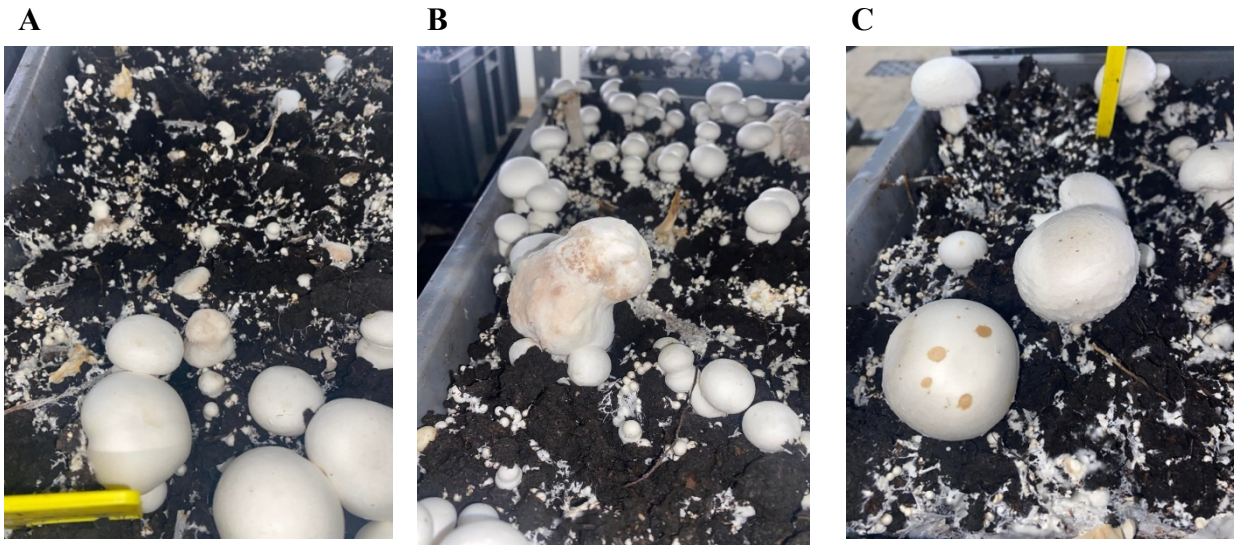


Figure 5.1 Symptoms of dry bubble disease. A: Early bubble mushroom development, B: Advanced bubble mushroom development and C: mushroom spotting symptom

If left untreated, dry bubble disease can result in severely damaged mushroom produce which will directly impact revenue of growers. One way to control disease levels is to implement strict integrated pest management (IPM) practices on the farm. The eight strategies of IPM include 1: prevention and suppression, 2: monitoring, 3: decision based on monitoring and thresholds, 4: non-chemical methods, 5: pesticide selection, 6: reduced pesticide use, 7: anti-resistance strategies and 8: evaluation (Barzman *et al.*, 2015). The use of personal protective equipment (gloves, hairnets etc), foot washes upon entry of growing rooms, and sterilisation of all equipment used is key to limiting disease spread. Mushroom houses must also be well maintained and fitted with door seals. Growers are advised to monitor their crops carefully and identify disease at an early stage before it has the chance to spread. Growers are also encouraged to have a salting routine which involves adding a layer of salt over diseased areas to limit the spread of pathogenic spores (Grogan & Gaze, 2008). Most growers also regularly apply preventative synthetic fungicides, which have been a key tool to growers who are dealing with difficult to control diseases. However fungicide use can have

significant effects on non-target organisms and negatively impact human health (Kim *et al.*, 2017; Brauer *et al.*, 2019; Zubrod *et al.*, 2019). Growers are also dealing with increased resistance levels to the fungicides (Grogan & Gaze, 2000; Grogan, 2006; Du *et al.*, 2021; Gea *et al.*, 2021). Prochloraz, a demethylation inhibitor fungicide, was a popular and effective treatment to control diseases in mushrooms crops. As of June 2023, approval for the use of this fungicide within the EU was removed. This left growers in the EU with only one approved fungicide, metrafenone. There is evidence of emerging *Cladobotryum* strains which are tolerant to metrafenone suggesting it will be less effective against cobweb disease (Clarke *et al.*, 2024) .

In recent years there has been a steady decline in the number of approved fungicides and this has created an urgent need for environmentally sustainable alternatives. This is supported by the European Commission (EC) which outlined a more sustainable approach to pest management in its Sustainable Use of Pesticides Directive (SUD) 2009/128/EC (Parliament, 2009). Biocontrol treatments exploit the antagonistic potential of bacterial strains which are naturally found in the environment (Lahlali *et al.*, 2022). *Bacillus velezensis* species have been investigated as biocontrol strains for several plant crops as they reduce the growth of pathogenic strains through the production of antimicrobial compounds, lytic enzymes or through competition for space and nutrition (Borriss, 2015; Rabbee *et al.*, 2019; Alenezi *et al.*, 2021). Biocontrol treatments have also been investigated in relation to mushroom crops (Preston *et al.*, 2019). Serenade (AgraQuest Inc.) is a commercially available biocontrol product which contains *B. velezensis* (strain QST 713) as its active agent (Pandin *et al.*, 2018). This product has been characterised as a potential treatment for several mushroom diseases (Pandin *et al.*, 2018; Potocnik *et al.*, 2018; Pandin *et al.*, 2019; Stanojević *et al.*, 2019). Another novel biocontrol strain included in this work is *B. velezensis* (strain Kos) which was originally isolated from mushroom casing (Kosanovic *et al.*, 2021). This strain has previously been shown to inhibit the pathogens of cobweb disease and dry bubble disease *in vitro* (Clarke *et al.*, 2022a; b) and has been investigated at a crop level for treatment of cobweb disease (Clarke *et al.*, 2024).

The aim of this work was to investigate the *in vitro* resistance levels of *L. fungicola* strains towards to prochloraz and metrafenone and to determine the efficacy of both

fungicide and biocontrol treatments to control dry bubble disease at a large scale. The optimum experimental inoculation rate which accurately represent disease levels on farms during disease crop trials was also investigated.

5.2 Materials and Methods

5.2.1 Fungal cultures

Two *L. fungicola* strains (Teagasc isolates 620 and 1722) were evaluated for their *in vitro* response to two fungicide active ingredients (a.i.s): prochloraz and metrafenone. Strain 620 was known to be sensitive to prochloraz and was isolated before metrafenone use. Strain 1722 was isolated in 2020, after metrafenone introduction. Isolate details are shown in **Table 5.1**. Strains were isolated from infected mushrooms and stored in liquid nitrogen at -80°C in the Teagasc Ashtown culture collection (Dublin, Ireland).

Table 5.1 *Lecanicillium* isolates used in *in vitro* experiments.

Isolate Number	Species	Year of isolation	Place of origin
620	*L. fungicola*	1997	Surrey, England
1722	*L. fungicola*	2020	Cavan, Ireland

5.2.2 Fungicides and biological control agents (BCAs)

The chemical fungicides prochloraz (Sporgon® 50 WP) (460 g a.i.s kg^{-1}) and metrafenone (Vivando®) (500 g a.i.s 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 contained a minimum of 1 x 10^{12} colony forming units (CFUs) per litre. A bacterial strain *B. velezensis* was originally isolated from mushroom casing (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 produced by inoculating 4 L of sterile nutrient broth (NB) with 140 hr *B. velezensis* Kos liquid culture (1ml/L). Flasks were grown for 96 hr (30°C at 120 rpm) and the CF was collected by centrifugation (1792 x g, 10 min). The CF was filtered using Miracloth (Merck) into sterile flasks (Duran).

5.2.3 Analysis of *in vitro* response of *Lecanicillium* isolates to fungicides

Plates of *L. fungicola* (isolate 1722 and 620) were washed with 5 ml PBS+ 0.1%v/v TWEEN and a spore suspension was collected. The concentration of the spore suspension was determined using a haemocytometer. The spore suspension was adjusted with dilutions so that each Sabouraud Dextrose Broth (SDB) flask (50 ml) had a final concentration of $1x10^5$cfu/ml. The flasks were then treated with either prochloraz or metrafenone (1, 10, 100 or 500 mg/kg^{-1}). Three replicate flasks were prepared per treatment/isolate combination. Untreated, inoculated flasks were included as a control. Flasks were grown at 25°C (100 rpm) for 72 hr. Fungal mycelium was separated from the liquid with Miracloth and the mycelial wet weight of each flask was determined.

To determine the effect the various concentrations of prochloraz and metrafenone have on sporulation and hyphae development of the 1722 and 620 isolates, flasks were set up according to the methods outlined above, but using 25 ml SDB and a final concentration of $5x10^5$cfu/ml. After 24 hr of growth at 25°C (100 rpm) evidence of sporulation and hyphal development was monitored using an Olympus microscope (40X). Both isolates were brought forward to be tested in crop trials, however this chapter will only discuss crop trial results from isolate 1722.

5.2.4 Mushroom cultivation

Crop trials were carried out in 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 x 600 mm x 300 mm) with a 0.2 m^2 internal crop surface area was filled with 16 kg (equivalent fill rate of 80kg/m^2) of commercially-sourced Phase III substrate, spawned with rye grains inoculated with *A. bisporus* strain Sylvan A15 (Carbury Compost Ltd., Carbury, Co. Kildare, Ireland). The crates of substrate were covered with a layer of commercial

peat-based mushroom casing (50 mm) (Harte Peat Ltd., Clones, Co. Monaghan, Ireland) on day 1 of the crop cycle and then placed onto shelves in the growing room. Crops were managed following standard operating procedures for mushroom crops in the environmentally controlled growing rooms 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 hr to 20°C and 21°C respectively (cool down pinning). This change in growing conditions triggers the *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 mushroom harvesting cycles (flushes). Six replicate crates were prepared for each treatment combination. Healthy mushrooms were harvested as predominantly closed cups over two/three flushes and recorded as kg plot^{-1}. Diseased or spotted mushrooms caps were recorded separately. The average number of bubble mushrooms which developed on each plot was recorded for each flush. For crop trial 1, a strict salting regime was undertaken. Once a bubble mushroom had been identified it was recorded, and the area was salted carefully before the crop was watered. If bubble mushrooms were too large to be covered by salt, they were very carefully removed before adding salt to the area on the bed where the bubble mushroom originated from. For crop trial 2 and 3, a separate salting treatment was included where salt was applied in the same manner as described for crop trial 1 only for these specific salted treatment plots. No salt was applied to the control or other treatment plots. In these non-salted plots, bubble mushrooms were recorded and removed carefully only at the end of the flush.

5.2.5 Crop trials:

Three crop trials were conducted to evaluate the efficacy of different fungicides and BCAs to control dry bubble disease. Crop trial 1 looked at the efficacy of fungicides and BCAs to control dry bubble disease at different rates of inoculation with *L. fungicola* 1722. Crop trial 2 looked at the efficacy of fungicides, BCAs and salting to control dry bubble disease at different rates of inoculation. Crop trial 3 was a repeat of the key treatments in Crop trials 1 and 2 that gave the most interesting results. Crop trials were set up in industry standard growing rooms at Teagasc, Ashtown centre.

There were 16, 12 and 12 treatments included, in crop trial 1, 2 and 3 respectively, summarised in **Table 5.2**.

Table 5.2 Details of treatments and inoculation rates used in crop trials 1, 2 and 3.

Crop trial 1: Efficacy of fungicides and BCAs to control dry bubble disease at different rates of inoculation				
Treatment	**Fungicide/BCA/ Treatment**	**Inoculation rate Treatment**	***L. fungicola* strain**	**Reps**
1: Control uninoculated	None	None	None	6
2: Control $1x10^6$cfu/m^2	None	$1x10^6$cfu/m^2	1722	6
3: Control $1x10^4$cfu/m^2	None	$1x10^4$cfu/m^2	1722	6
4: Control $1x10^2$cfu/m^2	None	$1x10^2$cfu/m^2	1722	6
5: Prochloraz uninoculated	Prochloraz	None	None	6
6: Prochloraz $1x10^6$cfu/m^2	Prochloraz	$1x10^6$cfu/m^2	1722	6
7: Prochloraz $1x10^4$cfu/m^2	Prochloraz	$1x10^4$cfu/m^2	1722	6
8: Prochloraz $1x10^2$cfu/m^2	Prochloraz	$1x10^2$cfu/m^2	1722	6
9: QST 713 uninoculated	QST 713 (*B. velezensis*)	None	None	6
10: QST 713 $1x10^6$cfu/m^2	QST 713 (*B. velezensis*)	$1x10^6$cfu/m^2	1722	6
11: QST 713 $1x10^4$cfu/m^2	QST 713 (*B. velezensis*)	$1x10^4$cfu/m^2	1722	6
12: QST 713 $1x10^2$cfu/m^2	QST 713 (*B. velezensis*)	$1x10^2$cfu/m^2	1722	6
13: Kos uninoculated	Kos (*B. velezensis*)	None	None	6
14: Kos $1x10^6$cfu/m^2	Kos (*B. velezensis*)	$1x10^6$cfu/m^2	1722	6
15: Kos $1x10^4$cfu/m^2	Kos (*B. velezensis*)	$1x10^4$cfu/m^2	1722	6
16: Kos $1x10^2$cfu/m^2	Kos (*B. velezensis*)	$1x10^2$cfu/m^2	1722	6
Crop trial 2: Efficacy of fungicides, BCAs and salting to control dry bubble disease at different rates of inoculation				
Treatment	**Fungicide/BCA/ Treatment**	**Inoculation rate Treatment**	***L. fungicola* strain**	**Reps**
1: Control uninoculated	None	uninoculated	None	6
2: Control $1x10^4$cfu/m^2	None	$1x10^4$cfu/m^2	1722	6
3: Control $1x10^2$cfu/m^2	None	$1x10^2$cfu/m^2	1722	6
4: Salted uninoculated	Salted	uninoculated	None	6
5: Salted $1x10^4$cfu/m^2	Salted	$1x10^4$cfu/m^2	1722	6
6: Salted $1x10^2$cfu/m^2	Salted	$1x10^2$cfu/m^2	1722	6
7: Metrafenone uninoculated	Metrafenone	uninoculated	None	6
8: Metrafenone $1x10^4$cfu/m^2	Metrafenone	$1x10^4$cfu/m^2	1722	6
9: Metrafenone $1x10^2$cfu/m^2	Metrafenone	$1x10^2$cfu/m^2	1722	6
10: QST 713 uninoculated	QST 713 (*B. velezensis*)	uninoculated	None	6
11: QST 713 $1x10^4$cfu/m^2	QST 713 (*B. velezensis*)	$1x10^4$cfu/m^2	1722	6
12: QST 713 $1x10^2$cfu/m^2	QST 713 (*B. velezensis*)	$1x10^2$cfu/m^2	1722	6
10: Kos uninoculated	Kos (*B. velezensis*)	uninoculated	None	6
11: Kos $1x10^4$cfu/m^2	Kos (*B. velezensis*)	$1x10^4$cfu/m^2	1722	6
12: Kos $1x10^2$cfu/m^2	Kos (*B. velezensis*)	$1x10^2$cfu/m^2	1722	6

Crop trial 3: Efficacy of fungicides, BCAs and salting to control dry bubble disease at different rates of inoculation				
Treatment	**Fungicide/BCA/ Treatment**	**Inoculation rate Treatment**	***L. fungicola* strain**	**Reps**
1: Control uninoculated	None	uninoculated	None	6
2: Control $1x10^4$cfu/m^2	None	$1x10^4$cfu/m^2	1722	6
3: Salted uninoculated	Salted	uninoculated	None	6
4: Salted $1x10^4$cfu/m^2	Salted	$1x10^4$cfu/m^2	1722	6
5: Metrafenone uninoculated	Metrafenone	uninoculated	None	6
6: Metrafenone $1x10^4$cfu/m^2	Metrafenone	$1x10^4$cfu/m^2	1722	6
7: QST 713 uninoculated	QST 713 (*B. velezensis*)	uninoculated	None	6
8: QST 713 $1x10^4$cfu/m^2	QST 713 (*B. velezensis*)	$1x10^4$cfu/m^2	1722	6
9: Kos uninoculated	Kos (*B. velezensis*)	uninoculated	None	6
10: Kos $1x10^4$cfu/m^2	Kos (*B. velezensis*)	$1x10^4$cfu/m^2	1722	6
11: Control $1x10^6$cfu/m^2	None	$1x10^6$cfu/m^2	1722	6
12: Control $1x10^4$cfu/m2	None	$1x10^6$cfu/m^2	1722	6

5.2.6 Fungicide and BCA application.

For treatment application, the commercial fungicide and BCAs were applied to plots on day 6 after casing (day 1) following the approved rates on the label. Prochloraz was applied at a rate of 1 g of product (Sporgon® 50WP) m^{-2}, metrafenone was applied at a rate of 1 ml of product (Vivando®) m^{-2} and *B. velezensis* QST 713 was applied at a rate of 0.8 ml of product (Serenade® ASO) m^{-2} (= 0.8 x 10^{12} cfu m^{-2}). *B. velezensis* Kos 96 hr 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. There were two further applications of the two BCA treatments: between 1st and 2nd flush and again between 2nd and third flush. Water was applied to control and fungicide plots. In crop trial 2 the fungicide Vivando (metrafenone) was used in place of the previously used Sporogon (prochloraz) as the fungicide control treatment. This decision was made due to the imminent expiration of Sporogon approval for use on mushroom crops in the EU from 30th June 2023.

5.2.7 Crop inoculation

For crop trial 1, 2 and 3, inoculum was prepared for *L. fungicola* isolates 1722. Subcultures of the isolate were grown on potato dextrose agar (PDA) at 25°C for 5 weeks. Plate cultures were washed with PBS +Tween 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 $1x10^6$/ml. This was further diluted in PBS+Tween, to give inoculum concentrations of $1x10^4$/ml and $1x10^2$/ml. A 50 ml aliquot of inoculum of isolate 1722 was applied to each 0.2 m^{-2} plot to give a final application rate of either $1x10^6$, $1x10^4$ or $1x10^2$ spores m^{-2} according to the crop plan (**Table 5.2**). Inoculation of plots took place on day 12 of the crop cycle for crop trial 1 and 2, and on day 11 for crop trial 3.

5.2.8 Disease data collection

During crop trial 1, a disease assessment for symptomatic bubble mushrooms on plots was carried out regularly over the course of each flush. Any bubble mushrooms found on plots were recorded and salt was carefully applied to cover the infected bubble to limit cross contamination between plots. For crop trial 2 and 3, the disease evaluation protocol was revised based on the results of crop trial 1. During these trials, a disease assessment for symptom bubble mushroom on plots were carried out only at the end of each flush allowing bubble to develop during the flush. Any sizeable bubble mushrooms found at the end of the flush were recorded and were removed carefully to limit cross contamination, but no salt was applied. For the salted treatments, bubble mushrooms development was monitored regularly. Any bubble mushrooms found were recorded and salt was carefully applied to cover the infected bubble. Disease incidence was represented by the average number of bubble mushrooms per treatment at the end of the crop trial. 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 the treated samples (Stanojević *et al*., 2019).

5.2.9 Statistical analysis

In the *in vitro* fungicide tests, after determining normality and equal variance, the data at each concentration were analysed using analysis of variance (ANOVA) in Minitab (version 20.04.00). Differences between treatments were determined using Tukey method and 95% confidence for pairwise comparisons ($p < 0.05$). In the crop trials treatment plots were arranged on shelves in a randomized block design. After determining normality and equal variance, data were analysed using ANOVA in Minitab (version 20.04.00). Differences between treatments were determined using

Tukey method and 95% confidence for pairwise comparisons ($p < 0.05$). During crop trial 2, one plot inoculated with *L. fungicola* 1722 $1x10^4$cfu/m^2 resulted in abnormal disease levels which were not in line with the other replicates. Therefore disease data analysis for the $1x10^4$cfu/m^2 plots in crop trial 2 were analysed using 5 replicates rather than 6 to remove this outlier.

5.3 Results:

5.3.1 Analysis of *in vitro* response of *Lecanicillium* isolates to fungicides

Prochloraz significantly reduced the growth of isolate 1722 in flask culture. At 1 mg/kg growth was reduced by 50% while at 10 mg/kg growth was reduced by 99%. No growth was recorded for isolate 1722 grown in the presence of 100 and 500 mg/kg prochloraz **(Figure 5.2A)**. Hyphal development at 24 hr was seen only in 1 and 500 mg/kg prochloraz **(Figure S1A)**. Metrafenone also significantly reduced the growth of isolate 1722 but growth was less severely affected compared to prochloraz treated flasks. Growth was reduced by 26%, 43%, 45% and 37% for 1,10, 100 and 500 mg/kg metrafenone respectively **(Figure 5.2 B)**. Sporulation and hyphal development were observed at all concentrations of metrafenone after 24 hr **(Figure S1A)**.

Prochloraz was also very effective at reducing the growth of isolate 620, similar to isolate 1722. At 1 mg/kg growth was reduced by 63% while at 10 mg/kg growth was reduced by 71%. No growth was recorded for isolate 620 grown in the presence of 100 and 500 mg/kg prochloraz **(Figure 5.2 C).** Hyphal development at 24 hr was seen only at 1mg/kg prochloraz. For isolate 620 treated with metrafenone, growth was reduced by 48%, 52%, 63% and 29% for 1,10, 100 and 500 mg/kg respectively **(Figure 5.2 D)** and sporulation and hyphal development was observed at all tested concentrations of metrafenone at 24hr **(Figure S1B)**.

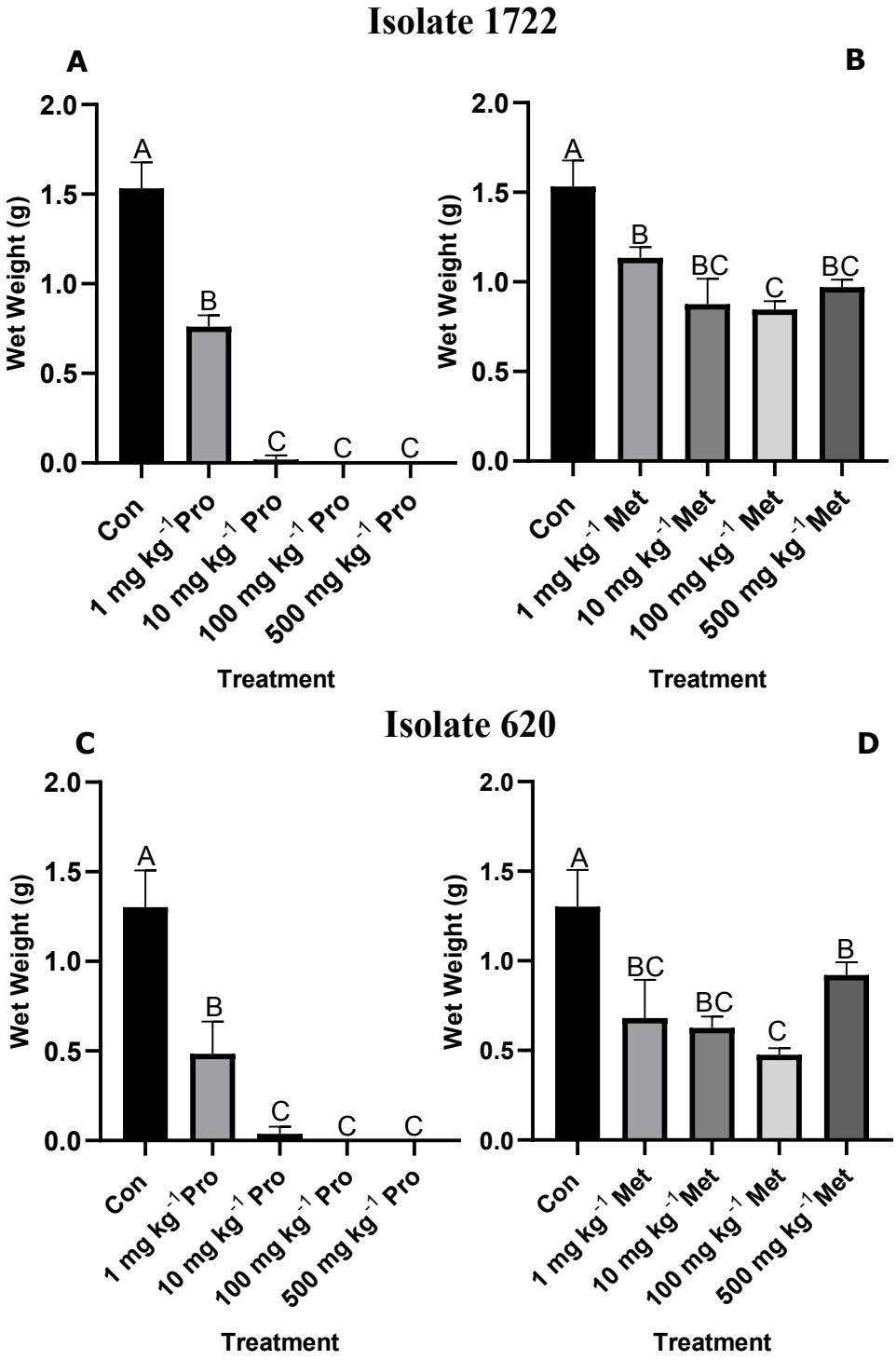


Figure 5.2 Growth of *Lecanicillium fungicola* in SDB liquid culture **A:** isolate 1722 with prochloraz (1, 10, 100 or 500 kg/mg), **B:** isolate 1722 with metrafenone (1, 10, 100 or 500 kg/mg), **C:** isolate 620 with prochloraz (1, 10, 100 or 500 kg/mg) and **D:** isolate 620 with metrafenone (1, 10, 100 or 500 kg/mg). Data represent the average wet weight of 3 replicates after 72 hr for each treatment. Error bars represent standard deviation. Data analysed by ANOVA, n = 3. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukeys pairwise comparisons test.

5.3.2 Crop trial 1: Efficacy of fungicides and BCAs to control dry bubble disease at different rates of inoculation

Yield: The average yield of healthy mushrooms for treatments 1-16, collected over three flushes in crop trial 1 can be seen in **Figure 5.3**. There was no statistically significant difference in yield between treatments during flush 1 and flush 2. The yield of flush 1 ranged from 3.9 to 4.5 kg $plot^{-1}$, while during flush 2 the yield was much lower ranging between 0.2 to 0.8 kg $plot^{-1}$. This may be due to a high number of smaller mushrooms being harvested during flush 1 which reflects the high yield recorded during this time, and may have negatively impacted the yield for flush 2. The yield for flush 3 ranged from 0.4 to 1.5 kg $plot^{-1}$ and at this point there was a significant difference in yield between treatments. The control inoculated at a rate of $1x10^6 cfu/m^2$ *L. fungicola* and all treatment plots inoculated at this rate were significantly reduced in yield compared to the uninoculated control ($P < 0.05$). For the control inoculated at the two lower inoculation rates ($1x10^4 cfu/m^2$ and $1x10^2 cfu/m^2$) and all treatment plots inoculated at these rates there was no significant reduction in yield compared to the uninoculated controls. Total yield over two flushes for the uninoculated controls across all treatments ranged from 5.9 to 6.73 kg $plot^{-1}$. The average yield of each treatment harvested during of trial 1 can be found in **Table S1**.

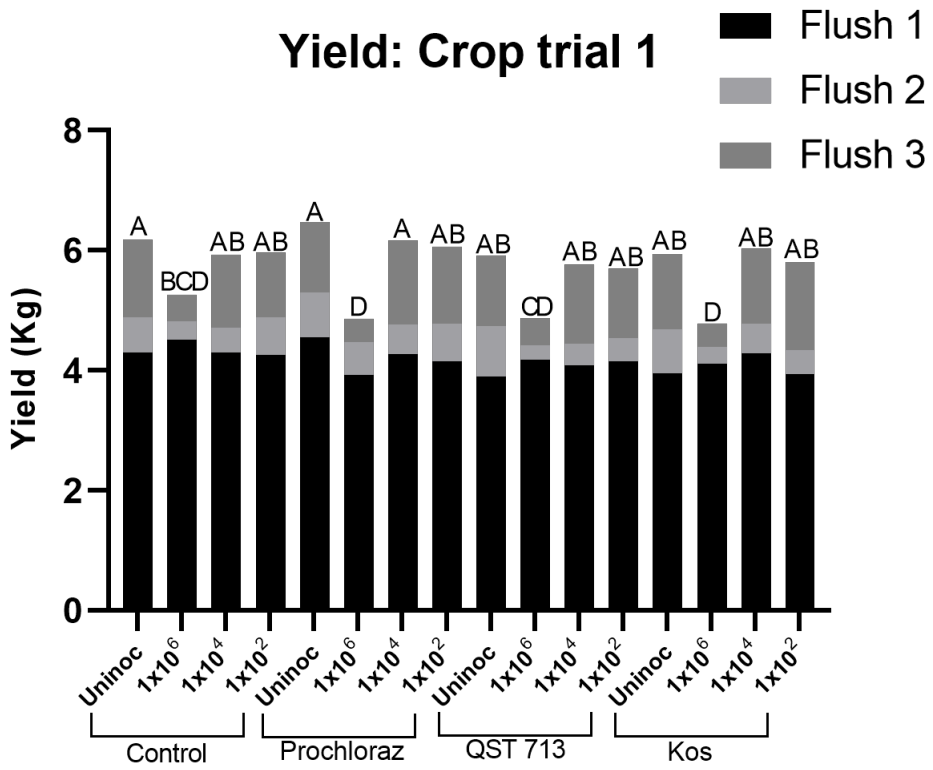


Figure 5.3 Average healthy yield of *A. bisporus* over three flushes following treatment with the fungicide prochloraz or the BCAs QST 713 or Kos, followed by inoculation with *L. fungicola* 1722 at inoculation rates of either $1x10^6$cfu/m^2, $1x10^4$cfu/m^2 or $1x10^2$cfu/m^2. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys pairwise comparisons test.

Dry bubble disease: In crop trial 1 at the end of the first flush, a small number of bubble mushrooms were present (≤ 4 bubbles/plot). These were predominantly on treatments inoculated with $1x10^6$cfu/m^2 *L. fungicola*. An occasional bubble mushroom was also detected on some $1x10^4$ cfu/m^2 inoculated plots at the end of the first flush but no bubble mushrooms were found on any $1x10^2$ cfu/m^2 inoculated or any uninoculated plots at this time (**Table S2**).

During flush 2, the number of bubble mushrooms observed on all $1x10^6$ cfu/m^2 inoculated plots had increased considerably but there was still no significant difference between the inoculated control and any of the treatments inoculated at the $1x10^6$ cfu/m^2 rate. The average number of bubble mushrooms developing ranged from 25 to 32 bubbles/plot. A few bubble mushrooms were present in both the $1x10^4$cfu/m^2 and $1x10^2$cfu/m^2 inoculated plots but their numbers were much lower (< 4 bubbles/plot)

compared to the $1x10^6$ cfu/m^2 rate (**Table S2**). Bubble mushrooms were found occasionally on uninoculated plots during flush 2, with <1 bubble/plot on average.

During flush 3, there was minimal bubble mushroom development for the entire crop. There was no significant difference between control treatments and any other treatment group at all three inoculation levels.

Over the three flushes of crop trial 1 there was significant disease development only on $1x10^6$cfu/m^2 inoculated plots. The inoculated control plots had a total average of 35 bubbles/plot at the end of the trial while the inoculated plots treated with different products had total averages of between 29 and 38 bubbles/plot. There was no significant difference in disease levels with any of the treatments at the $1x10^6$cfu/m^2 inoculation rate (**Figure 5.4**). The disease incidence on the $1x10^4$cfu/m^2 and $1x10^2$cfu/m^2 inoculated plots remained low in control plots at the end of crop trial 1. There was an average of 3 bubbles on control plots treated with $1x10^4$cfu/m^2 and no significant difference between control and treatment plots inoculated at the same rate. Control plots inoculated with $1x10^2$cfu/m^2 had an average of 5 bubbles/plot while inoculated treatment plots had averages of 2 bubbles/plot or less (**Figure S2)**. Disease development for crop trial 1 is summarised in **Table S2**.

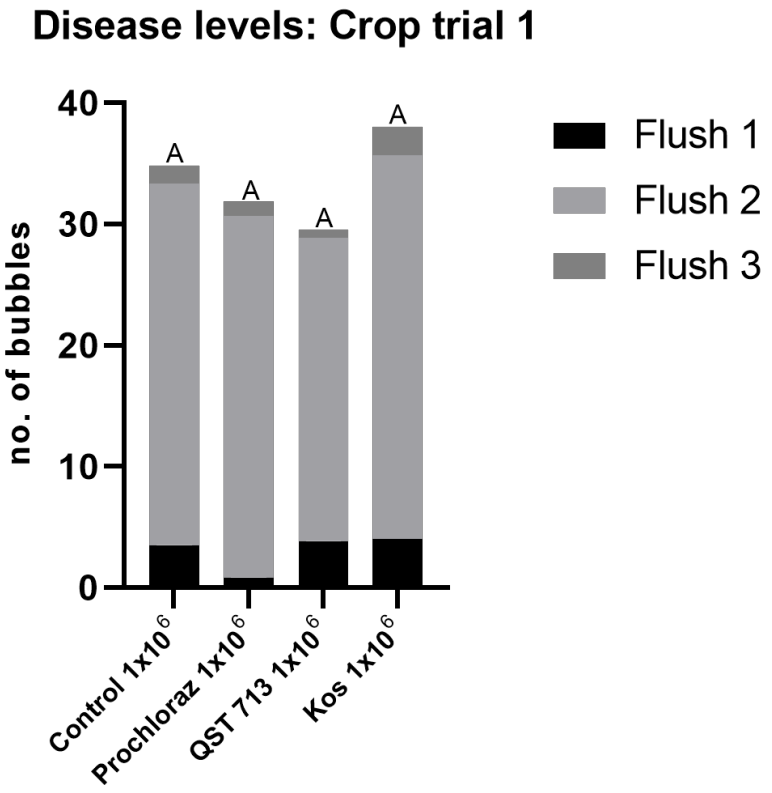


Figure 5.4 Average number of bubbles recorded at the end of crop trial 1 for plots treated with the fungicide prochloraz or the BCAs QST 713 or Kos, followed by inoculation with $1x10^6$cfu/m^2 *L. fungicola* 1722. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys pairwise comparisons test.

5.3.3 Crop trial 2: Efficacy of fungicides, BCAs and salting to control dry bubble disease at different rates of inoculation

Yield: The average yield of healthy mushrooms collected over three flushes following inoculation at rates of $1x10^4$cfu/m^2 and $1x10^2$cfu/m^2 *L. fungicola* 1722 during crop trial 2 can be seen in **Figure 5.5**. The average yield ranged from 2.4 to 2.85 kg $plot^{-1}$ for flush 1, 1.85 to 2.26 kg $plot^{-1}$ for flush 2 and 0.66 to 1.18 kg $plot^{-1}$ for flush 3. Over the course of this crop trial, there was no statistically significant difference in the yield harvested from the uninoculated control plots with any other treatment/inoculation combination used. Total yield over two flushes for the uninoculated controls across all treatments ranged from 5.56 to 5.97 kg $plot^{-1}$. The average yield of each treatment harvested at the end of trial 2 can be found in **Table S3**.

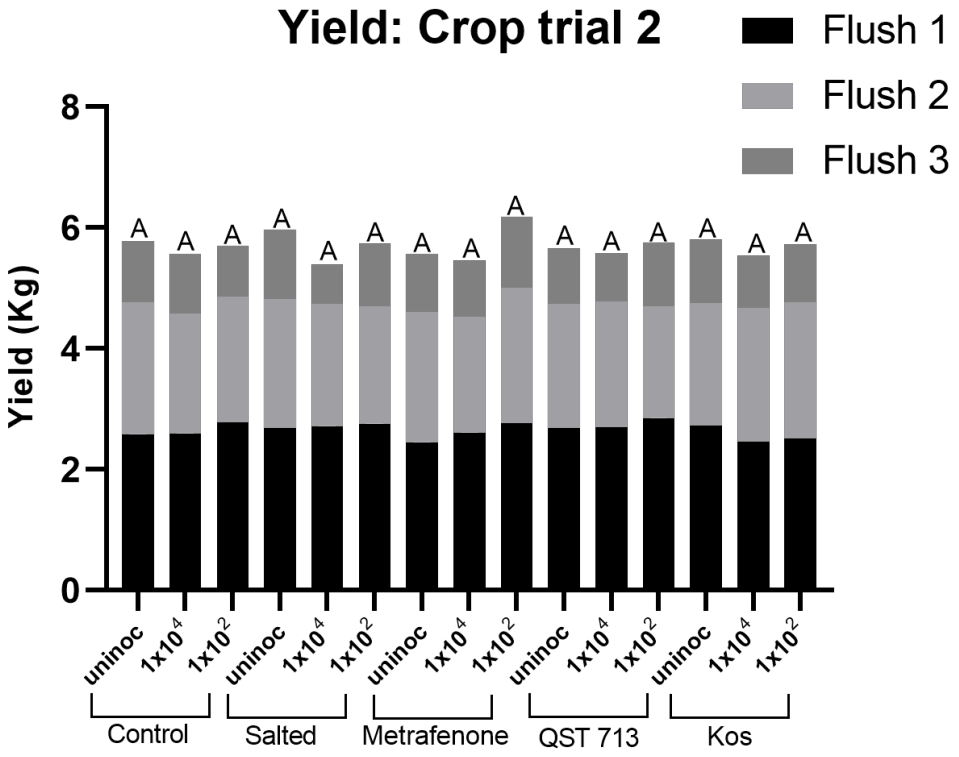


Figure 5.5 Average healthy yield of *A. bisporus* over three flushes following treatment with salt, the fungicide metrafenone or the BCAs QST 713 or Kos, followed by inoculation with *L. fungicola* 1722 at inoculation rates of either $1x10^4$cfu/m^2 or $1x10^2$cfu/m^2. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys pairwise comparisons test.

Dry bubble disease: No bubble mushrooms were recorded during the first flush of crop trial 2. For plots inoculated with $1x10^2$cfu/m^2 *L. fungicola*, very few bubble mushrooms developed and these were predominantly on the inoculated control plots in the third flush (average 1.5/plot). No bubble mushrooms were recorded for any salted, metrafenone, *B. velezensis* QST 713 or Kos treated plots inoculated at the same rate. Bubble mushrooms appeared on plots inoculated with $1x10^4$cfu/m^2 *L. fungicola* during flush 2 (**Table S4**). The highest average number of bubble mushrooms occurred on control plots inoculated with $1x10^4$cfu/m^2 *L. fungicola* (17 bubbles/plot) (**Figure 5.6**). The average numbers of bubble mushrooms on all treated plots inoculated at the same rate were significantly lower than the control ($p < 0.05$) at <5 bubbles/plot. The efficacy of the treatments ranged from 72% for salting, followed by 73% and 85% for *B. velezensis* Kos and QST 713, respectively, and 96% for metrafenone (**Table S4**).

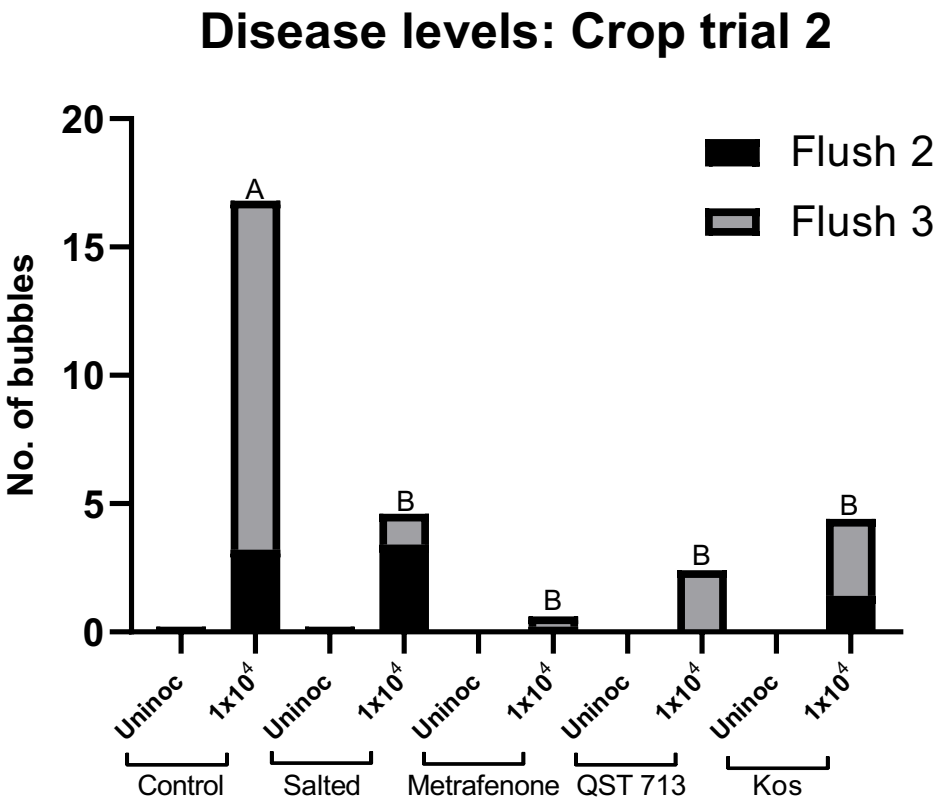


Figure 5.6 Average number of bubbles recorded at the end of crop trial 2 for plots treated with salt, the fungicide metrafenone or BCAs QST 713, Kos, followed by inoculation with $1x10^4$cfu/m^2 *L. fungicola* 1722. Data analysed by ANOVA, n = 5. Means sharing the same letter are not significantly different at P <0.05 by Tukeys pairwise comparisons test.

5.3.4 Crop trial 3: Efficacy of fungicides, BCAs and salting to control dry bubble disease

Crop trial 3 was a repeat of the key treatments in Crop trials 1 and 2 to confirm the results. The main treatments included were; Control (untreated and uninoculated) and Control inoculated at $1x10^2$cfu/m^2, $1x10^4$cfu/m^2 and $1x10^6$cfu/m^2 *L. fungicola* 1722; and the four treatments: salted, metrafenone, QST 713 and Kos, uninoculated and inoculated at 1 $x10^4$cfu/m^2 *L. fungicola* 1722 (**Table 5.2**).

Yield: This crop was not taken into a third flush due to the development of disease in uninoculated plots at the beginning of flush 3. Which was likely due to cross contamination from the extremely high number of bubble mushrooms on the $1x10^6$cfu/m^2 plots. The average yield of healthy mushrooms collected over two flushes

during crop trial 3 can be seen in **Figure 5.7**. The average yield ranged from 1.7 to 2.3 kg plot^{-1} for flush 1 and 0.85 to 2.6 kg plot^{-1} for flush 2. Over the course of this crop trial, the only plots that had a statistically significant reduction in their yield compared to the uninoculated control plots were the control plots inoculated at 1x10^{6}cfu/m^2, confirming earlier results. Total yield over two flushes for the uninoculated controls across all treatments ranged from 2.9 to 4.5 kg plot^{-1}. The average yield of each treatment harvested at the end of trial 3 can be found in **Table S5**.

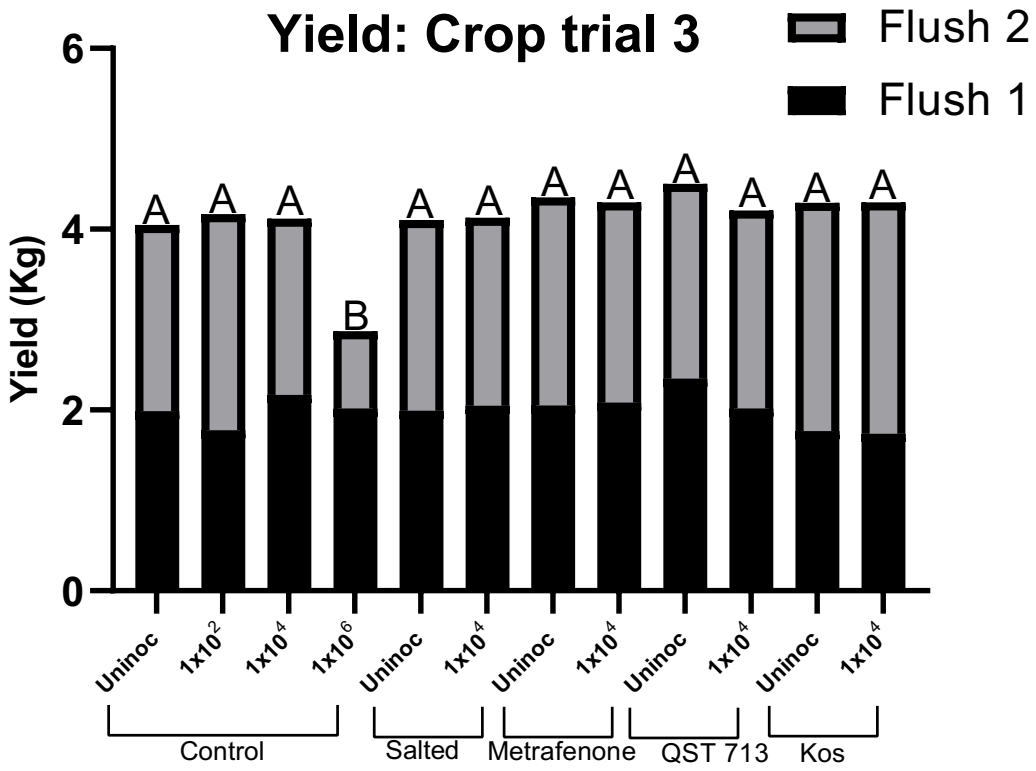
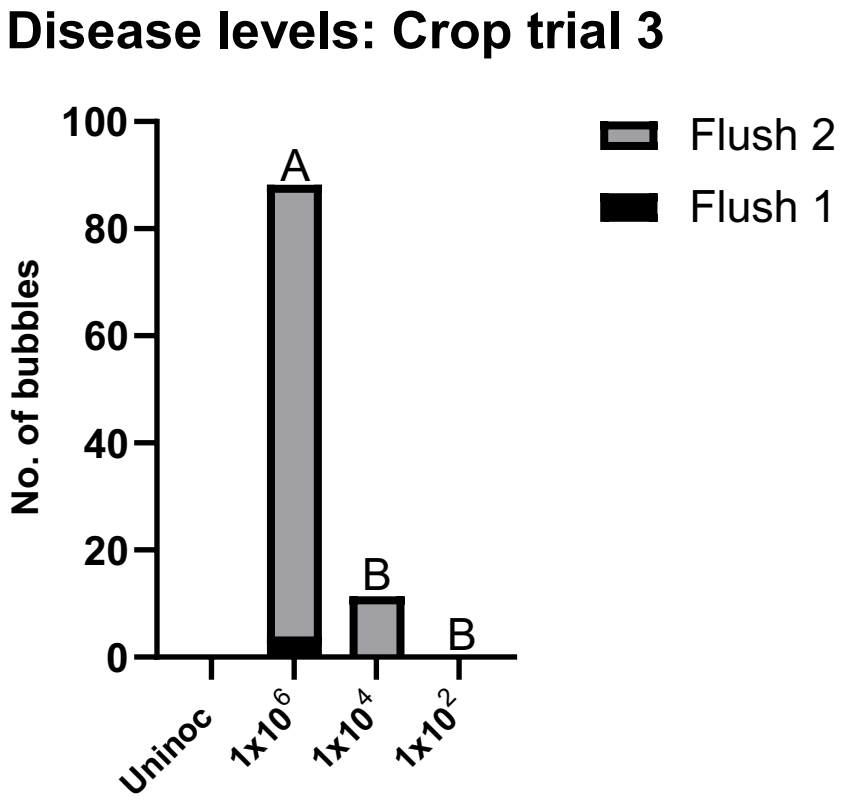


Figure 5.7 Average healthy yield of *A. bisporus* over two flushes following treatment with salting, the fungicide metrafenone, or the BCAs QST 713 or Kos, followed by inoculation with *L. fungicola* 1722 at inoculation rates of either 1x10^{6}cfu/m^2, 1x10^{4}cfu/m^2 or 1x10^{2}cfu/m^2. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys pairwise comparisons test.

Dry bubble disease: A few bubble mushrooms were present at the end of flush 1, with the majority being on the control plots inoculated at the 1x10^{6}cfu/m^2 rate. Very few bubble mushrooms were present in flush 1 on any treatment inoculated at the 1x10^{4}cfu/m^2 rate (**Table S6**). At the end of flush 2, the average number of bubbles in the control plots inoculated at the 1x10^{6}cfu/m^2 rate was 88, which was significantly

higher than disease development in either $1x10^2$cfu/m^4 or $1x10^4$cfu/m^2 inoculated plots, and which had an average of 11 and 0 bubble mushrooms respectively (**Figure 5.8 A**). This confirmed the results in crop trial 1. There were significantly more bubble mushrooms developing on control plots inoculated with $1x10^4$cfu/m^2 *L. fungicola* compared to the salted, metrafenone, *B. velezensis* QST 713 and Kos plots inoculated at the same concentration ($p < 0.05$) (**Figure 5.8 B**) and this also confirmed the results in crop trial 2. Disease development data for crop trial 3 is summarised in **Table S6**.



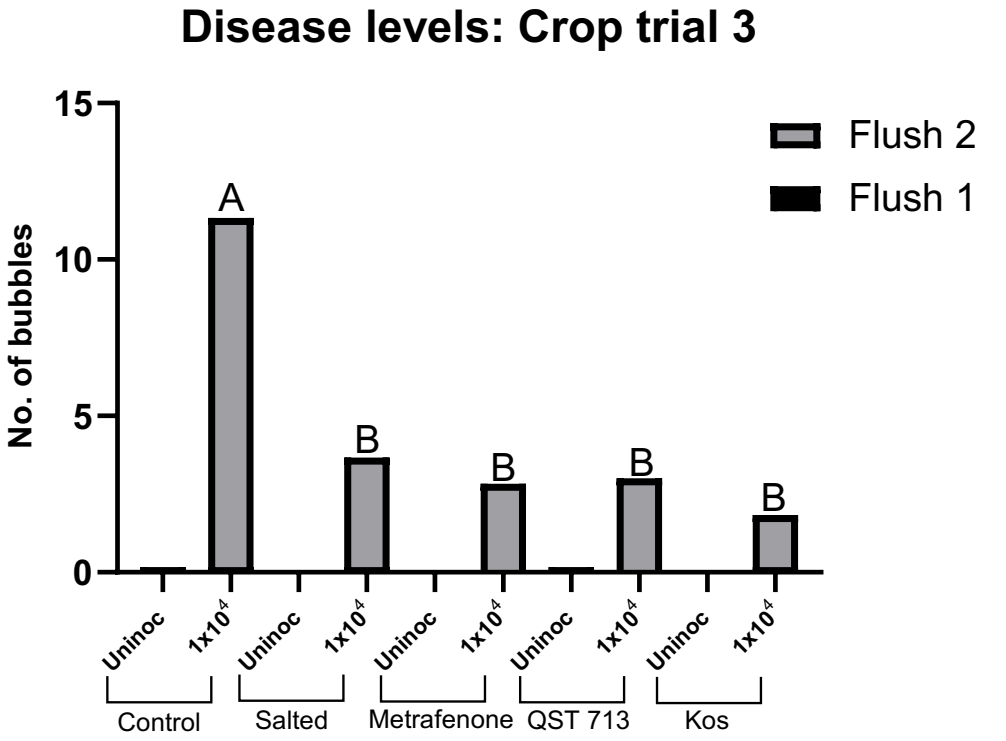


Figure 5.8 A: Average number of bubbles recorded at the end of crop trial 3 for plots inoculated with either $1x10^6$cfu/m^2, $1x10^4$cfu/m^2 or $1x10^2$cfu/m^2 *L. fungicola* 1722. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys pairwise comparisons test. **B:** Average number of bubbles recorded at the end of crop trial 3 for plots salted, treated fungicide metrafenone or the BCAs QST 713 or Kos, followed by inoculation with $1x10^4$cfu/m^2 *L. fungicola* 1722.

5.4 Discussion:

During this work, the treatment of dry bubble disease with fungicide and biocontrol treatments was investigated. *L. fungicola*, isolate 1722 and 620 were both significantly reduced when treated with the fungicides prochloraz and metrafenone *in vitro* with concentrations as low as 1 mg kg^{-1}. For both isolates, growth in the presence of 500 mg/kg metrafenone was higher than the growth recorded with lower metrafenone concentrations. This suggest that there is a threshold where inhibition of *Lecanicillium* isolates begins to decline. Previous work has shown that the culture filtrate from *B. velezensis* Kos and the biocontrol product Serenade$^{(R)}$, which contains *B. velezensis* QST 713 was also able to significantly reduce the growth of *L. fungicola*, isolate 1722 *in vitro* (Clarke *et al*., 2022b). Both isolates were brought forward to be tested with these treatments at a crop level. Only results from the 1722 isolate are discussed within this manuscript.

One of the aims of this work was to determine an inoculation rate which would reflect dry bubble disease conditions on mushroom farms. It has been seen that different inoculation levels used during *Trichoderma aggressivum* (green mould disease) crop trial experiments correlates to yield loss and disease symptom severity (O'Brien *et al.*, 2017). During this work, in both crop trial 1 and crop trial 3, inoculation with a rate of *L. fungicola* $1x10^6$cfu/m^2 in untreated control plots significantly increased bubble development compared to the uninoculated controls ($p < 0.05$). In crop trial 1, there was no significant difference between the bubble development in the untreated control plots and prochloraz*, B. velezensis* QST 713 or Kos treated plots inoculated at a rate of $1x10^6$cfu/ml. Bubble symptom also began to appear during the first flush of mushrooms. Growers generally report dry bubble disease occurring mid-crop, from about flush 2 onwards, which is supported by the results of a farm survey conducted between 2008-2010 (Piasecka *et al.*, 2011). In crop trial 3, bubble mushrooms also developed extremely quickly and at a high rate when plots were inoculated at $1x10^6$cfu/m^2. The yield of all treatments given $1x10^6$cfu/m^2 inoculation was statistically reduced compared to the uninoculated control and uninoculated treatment plots ($p < 0.05$) in both crop trial 1 and 3. These results suggested that *L. fungicola* 1722 at an experimental inoculation rate of $1x10^6$ cfu/m^2 was too high to be controlled by the fungicide, prochloraz or the biocontrol treatments examined in this work. The

results for prochloraz were surprising as normally this fungicide is generally reported as effective against dry bubble disease (Fletcher *et al.*, 1983; Gea *et al.*, 2014; Stanojević *et al.*, 2019). Similarly, the lack of development of mushroom bubbles in the third flush of the untreated inoculated control was unusual as again, the literature shows that disease usually develops rapidly once a crop is infected (Berendsen *et al.*, 2010). This is when we realised that the salting procedure, we used to minimise disease spread was actually very effective at preventing disease development. $1x10^6$ cfu/m^2 could represent extremely high disease levels that may not normally be seen on a farm with good disease monitoring and treatment practices in place. Prochloraz may have been expected to have better efficacy at this rate, but it has been suspected that this rate is too high for biocontrol treatments to suppress. Prochloraz is a popular fungicide treatment for several field crops. It can effectively inhibit pathogen growth by inhibition of the cytochrome P450-dependent 14a-demethylase but has been linked with high levels of toxicity (Vinggaard *et al.*, 2006). The effectiveness of prochloraz against dry bubble disease has been known for many decades (Van Zaayen & Van Adrichem, 1982; Fletcher *et al.*, 1983; Grogan *et al.*, 2000) and has been a popular treatment for growers to control disease. Stanojević *et al*., (2019) did find that an inoculation of $1x10^6$cfu/ml *L. fungicola* strain Sa_2V_6 isolated in Serbia, could be controlled by prochloraz. However there have been reports of reduced sensitivity of *L. fungicola* strains to this treatment (Gea *et al.*, 2005) and results of the work presented here suggest high inoculations of *L. fungicola* strain 1722 may be less sensitive to prochloraz. Regardless, the use of prochloraz on mushroom crops is no longer approved within the EU (Parliament, 2009).

The lower inoculation levels ($1x10^4$cfu/m^2 and $1x10^2$cfu/m^2) were expected to be more representative of disease pressure present on mushroom farms. There was also no significant difference in disease levels between the lower inoculation rates in control and treatment plots during crop trial 1. It was noted that bubble development was quite inconsistent between replicate plots. Extreme care was taken to salt bubbles to avoid cross contamination between plots during crop trial 1 and any bubble that did appear was salted immediately after identification. It is possible that the diligent salting of bubbles in the lower inoculated control plots was sufficient to prevent major bubble disease outbreak. It is also interesting to note that there was large bubble outbreak during flush 2, which appeared to be suppressed by flush 3, after salting was carried

out in crop trial 1. The disease levels in the untreated control plots may not have been representative of untreated disease progression, as the salt itself was acting as a type of treatment to suppress *Lecanicillium* spores from spreading. This could explain the inconsistencies in disease development on these plots.

To confirm this, we performed a second replicate trial with the two lower inoculation rates ($1x10^4$cfu/m^2 and $1x10^2$cfu/m^2). In this trial we included an unsalted control treatment as well as a separate salting treatment which was salted as in trial 1. Bubbles were left to develop without any interference during the flush in control, fungicide, and *B. velezensis* treated plots. During this second trial we found once again that disease was mostly absent from plots inoculated with 1722 $1x10^2$ cfu/m^2. This would suggest that this inoculation rate is too low for dry bubble disease to develop in an experimental setting. The scarce bubble that did develop from these plots, only appeared during the third flush, which would suggest that dry bubble in the third flush is likely to reflect low disease pressure on the farm. This was replicated in crop trial 3 as there was also no bubble development for the $1x10^2$ cfu/m^2 plots.

In crop trial 2, there was development of dry bubble disease in the plots inoculated with *L. fungicola* 1722 at a rate of $1x10^4$cfu/m^2 which was first identified on these plots during flush 2. By the end of crop trial 2, there were significantly higher bubble levels in the infected control plots compared to the salted, fungicide metrafenone and biocontrol *B. velezensis* QST 713 and Kos treated plots. This result was replicated in the third crop trial where once again, salting, metrafenone, QST 713 and Kos treatment significantly reduced bubble development on plots inoculated with *L. fungicola* at a rate of $1x10^4$cfu/m^2.

It was found that there were significantly higher levels of bubble on the control plots compared to the salted control plots in two replicate crop trials. Furthermore, bubble mushrooms which developed on $1x10^6$cfu/ml plots in crop trial 1 were salted. During crop trial 3, when no salt was applied bubble on $1x10^6$cfu/ml plots the average number of bubble mushrooms rose to 88 compared to an average of 31 in crop trial 1. These results confirm that carefully salting bubbles is effective as a treatment for bubble without any additional preventative treatment and is a useful and worthwhile technique for growers to employ on their farm.

The fungicide metrafenone preformed the best out of all treatments included in crop trial 2 and 3 with an efficacy value of 96% at the end of the three flushes showing that the only remaining fungicide for mushroom disease is effective against dry bubble. Due to the lack of any alternative fungicide, it is assumed that the development of metrafenone resistance strains will be difficult to avoid. Previous research has demonstrated how metrafenone treatment was effective for the treatment of cobweb diseases during crop trial experiments (Carrasco *et al.*, 2017). However, during recent crop trials carried out in this work, metrafenone tolerant isolates of *Cladobotryum* were identified (Clarke *et al.*, 2024).

Fortunately, biocontrol strains also performed well against dry bubble disease at this lower inoculation rate of $1x10^4$cfu/ml. *B. velezensis* QST 713 had the second highest efficacy of 85%, followed by *B. velezensis* Kos with an efficacy of 73%. Stanojević *et al*., 2019 also investigated the use of *B. velezensis* QST 713 to control dry bubble disease and found that although it did not perform as well as the prochloraz fungicide treatment, it did show a level of protection against a high inoculation rate of *L. fungicola*. We have previously shown that *B. velezensis* QST 713 and Kos can inhibit the growth of the *L. fungicola* pathogen *in vitro*. Proteomic analysis revealed that in response to the CF of the two strains, *L. fungicola* significantly reduces growth activities and increases activities involved with a stress response (Clarke *et al.*, 2022b). Several lytic enzymes, including subtilisin were also identified in the inhibitory CF fraction of *B. velezensis* Kos, which may contribute to the antagonistic potential of this strain (Clarke *et al.*, 2022a).

5.4.1 Conclusions/Final remarks

Using different inoculation levels in crop trials can allow various disease conditions to be tested. We can conclude that using a rate of $1x10^6$cfu/m^2 would represent extreme disease pressure which may be difficult to treat. An inoculation rate of $1x10^2$cfu/m^2 resulted in extremely low disease levels. It is therefore our recommendation that an inoculation rate of $1x10^4$cfu/m^2 would represent the optimum experimental inoculation rate of *L. fungicola* to represent a reasonable level of dry bubble disease conditions in an experimental setting.

Biocontrol treatments showed efficacy against *L. fungicola* infection when disease levels were low/moderate. The results from previous *in vitro* inhibition work (Clarke *et al.*, 2022b) and these large-scale crop trials, suggests that there is potential for the use of biocontrol treatments to treat dry bubble disease. Salting and early detection of symptomatic areas on mushroom beds can prevent significantly prevent disease spread when infection levels were low/moderate.

The future of mushroom disease control will need several IPM techniques working in combination. Biocontrol agents/treatments struggle to control high disease pressures, therefore, in order to maximise the effects of biocontrol treatment, it will need to be combined with other IPM techniques, such as salting, excellent hygiene, establishment of disease prevention practices and providing training for mushroom pickers to be able to identify disease symptoms early.

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5.6 Supplementary material

The following sections contain supplementary figures (5.6.1) and supplementary tables (5.6.2) which accompany Chapter 5 of this thesis.

5.6.1 Supplementary Figures

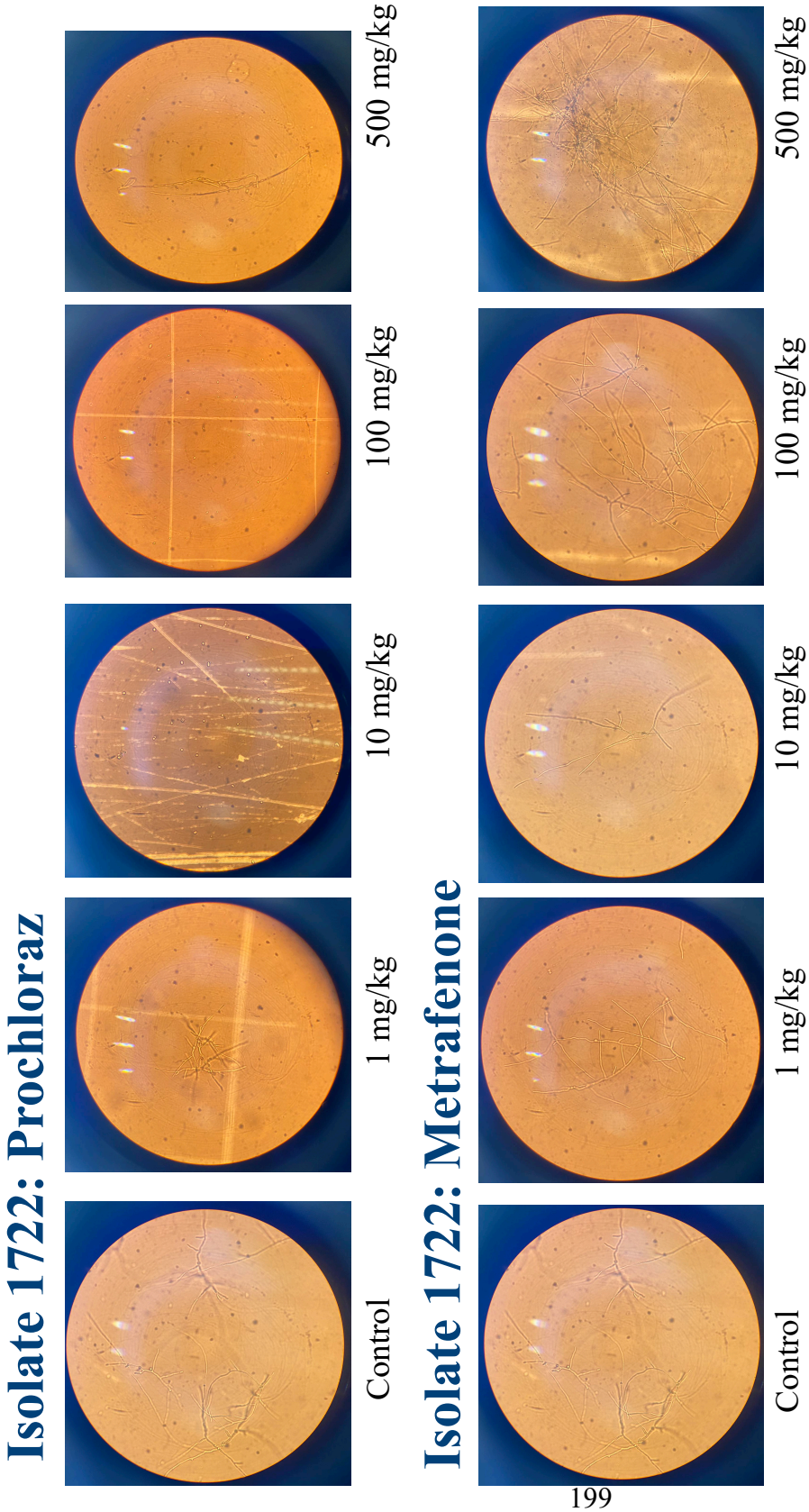

Figure S1A: Investigation of sporulation and hyphal development of *L. fungicola* 1722 when treated with fungicides metrafenone or prochloraz at 0, 1, 10, 100 and 500 mg/kg for 24 hr. Evidence of sporulation and hyphal development was monitored using an Olympus microscope (40X).

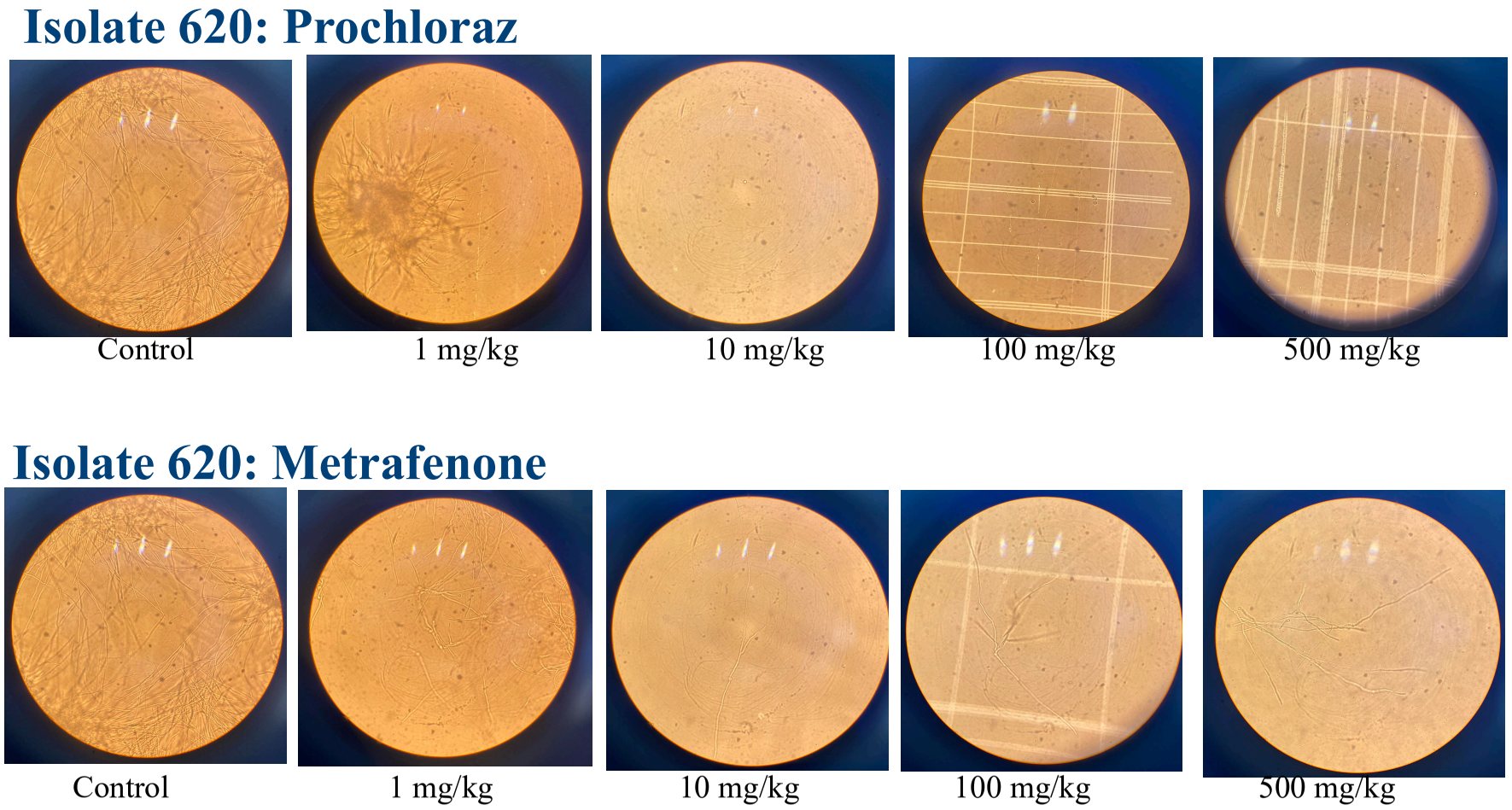

Figure S1B: Investigation of sporulation and hyphal development of *L. fungicola* 620 when treated with fungicides metrafenone or prochloraz at 0, 1, 10, 100 and 500 mg/kg for 24 hr. Evidence of sporulation and hyphal development was monitored using an Olympus microscope (40X).

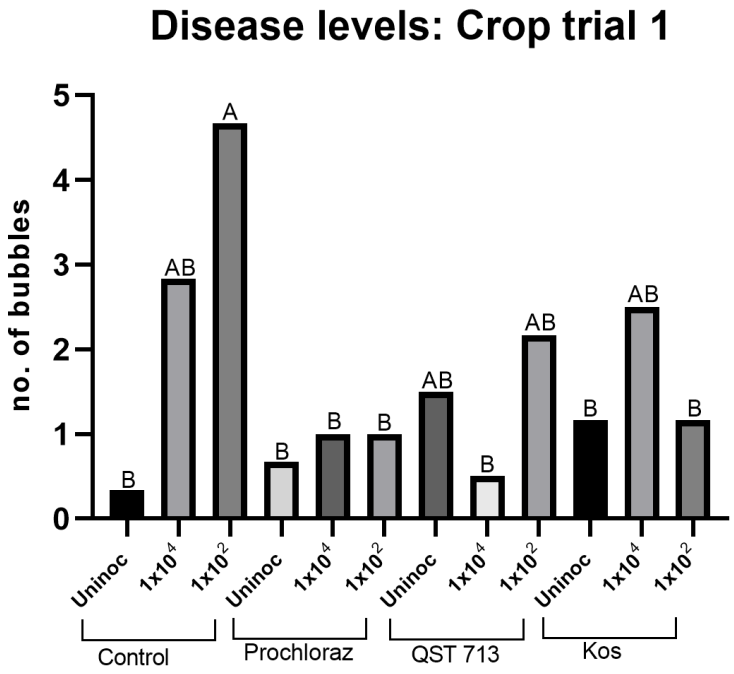

Figure S2: Average number of bubbles recorded at the end of crop trial 1 for plots treated with fungicide Prochloraz or the BCAs QST 713 or Kos, followed by inoculation with $1x10^4$cfu/m^2 and $1x10^2$cfu/m^2 *L. fungicola* 1722. Data analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at P <0.05 by Tukeys pairwise comparisons test.

5.6.2 Supplementary Tables

Table S1: Average healthy yield of healthy mushrooms, crop trial 1

Treatment	Average weight of healthy mushrooms Flush 1	Average weight of healthy mushrooms Flush 2	Average weight of healthy mushrooms Flush 3	Average weight of healthy mushrooms, End of Trial
Control uninoculated	4.30^{A}	0.59^{A}	1.30^{A}	6.15^{A}
Control $1x10^{6}cfu/m^{2}$	4.51^{A}	0.31^{A}	0.44^{C}	5.26^{BCD}
Control $1x10^{4}cfu/m^{2}$	4.3^{A}	0.41^{A}	1.22^{A}	5.93^{AB}
Control $1x10^{2}cfu/m^{2}$	4.26^{A}	0.63^{A}	1.07^{AB}	5.96^{A}
Prochloraz uninoculated	4.55^{A}	0.75^{A}	1.17^{A}	6.47^{AB}
Prochloraz $1x10^{6}cfu/m^{2}$	3.92^{A}	0.55^{A}	0.39^{C}	4.86^{D}
Prochloraz $1x10^{4}cfu/m^{2}$	4.28^{A}	0.49^{A}	1.40^{A}	6.17^{A}
Prochloraz $1x10^{2}cfu/m^{2}$	4.16^{A}	0.63^{A}	1.28^{A}	6.06^{AB}
QST 713 uninoculated	3.90^{A}	0.84^{A}	1.18^{A}	5.92^{AB}
QST 713 $1x10^{6}cfu/m^{2}$	4.19^{A}	0.24^{A}	0.46^{BC}	4.88^{CD}
QST 713 $1x10^{4}cfu/m^{2}$	4.09^{A}	0.36^{A}	1.31^{A}	5.76^{AB}
QST 713 $1x10^{2}cfu/m^{2}$	4.16^{A}	0.39^{A}	1.15^{A}	5.70^{ABC}
Kos uninoculated	3.96^{A}	0.72^{A}	1.25^{A}	5.94^{AB}
Kos $1x10^{6}cfu/m^{2}$	4.12^{A}	0.27^{A}	0.39^{C}	4.78^{D}
Kos $1x10^{4}cfu/m^{2}$	4.28^{A}	0.49^{A}	1.26^{A}	6.03^{AB}
Kos $1x10^{2}cfu/m^{2}$	3.94^{A}	0.40^{A}	1.46^{A}	5.80^{AB}

Data normally distributed and with equal variance analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukeys pairwise comparisons test.

Table S2: Disease levels, crop trial 1

$1x10^6 cfu/m^2$				
Treatment	**Average number of bubbles Flush 1**	**Average number of bubbles Flush 2**	**Average number of bubbles Flush 3**	**Total number of bubbles**
Control uninoculated	0	0.2	0.2	0.333
Control $1x10^6 cfu/m^2$	3.5	29.8^{A}	1.5^{A}	34.83^{A}
Prochloraz uninoculated	0	0.5	0.2	0.667
Prochloraz $1x10^6 cfu/m^2$	0.8	29.8^{A}	1.2^{A}	31.83^{A}
QST 713 uninoculated	0	0.7	0.8	1.5
QST 713 $1x10^6 cfu/m^2$	3.8	25^{A}	0.7^{A}	29.50^{A}
Kos uninoculated	0	0.3	0.8	1.167
Kos $1x10^6 cfu/m^2$	4	31.7^{A}	2.3^{A}	38^{A}
$1x10^4 cfu/m^2$ and $1x10^2 cfu/m^2$				
Treatment	**Average number of bubbles Flush 1**	**Average number of bubbles Flush 2**	**Average number of bubbles Flush 3**	**Total number of bubbles**
Control uninoculated	0	0.2	0.2	0.333
Control $1x10^4 cfu/m^2$	0	2.2	0.3	2.83^{AB}
Control $1x10^2 cfu/m^2$	0	3.2	1.5	4.66^{A}
Prochloraz uninoculated	0	0.5	0.2	0.667
Prochloraz $1x10^4 cfu/m^2$	0.3	0.7	0	1^{B}
Prochloraz $1x10^2 cfu/m^2$	0	0.8	0.2	1^{B}
QST 713 uninoculated	0	0.7	0.8	1.5
QST 713 $1x10^4 cfu/m^2$	0	0.5	0	0.5^{B}
QST 713 $1x10^2 cfu/m^2$	0	1.2	1	2.166^{AB}
Kos uninoculated	0	0.3	0.8	1.166
Kos $1x10^4 cfu/m^2$	0.5	1.3	1.3	2.5^{AB}
Kos $1x10^2 cfu/m^2$	0	0.7	0.5	1.166^{B}

Data normally distributed and with equal variance analysed by ANOVA, n = 6. Uninoculated plots not included in ANOVA analysis. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukeys pairwise comparisons test.

Table S3: Average healthy yield of healthy mushrooms, crop trial 2

Treatment	Average weight of healthy mushrooms Flush 1	Average weight of healthy mushrooms Flush 2	Average weight of healthy mushrooms Flush 3	Average weight of healthy mushrooms, End of trial
Control uninoculated	2.59 ^{A}	2.18 ^{A}	1.01 ^{A}	5.78 ^{A}
Control $1x10^{4}cfu/m^{2}$	2.60 ^{A}	1.98 ^{A}	1.0 ^{A}	5.57 ^{A}
Control $1x10^{2}cfu/m^{2}$	2.78 ^{A}	2.08 ^{A}	0.85 ^{A}	5.70 ^{A}
Salted uninoculated	2.68 ^{A}	2.14 ^{A}	1.15 ^{A}	5.97 ^{A}
Salted $1x10^{4}cfu/m^{2}$	2.72 ^{A}	2.02 ^{A}	0.66 ^{A}	5.40 ^{A}
Salted $1x10^{2}cfu/m^{2}$	2.75 ^{A}	1.95 ^{A}	1.04 ^{A}	5.73 ^{A}
Metrafenone uninoculated	2.45 ^{A}	2.17 ^{A}	0.95 ^{A}	5.56 ^{A}
Metrafenone $1x10^{4}cfu/m^{2}$	2.61 ^{A}	1.91 ^{A}	0.94 ^{A}	5.46 ^{A}
Metrafenone $1x10^{2}cfu/m^{2}$	2.76 ^{A}	2.24 ^{A}	1.18 ^{A}	6.18 ^{A}
QST 713 uninoculated	2.68 ^{A}	2.06 ^{A}	0.92 ^{A}	5.67 ^{A}
QST 713 $1x10^{4}cfu/m^{2}$	2.70 ^{A}	2.08 ^{A}	0.80 ^{A}	5.58 ^{A}
QST 713 $1x10^{2}cfu/m^{2}$	2.85 ^{A}	1.85 ^{A}	1.05 ^{A}	5.76 ^{A}
Kos uninoculated	2.72 ^{A}	2.03 ^{A}	1.06 ^{A}	5.81 ^{A}
Kos $1x10^{4}cfu/m^{2}$	2.46 ^{A}	2.22 ^{A}	0.86 ^{A}	5.54 ^{A}
Kos $1x10^{2}cfu/m^{2}$	2.5 ^{A}	2.26 ^{A}	0.96 ^{A}	5.72 ^{A}

Data normally distributed and with equal variance analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukeys pairwise comparisons test.

Table S4: Disease levels, crop trial 2

$1x10^4cfu/m^2$				
Treatment	**Average number of bubbles Flush 2**	**Average number of bubbles Flush 3**	**Total number of bubbles**	**Efficacy end of trial**
Control uninoculated	0	0.2	0.2	
Control $1x10^4cfu/m^2$	3.2^A	13.6^A	16.8^A	
Salted uninoculated	0	0.2	0.2	
Salted $1x10^4cfu/m^2$	3.4^A	1.2^B	4.6^B	72%
Metrafenone uninoculated	0	0	0	
Metrafenone $1x10^4cfu/m^2$	0.2^B	0.4^B	0.6^B	96%
QST 713 uninoculated	0	0	0	
QST 713 $1x10^4cfu/m^2$	0^B	2.4^B	2.4^B	85%
Kos uninoculated	0	0	0	
Kos $1x10^4cfu/m^2$	1.4^{AB}	3^B	4.4^B	73%
$1x10^2cfu/m^2$				
	Average number of bubbles Flush 2	**Average number of bubbles Flush 3**	**Total number of bubbles**	
Control uninoculated	0	0.2	0.2	
Control $1x10^2cfu/m^2$	0	1.5	1.5	
Salted uninoculated	0	0.2	0.2	
Salted $1x10^2cfu/m^2$	0	0	0	
Metrafenone uninoculated	0	0	0	
Metrafenone $1x10^2cfu/m^2$	0	0	0	
QST 713 uninoculated	0	0	0	
QST 713 $1x10^2cfu/m^2$	0	0	0	
Kos uninoculated	0	0	0	
Kos $1x10^4cfu/m^2$	0	0	0	

Data normally distributed and with equal variance analysed by ANOVA, n = 6. Uninoculated plots not included in ANOVA analysis. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukeys pairwise comparisons test.

Table S5: Healthy yield (kg), crop trial 3

Treatment	Average weight of healthy mushrooms Flush 1	Average weight of healthy mushrooms Flush 2	Average weight of healthy mushrooms, End of trial
Control uninoculated	1.99 ^{A}	2.06 ^{A}	4.05^{A}
Control $1x10^{2}cfu/m^{2}$	1.78 ^{A}	2.39 ^{A}	4.17^{A}
Control $1x10^{4}cfu/m^{2}$	2.17 ^{A}	1.95 ^{A}	4.12^{A}
Control $1x10^{6}cfu/m^{2}$	2.02 ^{A}	0.85 ^{B}	2.87^{B}
Salted uninoculated	1.99 ^{A}	2.11 ^{A}	4.10^{A}
Salted $1x10^{4}cfu/m^{2}$	2.05 ^{A}	2.08 ^{A}	4.13^{A}
Metrafenone uninoculated	2.05 ^{A}	2.30 ^{A}	4.35^{A}
Metrafenone $1x10^{4}cfu/m^{2}$	2.08 ^{A}	2.22 ^{A}	4.30^{A}
QST 713 uninoculated	2.35 ^{A}	2.16 ^{A}	4.50^{A}
QST 713 $1x10^{4}cfu/m^{2}$	2.02 ^{A}	2.19 ^{A}	4.21^{A}
Kos uninoculated	1.77 ^{A}	2.53 ^{A}	4.29^{A}
Kos $1x10^{4}cfu/m^{2}$	1.74 ^{A}	2.56 ^{A}	4.30^{A}

Data normally distributed and with equal variance analysed by ANOVA, n = 6. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukeys pairwise comparisons test.

Table S6: Disease levels, crop trial 3

Treatment	Average number of bubbles Flush 1	Average number of bubbles Flush 2	Total number of bubbles
Control uninoculated	0	0.167	0.167
Control $1x10^4$cfu/m^2	0.167	11.17^A	11.33^A
Salted uninoculated	0	0	0
Salted $1x10^4$cfu/m^2	0.167	3.5^B	3.67^B
Metrafenone uninoculated	0	0	0
Metrafenone $1x10^4$cfu/m^2	0	2.83^B	2.83^B
QST 713 uninoculated	0	0.167	0.167
QST 713 $1x10^4$cfu/m^2	0	3.0^B	3.0^B
Kos uninoculated	0	0	0
Kos $1x10^4$cfu/m^2	0.167	1.67^B	1.83^B
Control uninoculated	0	0.167	0.167
Control $1x10^6$cfu/m^2	3.83^A	84.33^A	88.2^A
Control $1x10^4$cfu/m^2	0.167^B	11.17^B	11.33^B
Control $1x10^2$cfu/m^2	0^B	0^B	0^B

Data normally distributed and with equal variance analysed by ANOVA, n = 6. Uninoculated plots not included in ANOVA analysis. Means sharing the same letter are not significantly different at $P < 0.05$ by Tukeys pairwise comparisons test.

Chapter 6

Population dynamics of mushroom casing over the course of *Agaricus bisporus* cultivation in the presence of *Bacillus velezensis* QST 713 and *Bacillus velezensis* Kos biocontrol agents.

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Author Contributions

Crop trial experimental design, set up and maintenance was performed by JC. With assistance from Brian McGuinness (Teagasc).

Crop sampling was performed by JC

DNA extractions were performed by JC

16S/ITS sequencing performed by BGI Genomics

16S/ITS sequencing analysis was performed by DF and JC

Manuscript writing was performed by JC and DF

Manuscript editing was performed by KK, HG

Abstract

The globally cultivated white button mushroom (*Agaricus bisporus*) is grown commercially at an industrial scale. Numerous pathogens pose a significant economic threat to its cultivation. Due to the emergence of resistance towards fungicide treatments, the future of mushroom disease treatment will need to move towards integrated pest management including the use of biological control agents (BCAs). In this study, we investigated the impact of the BCAs, *B. velezensis* QST 713 and Kos on the population dynamics of the microbiota of mushroom casing. Amplicon sequencing revealed that four Bacterial phyla Firmicutes, Proteobacteria, Bacteroidota and Actinobacteriota dominated at casing and the end of the crop cycle. The most dominant fungal genus detected at casing was *Agaricus* and after 7 days accounted for the vast majority of fungal species detected. The application of BCAs, *B. velezensis* QST 713 and *B. velezensis* Kos did not have a significant impact on the microbiota across the crop cycle and comparisons between control plots and plots treated with BCA showed no significant differences in their microbiome composition. This research contributes novel insights into the dynamics, composition, and structure of microbial communities within *A. bisporus* mushroom casing with and without the application of two BCAs.

Key words: White button mushroom, biocontrol agent, casing, 16S sequencing, ITS sequencing, microbiome, mushroom pathogens.

List of abbreviations:

BCA: Biological Control Agent

EC: European Commission

EU: European Union

IPM: Integrated Pest Management

SUD: Sustainable Use of Pesticides Directive

CF: Culture Filtrate

NA: Nutrient Agar

CFUs: Colony Forming Units

ASV: Amplicon Sequence Variants

PD: Phylogenetic Diversity

RH: Relative Humidity

6.1 Introduction

The white button mushroom (*Agaricus bisporus*) is one of several mushroom species which can be grown commercially at an industrial scale (Miles & Chang, 2004). Although mushrooms such as *Lentinus edodes* (shiitake), *Auricularia auricular* (wood ear), *Pleurotus* spp (oyster mushrooms) and *Flammulina filiformis* (enoki mushroom) are produced in higher quantities in the Asian market (Royse *et al*., 2017; Singh *et al*., 2020; Li & Xu, 2022). *A. bisporus* remains the most popular and cultivated species in Europe, Australia and Northern America (Royse *et al*., 2017). During 2018-2019, *A. bisporus* production accounted for 11% (4.7 million tonnes) of the global mushroom production (Singh *et al*., 2020). Cultivation requires prepared compost substrate (traditionally wheat straw, horse/poultry manure and gypsum) inoculated with spawn which contains the *A. bisporus* mycelium. The addition of a casing layer on top of the prepared substrate is required for *A. bisporus* fruitification initiation (Royse & Beelman, 2007). The casing layer is generally peat-based, 50 mm thick and provides optimum water holding capacity and the structural, physiochemical, and microbiological qualities needed for *A. bisporus* development (Pardo-Giménez *et al*., 2017; Dias *et al*., 2021). Although peat is the most widely used material for casing, alternative materials are under investigation as peat is non-renewable and not available easily in all mushroom growing countries (Dias *et al*., 2021).

The development of *Agaricus bisporus* mature fruiting bodies is a sophisticated process which is highly dependent upon the microbial community in its environment, including during substrate preparation and in the casing layer. For example, during the Phase II stage of substrate preparation, beneficial microbes are responsible for removing ammonia from the substrate, which is toxic to *Agaricus* growth and converting it to protein which can be utilised. Several bacterial species in the casing layer have also been implicated in triggering *A. bisporus* fruitification through the degradation of volatile compounds which block primordia formation, the most studied of these is *Pseudomonas putida* (Noble *et al*., 2003; Noble *et al*., 2009; Mcgee, 2018). There are many publications which review the microbial population dynamics associated with *A. bisporus* cultivation, beginning from substrate preparation right through to harvesting (Kertesz & Thai, 2018; Mcgee, 2018; Carrasco *et al*., 2019; Thai *et al*., 2022; Vieira & Pecchia, 2022). These studies detail the key species present at

each stage of mushroom cultivation and explain how microorganisms fulfil important roles to produce healthy *A. bisporus* crops. Alterations in casing population dynamics in response to fungicide application has been investigated. It was found that the application of chlorothalonil delayed *A. bisporus* colonization (Tello Martin *et al*., 2022).

Several fungal pathogens pose a huge threat to mushrooms growers. The four main fungal diseases which affect the commercial production of *A. bisporus* are cobweb disease (*Cladobotryum* spp.), dry bubble disease (*Lecanicillium fungicola*), wet bubble disease (*Mycogone perniciosa*) and green mould disease (*Trichoderma* spp.) (Fletcher & Gaze, 2008). These diseases display a range of symptoms but generally all of them directly reduce the yield of healthy mushrooms harvested from a crop which can impact revenue for growers. These diseases are controlled with very strict hygiene practices and through the preventative application of chemical fungicides (Van Zaayen & Van Adrichem, 1982; Bernardo *et al*., 2002; Grogan, 2006; Carrasco *et al*., 2017; Luković *et al*., 2020; Navarro *et al*., 2023). The severity of disease symptoms can be managed to a certain extent with early detection and treatment to prevent fungal spores spreading around growing rooms (Adie *et al*., 2006). This can be achieved with relatively simple methods such as strict use of personal protective equipment (gloves, hairnets etc), hand/foot wash stations upon entry, salting diseased areas promptly and completing steam cook-out at the end of each crop.

Historically, most growers have relied on chemical fungicides to prevent significant disease outbreaks and yield reductions. However there has been increasing pressure to reduce fungicide use due to environmental and health concerns (Grogan, 2008). Up until recently prochloraz and metrafenone were both commonly used on mushroom crops in Europe. As of June 2023, prochloraz is no longer approved for use (EC, 2021), which means that metrafenone is the only remaining fungicide approved for use on mushroom crops in Europe. Anecdotal evidence of metrafenone resistant strains has been common within the industry, and recent work has shown that *Cladobotryum* isolates are tolerant to metrafenone (Clarke *et al*., 2024). It is now accepted that the future of mushroom disease treatment will have to rely less on chemical fungicides and more on integrated pest management (IPM). An integral part of IPM strategies include the use of biological control agents (BCAs) (Barzman *et al*., 2015). BCAs are

being investigated as a potential alternative, and more sustainable treatments to use instead of chemical fungicides. Serenade(R) is a commercially available BCA product which contains *Bacillus velezensis* QST 713 as its active agent. The application of *B. velezensis* QST 713 has varied effectiveness in its ability to reduce the growth of disease-causing mushroom pathogens. Studies have highlighted the effectiveness of *B. velezensis* QST 713 for treating green mould disease (*Trichoderma aggressivum*) in France (Pandin *et al*., 2018). However, other studies have reported that *B. velezensis* QST 713 showed only limited efficacy for wet bubble disease (*Mycogone perniciosa*) (Navarro *et al*., 2023). Previous work has also shown that QST 713 is not effective against *C. mycophilum* (strain 1546) which causes cobweb disease on mushroom crops (Clarke *et al*., 2024). The novel strain, *B. velezensis* Kos has previously been shown to have potential as a BCA for mushroom disease such as cobweb disease, green mould disease and dry bubble disease (Clarke *et al*., 2022b).

The aim of this project was to characterise the population dynamics of bacterial and fungal microorganisms within the casing soil over the course of *A. bisporus* cultivation. Biocontrol species can target crop pathogens through several activities, including competition for space and nutrients as well as the production of secondary metabolites and lytic enzymes (Tyagi *et al*., 2024). We wished to determine if these activities, which result from BCA application had any impact on the non-target casing microbiota. The results presented here enrich our knowledge with respect to the evolution of microbial communities in casing in the presence and absence of the BCAs *B. velezensis* QST 713 and Kos.

6.2 Materials and Methods

6.2.1 Biological control agents

The commercially available biocontrol product Serenade® ASO (*B. velezensis* QST 713) was supplied by Bayer CropScience Ltd. and contains a minimum of 1 x 10^{12} colony forming units (CFUs) per litre. An additional *B. velezensis* strain (designated here as *B. velezensis* Kos), has previously been isolated from mushroom casing (Kosanovic *et al*., 2021) and was obtained for this work from liquid nitrogen stores at Maynooth University (Kildare, Ireland).

6.2.2 Growth trial set up

The crop trial was carried out in environmentally controlled mushroom growing rooms at the Mushroom Research Unit at Teagasc Ashtown Research Centre (Dublin, Ireland). Metal trays (external l x b x h dimensions of 0.8 m x 1.29 m x 0.2 m) with a 1 m^2 internal crop surface area were filled with 80 kg of commercially-sourced Phase III substrate, spawned with rye grains inoculated with *A. bisporus* strain Sylvan A15 (Carbury Compost Ltd., Carbury, Co. Kildare, Ireland). 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 0 of the crop cycle and then placed onto shelves in the growing room. Spawn run compost (approximately 1% w/w) was mixed through the casing layer after casing application. This treatment is known as compost at casing (CAC-ing), which speeds up casing colonisation by *A. bisporus*. Crops were managed following standard operating procedures for mushroom crops in the environmentally controlled growing rooms 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 hr 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 mushroom harvesting cycles (flushes). Three replicate crates were prepared for each treatment. Healthy mushrooms were harvested as predominantly

closed cups over three flushes but due to the purposes of this experiment, yield weights were not recorded for this trial.

6.2.3 Treatment application

Four treatments were included in this work, Control, *B. velezensis* QST 713 (cell suspension), *B. velezensis* Kos (culture filtrate (CF)) and *B. velezensis* Kos (cell suspension). The BCAs were applied to the relevant plots on days 5, 19 and 26 of the crop trial, corresponding to 12, 2 and 2 days before flush 1 (T3), flush 2 (T5) and flush 3 (T7) respectively (Table 6.1). *B. velezensis* QST 713 was applied according to the approved rates on the label, at a rate of 0.8 ml of product in 1L (Serenade® ASO) m^{-2} (= 0.8 x 10^{12} cfu m^{-2}). *B. velezensis* Kos 96 hr cell suspension (= 1 x 10^{12} cfu m^{-2}) and culture filtrate (CF) treatment was prepared fresh on the morning of treatment application. Liquid cultures of *B. velezensis* Kos were grown for 96 hr (30°C, 120 rpm). Cultures were centrifuged (1792xg) for 10 min to pellet cells. The culture filtrate was passed through Miracloth into sterile flasks (Duran) to be used for CF treatment. The pelleted cells were resuspended in ddH_2O and were applied as the cell suspension treatment. A sample of the *B. velezensis* Kos cell suspension and CF used for the treatment were plated out on nutrient agar (NA) to determine concentration. 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 each flush of mushrooms had been harvested, an additional application of the BCA treatments was applied. Water was applied to control plots.

6.2.4 Casing sample collection

Casing samples were collected at eight different timepoints over the course of the crop trial (T0-T7) and the crop stage corresponding to each timepoint is presented in **Table 6.1**. Casing samples taken after each treatment application (T2, T4, T6) were always collected two days after the treatment was applied to the crop to allow any effect to take place. On each day of sampling, 33.3g of casing soil was uniformly collected from three randomly chosen sections for each treatment plot, with a vertical sampling depth of 50 mm. The casing from the three sectors were combined in a plastic bag and homogenised by hand. This meant approximately 100 g of casing was collected per replicate per timepoint. Samples were transported the short distance to Maynooth

University and stored at -70°C within 1 hr of sampling. Three replicates of each treatment/timepoint combinations were sampled (n=3) resulting in a total of 96 samples (8 time points x 4 treatments x 3 replicates).

Table 6.1 Casing sampling timepoints

Sampling Timepoint	Day	Crop stage
T0	0	Freshly cased crates placed in growing room
T1	3	Pre-treatment applications
	5	Biocontrol application 1
T2	7	Post application 1
T3	17	Beginning of flush 1
	19	Biocontrol application 2
T4	21	Post application 2
T5	24	Beginning of flush 2
	26	Biocontrol application 3
T6	28	Post application 3
T7	31	Beginning of flush 3

6.2.5 DNA extractions and amplicon sequencing

Genomic DNA was extracted from 250 mg starting material of the casing samples using the DNeasy PowerSoil Pro Kit (QIAGEN) according to manufacturer's instructions. DNA concentration and purity values were analysed with a Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific). Library preparation and sequencing of 300bp paired-end reads was carried out in BGI-Shenzhen using the DNBSEQ-G400 sequencing platform. The bacterial community was determined by amplification and sequencing of the 16S V3/V4 region while the ITS2 region was amplified and sequenced to determine the fungal community. All raw sequences have been deposited to the NCBI under Bioproject number PRJNA1095552.

6.2.6 Microbiome sequence analyses

Demultiplexed paired FASTQ sequences were imported and analysed with QIIME2 v2023.7 (Bolyen *et al*., 2019). For the ITS2 reads, ITSexpress (Rivers *et al*., 2018) was used as a plugin in QIIME2 to trim reads. Quality control was carried out using the DADA2 pipeline (Callahan *et al*., 2016) incorporated into QIIME2. DADA2 undertakes specific quality control by merging paired-end reads and filtering for chimeras. An array of truncation lengths were trialled in DADA2 and a forward and reverse read truncation length of 270 and 180 nucleotides was selected for the 16S data as this maximised the number of ASVs per sample. No truncation of ITS reads was required. Very low abundant ASVs with total abundance below 3 were removed from both datasets. Taxonomy was assigned to the representative unique sequences for each ASV with sklearn classifiers implemented in QIIME2. Taxonomic annotation for bacteria was obtained using SILVA v138 database (Quast *et al*., 2013). Taxonomic annotation for fungi was obtained using UNITE v8.2 2020 database (Nilsson *et al*., 2019). ASVs classified as mitochondria or chloroplast were filtered out. Alpha rarefaction curves were analysed, to assess if sampling depth was enough to observe the full community diversity (Weiss *et al*., 2017). Alpha diversity was analysed using Faith's phylogenetic diversity (Faith, 1992), Pielou's species evenness (Pielou, 1966) and Shannon's index (Shannon & Weaver, 1949). Non-parametric Kruskal-Wallis comparisons were performed to determine if there is significant differences in alpha diversity (Kruskal & Wallis, 1952). Beta diversity was analysed using Bray–Curtis distance (abundance without phylogeny) (Sørensen, 1948) and unweighted UniFrac distance (presence and absence of OTUs with phylogeny) (Lozupone & Knight, 2005). QIIME2 commands for the amplicon analysis can be found in **Appendix 9.3**.

6.3 Results

6.3.1 Experimental procedure for Amplicon analysis

Casing microbial community diversity and dynamics were examined by amplifying the V3-V4 region of the 16S rRNA gene and the eukaryotic ITS2 region. In total there were eight specific timepoints (T0–T7) and each timepoint had four treatments, Control (C), QST 713 (Q), Kos cells (K) and Kos culture filtrate (CF). Each treatment at each timepoint was sequenced in triplicate resulting in 96 individual samples for the downstream 16S and ITS2 analysis respectively.

When filtering, denoising and merging the 16S sample paired end reads in QIIME2, an array of alternative read truncation lengths were performed. A forward and reverse read truncation length of 270 and 180 nucleotides was selected as this maximised the number of features per sample (**Table S1A**). After filtering, the total number of Amplicon Sequence Variants (ASVs) across all 96 16S samples was 2,272,248 (ranging from 13,926 to 39,023) and 6,571 of these were unique features. Across all 16S samples, 38 Bacterial phyla corresponding to 97 Families and 782 Genera were identified, 43.04% of ASVs were not assigned at the species level (Table S2).

The 96 ITS paired end reads were initially trimmed using ITSxpress before filtering, denoising and merging in QIIME2. After filtering the total number of ASVs across all ITS samples was 5,678,374 (ranging from 44,074 to 72,796, **Table S1B**) and 5,758 of these were unique features. In total, 8 Fungal phyla corresponding to 112 families and genera were identified, 21.3% of ASVs were not assigned at the species level (Table S2).

To ensure sampling depth was sufficient to capture the full community diversity Alpha rarefaction curves were undertaken. Both 16S and ITS rarefaction curves reached a plateau suggesting that our samples contained most of the potential community richness (**Figure S1**). For each of the 9 individual datasets analysed (controls through crop cycle and comparison of treatments at timepoints T0-T7), within sample diversity (alpha diversity) was examined using Pielou's evenness, Faiths' phylogenetic diversity (PD) and Shannon's index. Between sample diversity (beta diversity) was examined using Bray-Curtis and unweighted UniFranc tests (**Table 6.2, Figure S2&3**). ANCOM

tests were undertaken to compare the microbiome composition within bacterial populations across samples.

Table 6.2 (A) 16S Diversity statistics

			Alpha Diversity			Beta Diversity	
Description	**Timepoint**	**Day**	**Pielou's eveness**	**Faith's PD**	**Shannon's index**	**Bray curtis**	**Unifrac**
Casing application	**T0**	**0**	0.11	0.715	0.53	0.277	0.177
Pre-treatment application	**T1**	**3**	0.727	0.376	0.932	0.088	0.28
Post treatment 1	**T2**	**7**	0.281	0.826	0.679	0.369	0.913
Beginning of flush 1	**T3**	**17**	0.588	0.764	0.862	0.036*	0.165
Post treatment 2 after first flush harvest	**T4**	**21**	0.407	0.496	0.875	0.863	0.181
Beginning of flush 2	**T5**	**24**	0.91	0.086	0.715	0.193	0.115
Post treatment 3 after second flush harvest	**T6**	**28**	0.312	0.264	0.287	0.028*	0.017*
Beginning of flush 3	**T7**	**32**	0.727	0.168	0.862	0.142	0.204
Controls (T0-T7)	**Controls**	**0-32**	0.182	0.194	0.48	0.001	0.001

Table 6.2: (B) ITS Diversity statistics

			Alpha Diversity			Beta Diversity	
Description	**Timepoint**	**Day**	**Pielou's eveness**	**Faith's PD**	**Shannon's index**	**Bray curtis**	**Unifrac**
Casing application	**T0**	**0**	0.339	0.477	0.339	0.051	0.033
Pre-treatment application	**T1**	**3**	0.988	0.064	0.994	0.015	0.036
Post treatment 1	**T2**	**7**	0.432	0.407	0.556	0.061	0.159
Beginning of flush 1	**T3**	**17**	0.148	0.13	0.1628	0.007	0.034
Post treatment 2 after first flush harvest	**T4**	**21**	0.319	0.681	0.674	0.032	0.734
Beginning of flush 2	**T5**	**24**	0.311	0.147	0.258	0.046	0.299
Post treatment 3 after second flush harvest	**T6**	**28**	nan	0.0412	0.0597	0.011	0.01
Beginning of flush 3	**T7**	**32**	0.667	0.776	0.667	0.002	0.308
Controls (T0-T7)	**Controls**	**0-32**	0.0894	0.006	0.01	0.001	0.001

6.3.2 Microbiome dynamics of casing controls samples through the crop cycle

In order to examine change in community structure along the crop cycle, casing samples were taken in triplicate from control plots at eight different time points (T0-T7). These control plots have not been treated with a biocontrol agent. The relative abundance of Bacterial phyla for these control plots at the different time points were visualised (**Figure 6.1**). For clarity, relative abundance is a quantitative measure and corresponds to the total number (abundance) of ASVs of a particular kind which is present in a sample, relative to the total number of ASVs in that sample.

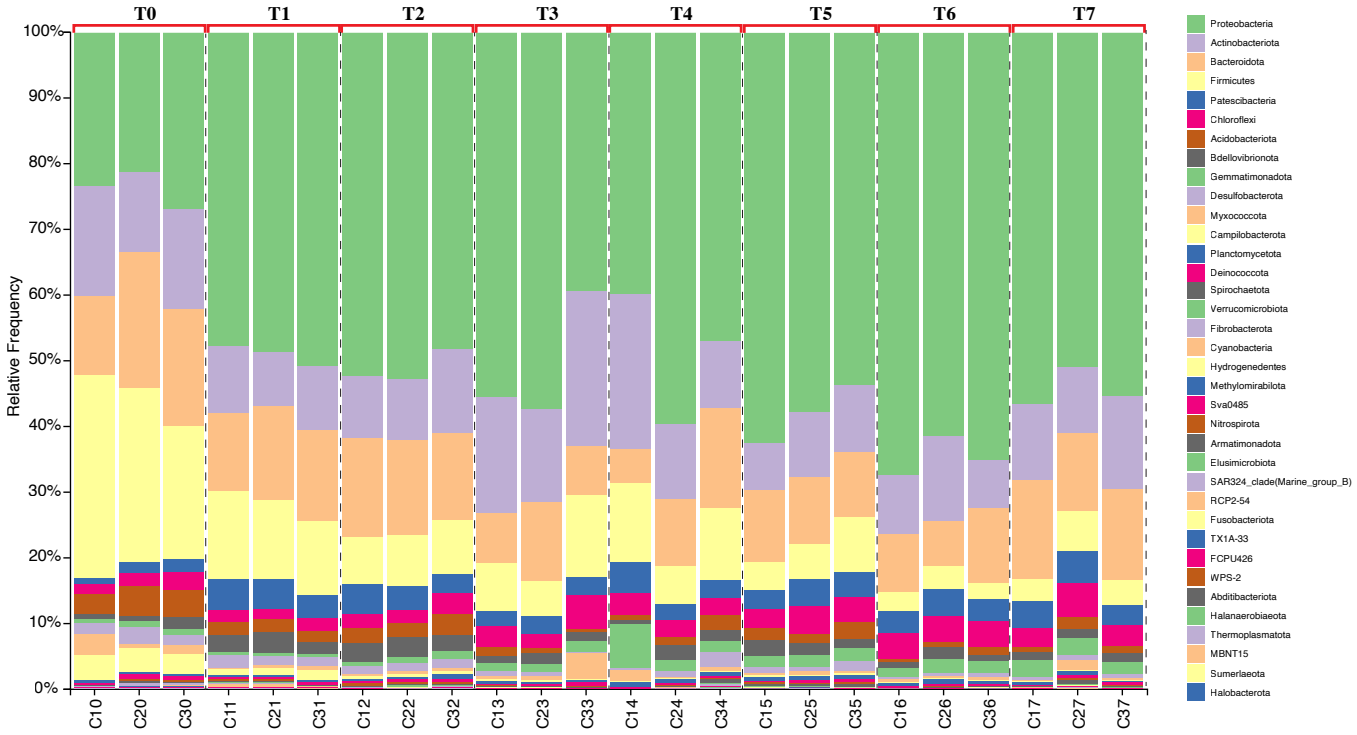

Figure 6.1 Phylum level relative abundance of Bacterial ASVs found in control samples (not treated with BCA) of mushroom casing across the crop cycle. T0: casing application, T1: pre-treatment, T2: post treatment 1, T3: Beginning Flush 1, T4: Post treatment 2, T5: Beginning Flush 2, T6: Day Post treatment 3 T7: Beginning Flush 3.

At casing (T0), four Bacterial phyla (Firmicutes (25.93% +/- 5.38 SD), Proteobacteria (24%, +/-2.86 SD), Bacteroidota (16.88% +/- 4.40 SD) and Actinobacteriota (14.62% +/-2.34 SD)) dominated accounting for ~81% of all ASVs identified, increasing to ~84% by T7 (**Figure 6.1, TableS2**). Over the crop cycle, Proteobacteria became the dominant phylum accounting for 54.41% (+/-2.93 SD) of all ASVs by T7, while

Firmicutes decreased to 4.37% (+/- 1.48 SD). The ANCOM test that compares the composition of the microbiome between samples only identified a single phylum, Campilobacterota as having a significantly different abundance profile over the course of the trial decreasing from a high of 3.41% (+/- 0.40 SD) to 0.08% (+/- 0.03 SD) at T7. At the genus level, *Trichococcus* was most dominant at T0 (9.22% +/- 3.08) but decreased over the crop cycle to 0.53% (+/-0.22) of all ASVs at T7. Similarly, species belonging to the *Thermobifida* genus, were found to be the second most abundant genus in casing at T0 (4.50%, +/-1.83 SD) but decreased in abundance to 0.30% (+/- 0.09 SD) by the time the crop cycle had reached T7 (**Table S2**). Conversely the *Devosia* and *Comamonadaceae* genera became more dominant along the crop cycle increasing their relative abundances at T0 from 0.35% and 0.70% (+/- 0.32 SD & 0.52 SD) to 6.84% and 5.24% (+/-3.20 SD & +/-0.90 SD) respectively at T7. The ANCOM test highlighted the compositional change in the *Devosia* genus as being significant along with changes to the *Lactobacillus* and *Candidatus Kaiserbacteria* genera. These differences correlate with statistically significant beta diversity measures (between sample differences) for Bray Curtis ($p = 0.001$) and unweighted uniFrac ($p = 0.001$) measures (**Table 6.2A**).

With respect to the Fungal community present in our casing samples, the Basidiomycota 84.09% (+/-18.73 SD) and Ascomycota 14.63% (+/- 17.10 SD) phyla dominate at T0 (**Figure S4, Table S2**). The most dominant genus detected was *Agaricus* (78.18% +/-24.62 SD) which may have been present at this timepoint due to the application of colonized compost to the casing layer during CAC-ing. By T2 the Basidiomycota phylum and *Agaricus* genus accounts for the majority of detected ASVs, 99.85% (+/-0.16 SD) and 99.81% (+/- 0.16 SD) respectively. A trend that is maintained across the crop cycle. The ANCOM test highlighted the compositional change in the Basidiomycota phylum as being significant. This observation correlates with the statistically significant different beta diversity measures, Bray Curtis $p = 0.001$ and unweighted uniFrac $p = 0.001$ (**Table 6.2B**). Alpha diversity measures also highlight significant within sample differences (Faith's phylogenetic distance $p=0.006$ & Shannon's index $p=0.01$) which is unsurprising due to the fact the *Agaricus* genus accounts for the vast majority of ASVs observed after T1.

6.3.3 The effect of biocontrol agents on the casing microbiome through the crop cycle

To examine possible changes in the casing microbiome before and after the application of biocontrol agents, casing samples were taken in triplicate from control (see above) and treated plots at eight different time points (T0-T7). Treated plots have had a biocontrol agent (QST 713 (Q), Kos cells (K) or Kos culture filtrate (CF)) applied.

The relative abundances of Bacterial ASVs at the phylum level for each time point were plotted (**Figure 6.2 & Figure S5**). At T0, four Bacterial phyla, Firmicutes (which includes *Bacillus* species), Proteobacteria, Bacteroidota and Actinobacteriota dominated in all treatments. Over the crop cycle, Proteobacteria became the dominant phylum (**Figure 6.2**). Similarly the relative proportion of Firmicutes species decreased over the crop cycle. This trend was observed for all samples regardless of treatment (**Figure 6.2 & Figure S5**). Alpha and beta diversity measures were undertaken for the eight time points and no significant differences were observed for the three alpha diversity measures indicating no differences in species richness or evenness between treatment plots (**Table 6.2A**). Beta diversity measures indicated no significant differences between treatments at T0, T1, T2, T4, T5 or T7. At T3 the Bray Curtis measure indicated a significant difference between all four samples ($p=0.036$) however inspection of sample pairwise comparisons did not reveal any significant differences between individual groups. Furthermore the ANCOM test did not highlight any compositional change at the phylum or genus level. At T6 both Bray Curtis and unweighted UniFrac measures indicated a significant difference between samples ($p=0.028$ & $p=0.017$) however inspection of sample pairwise comparisons did not identify any significant differences between individual groups (**Table 6.2A**). The ANCOM test did not highlight any compositional change at the phylum level but does highlight a difference in the abundance of the genus *Alcaligenes* in the plots treated with Kos-cells (**Table S2**, 3.43%, +/-0.02 SD). Interestingly, the *Alcaligenes* genus is only detected in Kos culture filtrate and Kos cell treatments from T2 onwards. Its relative abundance also peaks at T2 and T6 which corresponds to post treatment time points.

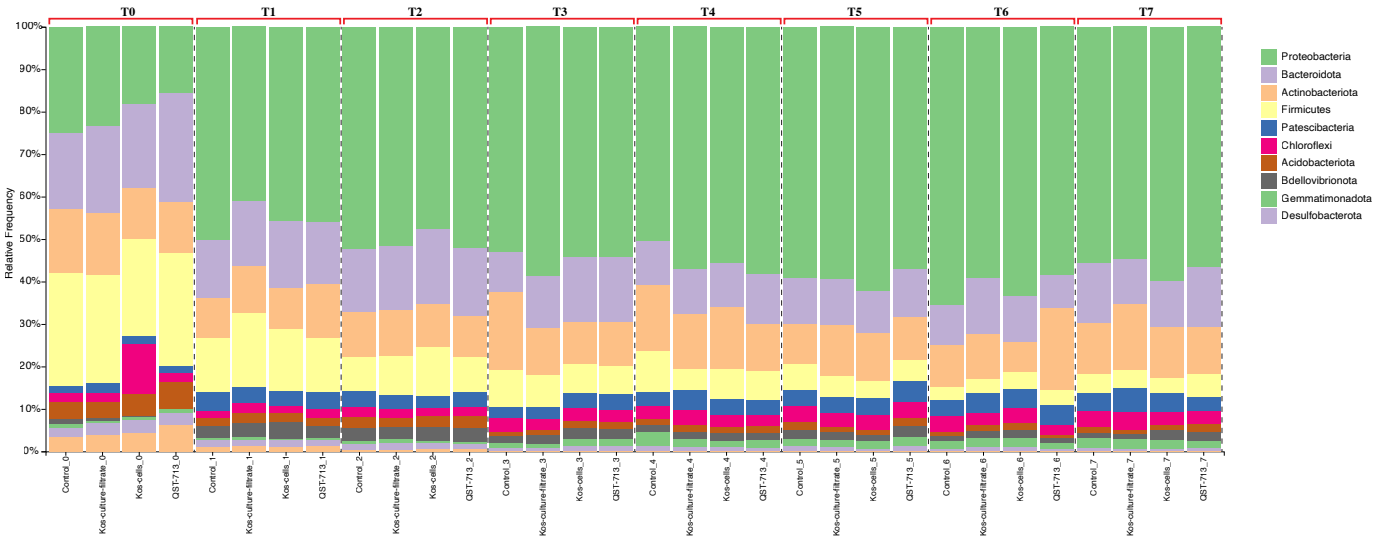

Figure 6.2 Phylum level relative abundance of Bacterial ASVs found in casing across the crop cycle. To help visualization, biological replicates (3) have been merged for each time point and only the top 10 Phyla are listed in the Phylum legend. For each time point, control samples as well as the three BCAs, Kos Culture Filtrate, Kos cells and QST 713 cells (Serenade) are shown. T0:Day 0, T1:Day 3, T2:Day 7, T3:Day 17, T4:Day 21, T5:Day 24, T6:Day 28, T7:Day 31

Next we investigated the fungal community present in treated and untreated casing samples, throughout the crop cycle. The Basidiomycota and Ascomycota phyla dominate at T0 (**Figure S6, Table S2**) with the most dominant genus being the *Agaricus*. By T2, regardless of treatment the Basidiomycota phylum and *Agaricus* genus accounts for the vast majority of detected ASVs. A trend that is maintained across the crop cycle. Beta diversity measures indicate between sample diversity at different time points but low number of ASVs in transient species most likely account for this (**Table 6.2B**). For example in T0 & T1, the ANCOM test highlight the compositional change of the *Nadsonia* genus as being significant. At T0 members of the *Nadsonia* are only detected in the control samples while at T1 they are only detected in K and CF treated plots (**Table S2**).

6.3.4 Persistence and Bioaccumulation of Biocontrol agents

Two of the treatments involved the addition of biocontrol agents, namely *B. velezensis* (either QST 713 (Q) or Kos (K)). To determine if the application of biocontrol agents led to a change in the *Bacillus* population we investigated the relative abundance of the Firmicutes phylum and *Bacillus* genus through the crop cycle in the different treatment plots (**Figure 6.3A & Table S3**). Timepoints T2, T4 and T6 occurred 2 days after the application of the biocontrol agents. The relative abundance of the Firmicutes population at these time points is similar amongst all treatments. For example at T2 there is no significant difference between the average relative abundance of C plots (7.72% +/-0.62 SD) and the plots treated with either Q (11.51% +/-3.67 SD) or K (8.23% +/-0.49 SD). At T4, the average relative abundance of Firmicutes species in the C plots (9.54% +/-3.29 SD) is actually greater than that observed in the Q (6.93% +/-2.40 SD) and K plots (6.62% +/-2.04 SD). The relative abundance of species belonging to the *Bacillus* genus was also investigated. The average relative abundance of Bacilli was <1% in all treatment plots (except for CF at T0, (**Figure 6.3B**). Again, no significant differences in relative abundances were observed between treatments at any time point. For example at T2 the average relative abundance of C plots (0.430% +/-0.05 SD) and the plots treated with either Q (0.52% +/-0.34 SD) or K (0.29% +/-0.08 SD) are very similar (**Figure 6.3B & Table S3**). Unsurprisingly the ANCOM test did not highlight either the Firmicutes phylum or the *Bacillus* genus as having a significantly different abundance profile between treatments at individual timepoints.

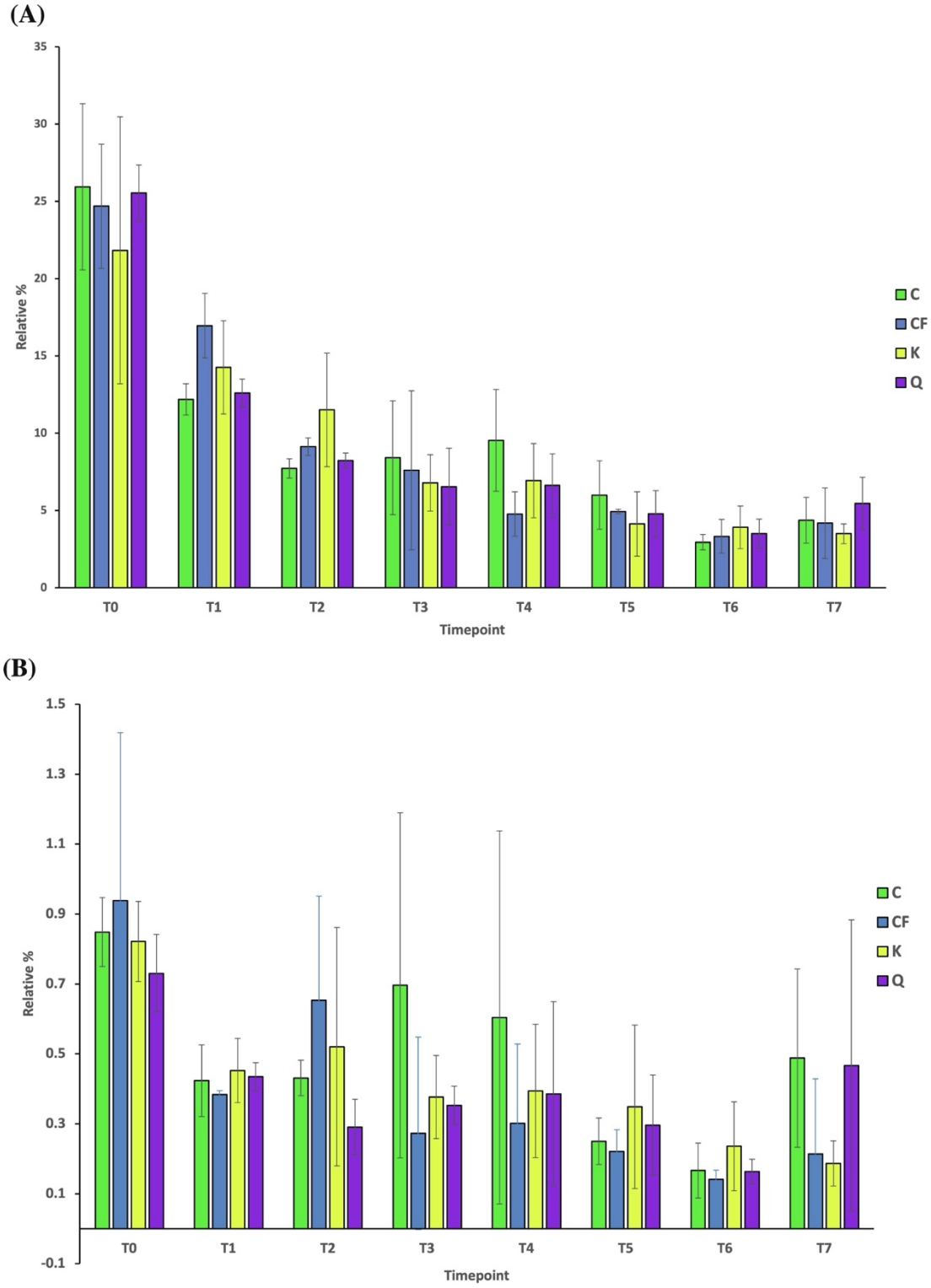

Figure 6.3 Relative abundance of the Firmicutes phylum (A) and *Bacillus* genus (B) through the crop cycle in the different treatment plots. C = control, CF = Kos culture filtrate, K = Kos cells, Q = QST 713. Timepoints T2,T4 and T6 occurred 3 days after the application of the biocontrol agents. There is no significant difference in the abundance of either the Firmicutes phylum or *Bacillus* genus at any timepoint regardless of treatment.

6.4 Discussion

Biocontrol agents represent an environmentally friendly alternative for the treatment of mycopathogens on mushroom farms (Preston *et al*., 2019). In this work, we wished to investigated if the application of BCAs including Serenade ® (*B. velezensis* QST 713), *B. velezensis* Kos or *B. velezensis* Kos culture filtrate had any impact on the natural population dynamics of the mushroom casing. The application of *B. velezensis* QST 713 and *B. velezensis* Kos are analogous as they both involve the direct application of microbial cells and are therefore the primary comparison in this work. However, we have previously shown that *Bacillus velezensis* culture filtrate inhibits *Cladobotryum mycophilum* biomass accumulation, most likely due to the activities of lytic enzymes (Clarke *et al*., 2022a). Therefore we examined the impact of this application also.

In control crops at casing, four Bacterial phyla dominated and accounted for the majority of all ASVs identified. The dominance of the four phyla in question, the Firmicutes, Proteobacteria, Bacteroidota and Actinobacteria is in broad agreement with previous studies (Siyoum *et al*., 2016; Tello Martin *et al*., 2022). Previous research has also reported that over the crop cycle, the relative proportion of Proteobacteria increased while the relative proportion of the Firmicutes decreased (Pecchia *et al*., 2014; Tello Martin *et al*., 2022), and our control crops displayed a similar change in phylum dynamics (**Figure 6.1**). At the genus level, the *Trichococcus* genus dominated at casing in our control crops and decreased in abundance as the crop cycle goes on. This observation is congruent with previous research that has shown *Trichococcus collinsii* is a cultivatable bacterial isolate throughout the mushroom cropping cycle that decreases in relative abundance over the course of the crop cycle (Siyoum *et al*., 2016). Species belonging to the *Thermobifida* genus (thermophilic Actinobacteria), were found to be the second most abundant genus in casing at T0 but decreased in abundance during the crop cycle (**Table S2**). *Thermobifida* species are important in multiple composting systems (Lin & Stutzenberger, 1995; Goodfellow *et al*., 2005; Vajna *et al*., 2012) and have previously been found among the most abundant of thermophilic Actinobacteria species in mushroom compost substrate (Song *et al*., 2021; Thai *et al*., 2022). It is likely that this species originated from compost applied during CAC-ing treatment at the beginning of the crop trial. The decrease in

Thermobifida species could coincide with the lower temperatures used as the crop progressed. Members of the *Thermobifida* genus are known for their cellulose degrading enzymes and species such as *Thermobifida cellulolytica* can completely degrade cellulose (Kukolya *et al*., 2002). A reduction in the abundance of these cellulose degrading members in tandem with nutrient depletion in mushroom compost throughout the crop cycle could be correlated with a reduction in mushroom yields which is frequently observed after the second flush. A previous investigation of microbiome dynamics in mushroom casing found that the *Flavobacterium* genus belonging to the Bacteroidetes phylum and the genus *Devosia* (Proteobacteria phylum) are the most abundant at casing (Carrasco *et al*., 2020), our results are also in agreement with this finding (**Table S2**). Conversely, numerous studies have reported an increase in the abundance of species belonging to the *Pseudomonas* genus along the crop cycle (Carrasco *et al*., 2019; Tello Martin *et al*., 2022), while another showed a peak after the first flush of mushrooms (McGee *et al*., 2017), *Pseudomonas* species are believed to play an important role in mushroom development during the crop cycle, specifically metabolising volatile compounds that may be inhibitors of primordia formation (Noble *et al*., 2009; Zhang *et al*., 2016). However, our results revealed a relatively stable abundance for *Pseudomonas* species (**Table S2**). With respect to the Fungal community in the control casing samples, at T0, members of the Basidiomycota and Ascomycota phyla dominated. Other studies have shown that Ascomycete species dominate initially but are superseded by Basidiomycetes throughout the crop cycle (Gandy & Spencer, 1978; Carrasco *et al*., 2020; Tello Martin *et al*., 2022). While we did observe a decrease in Ascomycetes throughout the crop cycle they were never observed as the dominant phylum (even at T0). The main genera of Ascomycota fungi observed at T0 were *Nadsonia* and *Candida* respectively but their relative abundance was negligible and as the crop cycle progressed the *Agaricus* genus was found to account for the vast majority of species (>91-99%). Therefore in our control casing samples, we detected very low levels of fungal diversity due to the fact that *A. bisporus* quickly replaces the native mycota in casing after spawn inoculation. Furthermore, previous studies have detected low levels of mycoparasites such as *Lecanicillium fungicola* in casing, even when the crop is healthy (Carrasco *et al*., 2019). Our analyses did not detect the presence of *L. fungicola* or other common mycoparasites such as *Mycogone perniciosa.*

The microbiome dynamics of plots treated with one of three biocontrol agents were determined. As with the control plots above, the four most abundant phyla observed at T0 in the casing were the Firmicutes, Proteobacteria, Bacteroidota and Actinobacteria and as the crop cycle progressed Proteobacteria became the dominant phylum regardless of treatment (**Figure 6.2**). Based on alpha and beta diversity analyses there were no significant differences between control and BCA treated plots at specific time points. Therefore, the addition of biological treatments did not significantly impact the composition of bacterial or fungal communities present in the casing layer during *A. bisporus* cultivation. There are limited studies which investigate the impact of biocontrol products on casing soil but a previous study did compare *B. velezensis* QST 713 treated compost to untreated control compost. The authors also found no significant differences in microbial populations during the fruitification stages meaning the biocontrol treatment did not impact compost microbiota either (Pandin *et al*., 2018).

From numerous crop trial studies, we know that the application of biocontrol treatments can be effective at reducing disease levels (Pandin *et al*., 2019; Navarro *et al*., 2023; Clarke *et al*., 2024). Biocontrol strains may employ several strategies to reduce pathogen growth and therefore the exact mode of action is not certain. For example *B. velezensis* QST 713 has been shown to express genes involved in biofilm formation and antimicrobial compound production (Pandin *et al*., 2019). It has previously been shown that *B. velezensis* Kos CF contains a number of lytic enzymes including subtilisin (Clarke *et al*., 2022a). These strains are known to reduce the impact of mycopathogens but their persistence in casing had never been previously investigated. It is interesting to note that none of our ASVs were classified as originating from *B. velezensis* strains, similarly ~43% of our ASVs could not be classified at the species level indicating a lack of phylogenetic resolution for our amplicon reads. Therefore to investigate if the BCA persisted after application we measured the relative abundances at the Phylum (Firmicutes) and genus level (*Bacillus*) throughout the crop cycle (**Figure 6.3**). Even at time points close to BCA application (T2, T4 & T6) we did not observe a significant increase in either abundance indicating BCA persistence is short lived and does not impact the relevant abundance at the Phylum or Genus level in agreement with our alpha and beta diversity measures (**Table 6.2**). Therefore while previous work has shown that BCA application has a

positive impact in fighting disease, our results show that these species are not persisting in the casing layer, indicating that the biopesticides are most likely compounds or enzymes produced by the microbes, rather than the microbial BCA itself.

6.4.1 Conclusion

The results presented here are in broad agreement with other studies that have investigated the microbial population dynamics within mushroom casing throughout the crop cycle (Siyoum *et al*., 2016; Yang *et al*., 2019; Tello Martin *et al*., 2022). Furthermore, the application of two *B. velezensis* BCAs with demonstrable mushroom pathogen supressing qualities do not alter the natural microbiota of the mushroom casing. We also did not see any evidence for bioaccumulation of the BCAs at the end of the crop cycle or in periods soon after their application. Research by others has also shown that biocontrol agents have no effect or minor and transient effects on the soil microflora and microfauna (Pandin *et al*., 2018). This is because many BCAs are highly specific against pathogens and their persistence is limited.

We have shown in previous work that these BCAs are extremely effective at significantly inhibiting mushroom pathogen growth *in vitro* (Clarke *et al*., 2022a; Clarke *et al*., 2022b). When tested in disease crop trials, the BCAs did show efficacy against the pathogens, but they did not perform at the same level seen in the *in vitro* studies (Clarke *et al*., 2024). Navarro *et al*., 2023 also found that the BCA's used in crop trial experiments had limited efficacy. The *in vitro* studies do not mimic the very complex microbial interactions which occur within the casing and compost. Perhaps the intense competition with other microorganism could explain why we do not see the BCAs persisting in the casing. This may be a factor which limits the BCAs ability to exert the antagonistic affects. *B. velezensis* Kos was originally isolated from a mushroom crop (Kosanovic *et al*., 2021), yet it did not proliferate when applied back to the casing during this work. This highlights how tightly controlled and regulated the population dynamics within the casing layer can be. Finding BCA strains which can compete in the casing may result in BCA treatments with higher efficacy. However, you would have to ensure that the BCA strain did not impact or alter the casing population dynamics to a point where *A. bisporus* cultivation was compromised. The use of BCAs can improve an agronomic strategy and they are a sustainable tool in the

reduction of casing pathogens and can improve casing and mushroom health without altering the natural microbiota of the mushroom casing. However, additional research into the interaction of the BCAs with the pathogens, but also the microbial casing populations is required to enhance our understanding of BCAs and how they might contribute to effective disease control in the future.

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6.6 Supplementary material

The following sections contain supplementary figures (6.6.1) and supplementary tables (6.6.2) which accompany Chapter 6 of this thesis.

6.6.1 Supplementary Figures

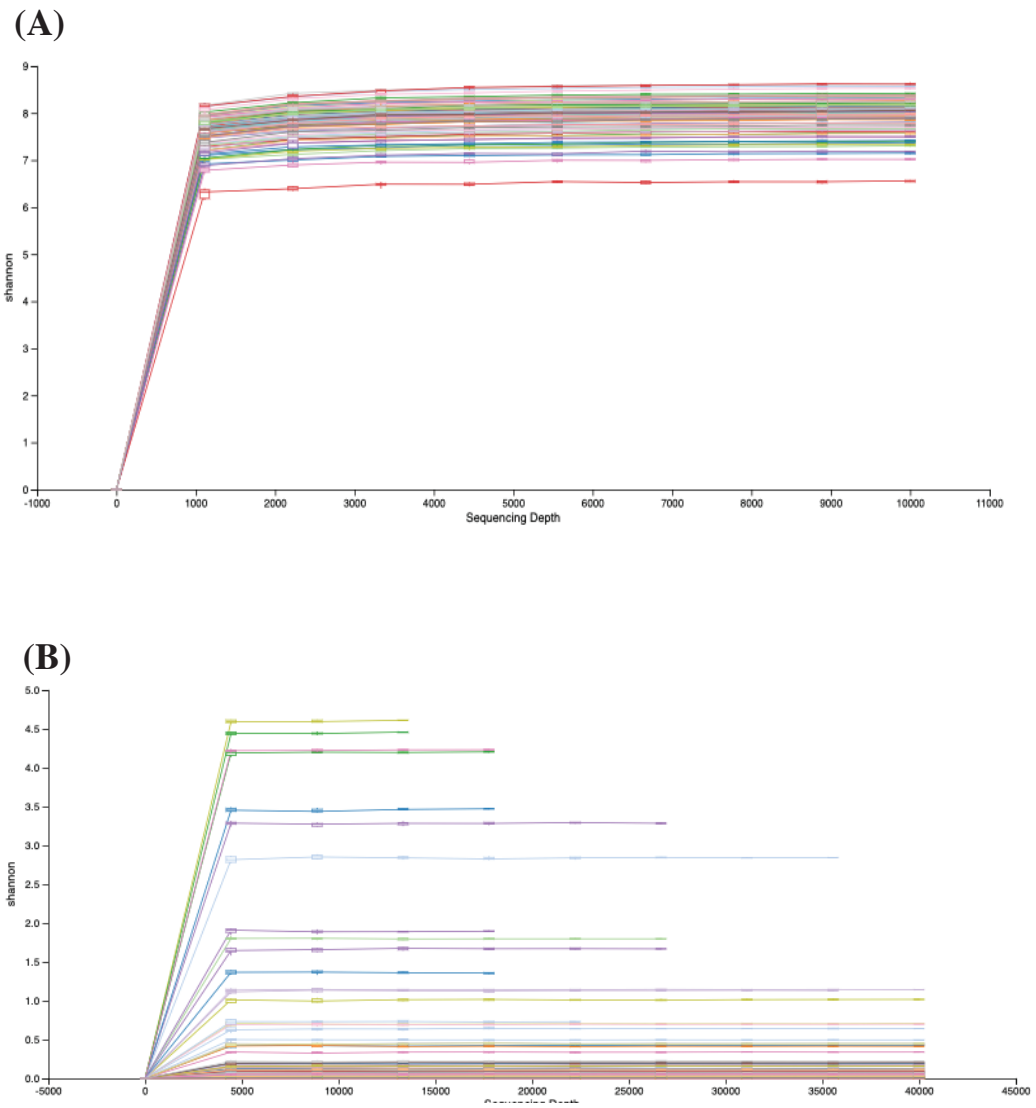

Figure S1: Rarefaction plots for all 16S (A) and ITS2 (B) samples. Shannon's index shown on Y axis.

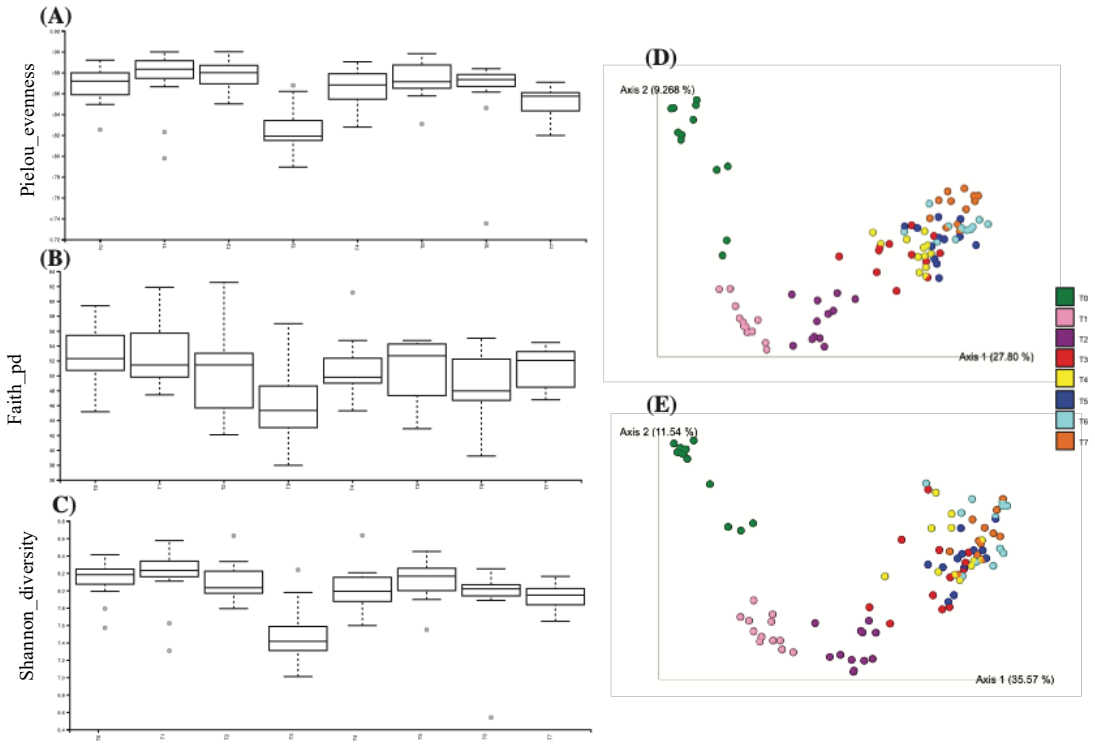

Figure S2: Diversity boxplots for 16S analysis by Time point (T0-T7). With Pielou's species evenness (**A**), Faith Phylodiversity (**B**), and Shannon's index (**C**). Two-dimensional PCoA built using the Bray Curtis distance matrix (**D**) and the unweighted UniFrac distance matrix (**E**).

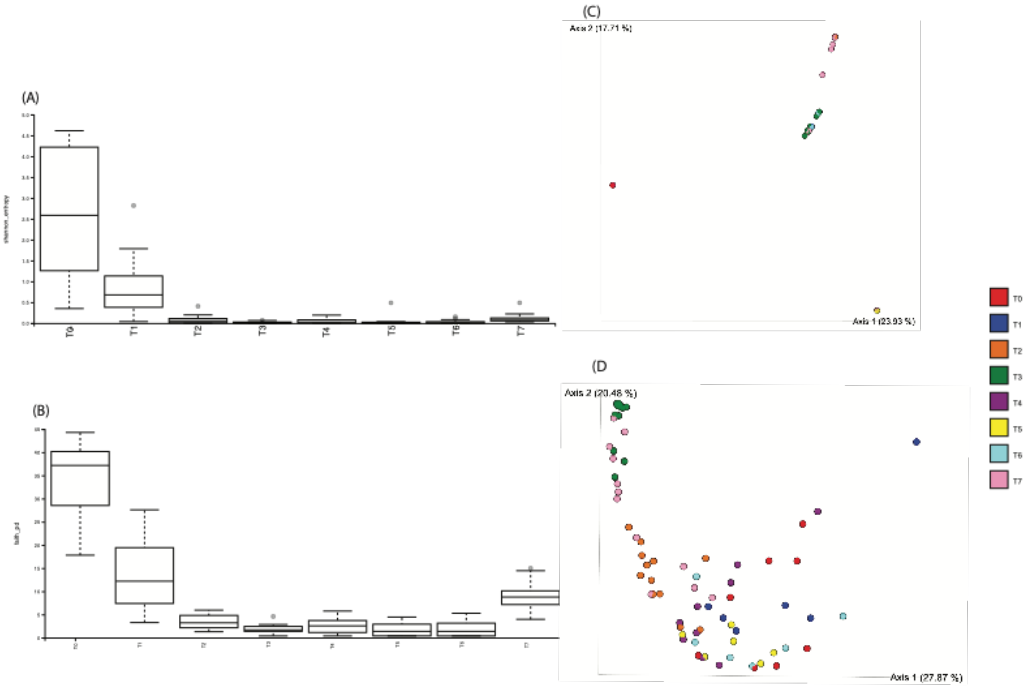

Figure S3: Diversity boxplots for ITS2 analysis by Time point (T0-T7). With Shannon's index (**A**) and Faith Phylodiversity (**B**). Two-dimensional PCoA built using the Bray Curtis distance matrix (**C**) and the unweighted UniFrac distance matrix (**D**).

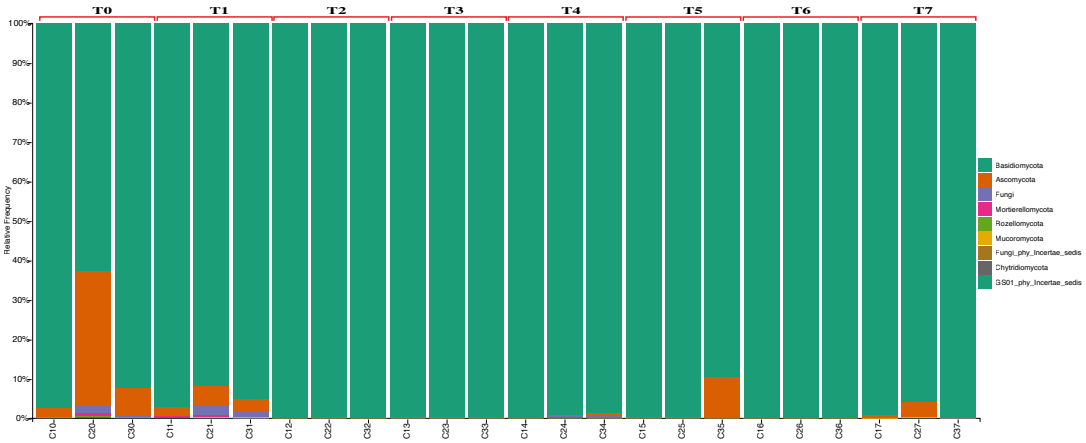

Figure S4: Phylum level relative abundance of Fungal ASVs found in control samples (not treated with BCA) of mushroom casing across the crop cycle. T0:Day0, T1:Day3, T2:Day7, T3:Day17, T4:Day21, T5:Day24, T6:Day28, T7:Day31

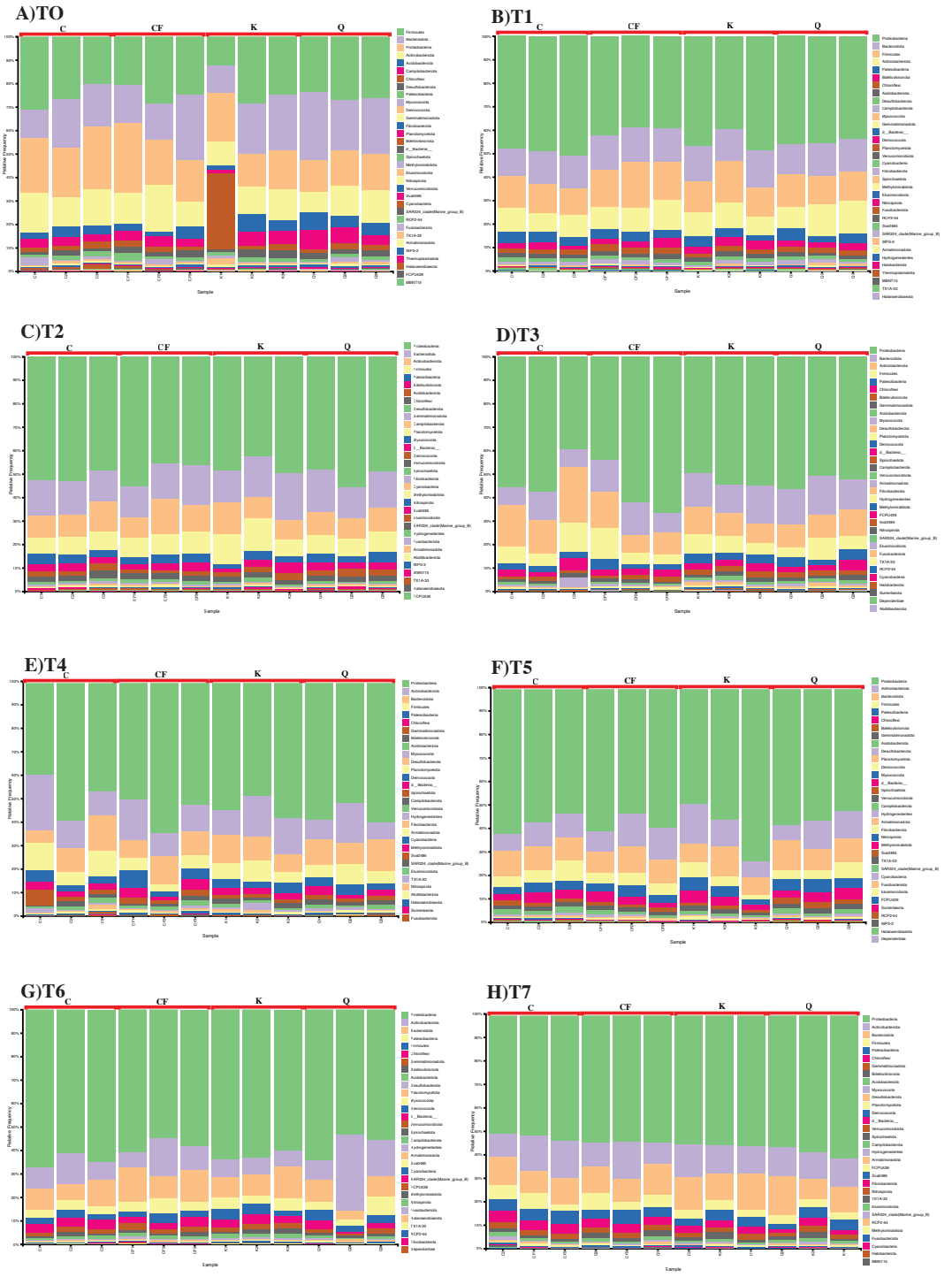

Figure S5: Phylum level relative abundance of Bacterial ASVs found in control and treated samples plots of mushroom casing across the crop cycle. C=Control, CF= Kos Cell Filtrate, K=Kos Cells, Q=QST-317 cells (Serenade). T0:Day0, T1:Day3, T2:Day7, T3:Day17, T4:Day21, T5:Day24, T6:Day28, T7:Day31

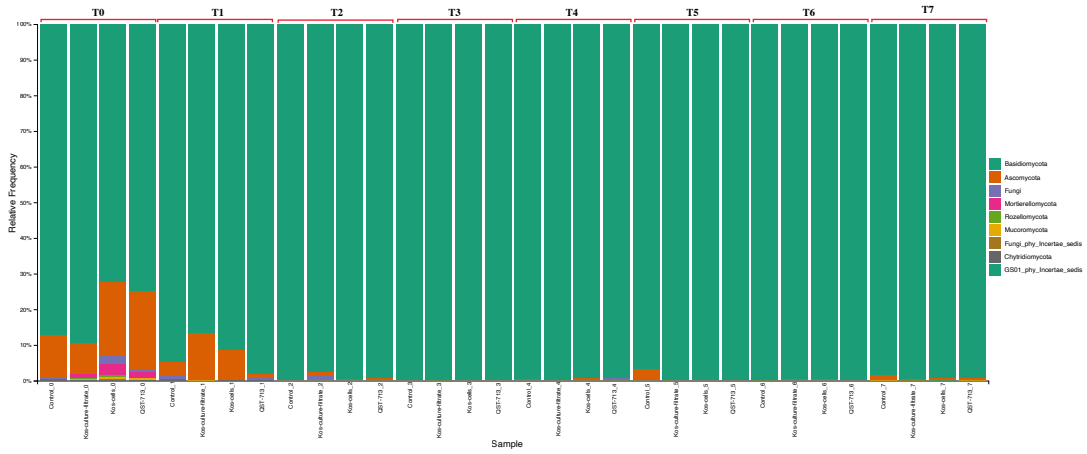

Figure S6: Phylum level relative abundance of Fungal ASVs found in casing across the crop cycle. To help visualization, biological replicates (3) have been merged for each time point. For each time point, control samples as well as the three BCAs, Kos Culture Filtrate, Kos cells and QST-713 cells (Serenade) are shown. T0:Day0, T1:Day3, T2:Day7, T3:Day17, T4:Day21, T5:Day24, T6:Day28, T7:Day31

6.6.2 Supplementary Tables

Table S1A: 16S samples

Note: For sample ID. First number after letter is replicate number and second number is timepoint. E.g. C10=Control replicate 1 at time), CF24=Culture filtrate replicate 2 at T4 etc.								
sample-id	input	filtered	percentage of input passed filter	denoised	merged	percentage of input merged	non-chimeric	percentage of input non-chimeric
C10	79495	38808	48.82	31963	24439	30.74	21455	26.99
C11	78657	49137	62.47	42290	32920	41.85	28821	36.64
C12	78655	43493	55.3	36497	27212	34.6	24430	31.06
C13	78986	51367	65.03	44432	33655	42.61	29630	37.51
C14	78941	43318	54.87	36797	27817	35.24	24093	30.52
C15	79641	36778	46.18	30533	22651	28.44	20184	25.34
C16	78533	44973	57.27	37935	26535	33.79	22797	29.03
C17	79034	46360	58.66	40062	29943	37.89	26740	33.83
C20	79481	47623	59.92	39132	30035	37.79	26870	33.81
C21	79083	47086	59.54	39757	30463	38.52	26461	33.46
C22	78729	43014	54.64	35402	25991	33.01	22952	29.15
C23	79540	46753	58.78	40095	29733	37.38	26549	33.38
C24	78968	45726	57.9	38909	28359	35.91	24673	31.24
C25	79934	43292	54.16	35951	26216	32.8	23565	29.48
C26	78690	42421	53.91	35361	24996	31.77	21988	27.94
C27	79340	44681	56.32	36195	26471	33.36	24376	30.72
C30	79709	46317	58.11	37092	27377	34.35	25109	31.5
C31	78458	42735	54.47	35252	26436	33.69	22640	28.86
C32	79046	39212	49.61	31328	22564	28.55	20365	25.76
C33	78786	43457	55.16	36025	26925	34.17	23769	30.17
C34	79446	37641	47.38	29774	21421	26.96	19452	24.48
C35	79090	41397	52.34	33786	24460	30.93	22335	28.24
C36	78933	40159	50.88	33486	23750	30.09	20798	26.35
C37	78601	41965	53.39	35341	25917	32.97	22742	28.93
CF10	78555	42030	53.5	34164	26258	33.43	24017	30.57
CF11	79243	46177	58.27	38829	29504	37.23	26323	33.22
CF12	78359	51907	66.24	47365	40614	51.83	39023	49.8
CF13	79809	44642	55.94	36463	26886	33.69	24362	30.53
CF14	79255	41961	52.94	35031	25611	32.31	23696	29.9
CF15	79831	35875	44.94	29383	21015	26.32	18910	23.69
CF16	78737	43722	55.53	36849	26957	34.24	23828	30.26
CF17	79767	42334	53.07	34552	24999	31.34	22972	28.8
CF20	78753	40354	51.24	32965	25523	32.41	23148	29.39
CF21	78417	44286	56.47	36325	27483	35.05	24647	31.43
CF22	79107	38390	48.53	27243	16978	21.46	13926	17.6
CF23	78961	41910	53.08	35623	26099	33.05	23195	29.38
CF24	79368	40529	51.06	34837	26113	32.9	22992	28.97
CF25	79198	40792	51.51	33605	24170	30.52	21985	27.76
CF26	79085	37019	46.81	29472	21129	26.72	19193	24.27
CF27	79033	38970	49.31	31422	22054	27.9	19800	25.05
CF30	78622	44614	56.74	37081	29306	37.27	25976	33.04
CF31	78385	46091	58.8	38619	29439	37.56	25654	32.73
CF32	79088	43425	54.91	36334	27389	34.63	24054	30.41
CF33	78871	45005	57.06	39385	30272	38.38	25297	32.07
CF34	79607	38454	48.3	31372	22783	28.62	21067	26.46
CF35	79052	45269	57.26	39578	30881	39.06	26005	32.9
CF36	79073	39384	49.81	32777	23973	30.32	21638	27.36
CF37	79099	40892	51.7	33482	23839	30.14	20734	26.21
K10	78855	46750	59.29	38823	30714	38.95	26653	33.8
K11	78793	41787	53.03	34558	25953	32.94	23332	29.61
K12	78812	41109	52.16	33530	24705	31.35	22519	28.57
K13	79244	39896	50.35	32050	23486	29.64	21044	26.56

K14	79104	40884	51.68	33909	24846	31.41	22424	28.35
K15	79662	37847	47.51	30508	22214	27.89	20393	25.6
K16	78731	41081	52.18	33794	23650	30.04	20658	26.24
K17	79545	33833	42.53	27045	18743	23.56	16846	21.18
K20	79117	45013	56.89	37140	28845	36.46	25208	31.86
K21	78708	46148	58.63	38658	29557	37.55	26350	33.48
K22	78723	44058	55.97	35947	26755	33.99	23831	30.27
K23	79363	34297	43.22	27472	20068	25.29	18085	22.79
K24	79066	43089	54.5	35115	25284	31.98	22941	29.02
K25	79713	39579	49.65	32814	23870	29.94	21640	27.15
K26	78522	45584	58.05	37629	26502	33.75	23533	29.97
K27	79476	36137	45.47	29153	20963	26.38	18586	23.39
K30	78150	47463	60.73	39259	30565	39.11	26818	34.32
K31	78685	50866	64.65	44279	34850	44.29	29801	37.87
K32	78577	47416	60.34	40596	30678	39.04	26583	33.83
K33	79206	49145	62.05	42225	31715	40.04	28200	35.6
K34	79267	40395	50.96	34173	24927	31.45	22168	27.97
K35	79603	47882	60.15	42437	32106	40.33	25876	32.51
K36	78700	40980	52.07	33844	24460	31.08	21894	27.82
K37	79502	41868	52.66	34890	25351	31.89	22826	28.71
Q10	78783	45056	57.19	37314	29163	37.02	25169	31.95
Q11	78694	41994	53.36	33829	24215	30.77	20810	26.44
Q12	79164	39746	50.21	32579	24026	30.35	20715	26.17
Q13	78735	46428	58.97	39182	29191	37.07	25945	32.95
Q14	79541	39121	49.18	32310	23692	29.79	21121	26.55
Q15	79058	44216	55.93	37785	28364	35.88	25248	31.94
Q16	79101	40767	51.54	34007	24063	30.42	21210	26.81
Q17	78774	40866	51.88	33226	24247	30.78	21857	27.75
Q20	78863	45014	57.08	37096	28819	36.54	25322	32.11
Q21	78394	44446	56.7	37238	28784	36.72	25620	32.68
Q22	79217	44570	56.26	38206	29063	36.69	25032	31.6
Q23	79010	42588	53.9	35108	25818	32.68	23289	29.48
Q24	79464	46542	58.57	39309	28494	35.86	25145	31.64
Q25	79197	41463	52.35	34712	25205	31.83	22629	28.57
Q26	78770	49337	62.63	44263	34162	43.37	25716	32.65
Q27	78728	43899	55.76	37256	27777	35.28	24699	31.37
Q30	78763	48107	61.08	40595	32089	40.74	27905	35.43
Q31	78504	46171	58.81	39121	29947	38.15	26805	34.14
Q32	79654	43100	54.11	35369	26107	32.78	23323	29.28
Q33	78866	46154	58.52	38105	27532	34.91	25298	32.08
Q34	79928	42933	53.71	36314	26885	33.64	23885	29.88
Q35	79045	47168	59.67	39230	28913	36.58	26338	33.32
Q36	79208	47115	59.48	40576	29590	37.36	25523	32.22
Q37	79107	39595	50.05	31810	22481	28.42	20199	25.53

Table S1B: ITS samples

Note: For sample ID. First number after letter is replicate number and second number is timepoint. E.g. C10=Control replicate 1 at time), CF24=Culture filtrate replicate 2 at T4 etc.								
sample-id	input	filtered	percentage of input passed	denoised	merged	percentage of input merged	non-chimeric	percentage of input non-
C10	72898	65718	90.15	65406	64703	88.76	64481	88.45
C11	73085	67951	92.98	67773	67224	91.98	67175	91.91
C12	69157	55256	79.9	55219	55152	79.75	55152	79.75
C13	67975	56347	82.89	56291	56210	82.69	56210	82.69
C14	65136	48283	74.13	48236	48187	73.98	48187	73.98
C15	69858	58544	83.8	58483	58223	83.34	58223	83.34
C16	64795	48806	75.32	48760	48686	75.14	48686	75.14
C17	76401	73201	95.81	73054	72796	95.28	72796	95.28
C20	73045	64865	88.8	64555	63245	86.58	63095	86.38
C21	73579	67874	92.25	67656	67031	91.1	66720	90.68
C22	69645	56648	81.34	56559	56352	80.91	56352	80.91
C23	70472	59437	84.34	59410	59380	84.26	59380	84.26
C24	66990	54194	80.9	54131	54011	80.63	54011	80.63
C25	69836	58444	83.69	58372	58030	83.09	58030	83.09
C26	69089	57070	82.6	57012	56762	82.16	56762	82.16
C27	75742	72124	95.22	71922	71581	94.51	71536	94.45
C30	71264	60433	84.8	60125	59339	83.27	59274	83.18
C31	73111	67268	92.01	66984	66351	90.75	66161	90.49
C32	68060	52042	76.46	51983	51836	76.16	51836	76.16
C33	69448	57097	82.22	57051	56940	81.99	56926	81.97
C34	67230	54675	81.33	54552	54205	80.63	54205	80.63
C35	69458	56909	81.93	56792	56445	81.26	56445	81.26
C36	70040	57851	82.6	57792	57685	82.36	57685	82.36
C37	76091	72478	95.25	72402	72324	95.05	72324	95.05
CF10	72115	64595	89.57	64326	62988	87.34	62569	86.76
CF11	70760	60705	85.79	60620	60343	85.28	60343	85.28
CF12	69707	57286	82.18	57230	57175	82.02	57175	82.02
CF13	69256	56517	81.61	56461	56422	81.47	56422	81.47
CF14	67539	55067	81.53	55025	54965	81.38	54965	81.38
CF15	68247	54804	80.3	54734	54522	79.89	54522	79.89
CF16	65576	52744	80.43	52690	52636	80.27	52636	80.27
CF17	75267	71791	95.38	71665	71528	95.03	71528	95.03
CF20	74037	65603	88.61	65315	64814	87.54	64720	87.42
CF21	70257	58560	83.35	58386	57719	82.15	57464	81.79
CF22	69163	54147	78.29	54029	53699	77.64	53699	77.64
CF23	69797	56160	80.46	56130	56117	80.4	56117	80.4
CF24	70254	58122	82.73	58102	58070	82.66	58070	82.66
CF25	68435	54001	78.91	53865	53143	77.65	53143	77.65
CF26	68362	54460	79.66	54385	54087	79.12	54087	79.12
CF27	75134	71501	95.16	71387	71267	94.85	71267	94.85
CF30	73220	66561	90.91	66296	65228	89.08	64806	88.51
CF31	69826	56517	80.94	56329	53740	76.96	53680	76.88
CF32	69751	58845	84.36	58733	58205	83.45	58155	83.38
CF33	67492	54734	81.1	54689	54612	80.92	54612	80.92
CF34	70236	59673	84.96	59589	59286	84.41	59286	84.41
CF35	67771	55555	81.97	55514	55458	81.83	55458	81.83
CF36	68284	55741	81.63	55670	55389	81.12	55389	81.12
CF37	75370	72494	96.18	72365	72218	95.82	72218	95.82
K10	70236	62734	89.32	62499	61648	87.77	61559	87.65
K11	69963	57310	81.91	57163	56537	80.81	56537	80.81
K12	70351	57540	81.79	57491	57393	81.58	57393	81.58
K13	69482	56166	80.84	56061	55515	79.9	55515	79.9
K14	69016	56844	82.36	56790	56628	82.05	56628	82.05

K15	69374	56338	81.21	56253	55906	80.59	55906	80.59
K16	69140	56115	81.16	55927	54550	78.9	54550	78.9
K17	76002	72822	95.82	72670	72589	95.51	72589	95.51
K20	73430	64733	88.16	64508	63414	86.36	63198	86.07
K21	69620	58551	84.1	58373	57513	82.61	57473	82.55
K22	66705	53135	79.66	53041	52627	78.9	52627	78.9
K23	69312	57691	83.23	57610	57419	82.84	57419	82.84
K24	66894	54706	81.78	54577	54191	81.01	54191	81.01
K25	69859	58723	84.06	58664	58597	83.88	58597	83.88
K26	69541	58130	83.59	58057	57958	83.34	57958	83.34
K27	75676	72153	95.34	72033	71858	94.95	71858	94.95
K30	72869	64503	88.52	64174	62671	86.01	61930	84.99
K31	68966	56801	82.36	56617	54220	78.62	54138	78.5
K32	69700	56038	80.4	55975	55777	80.02	55777	80.02
K33	68688	55325	80.55	55268	55105	80.23	55105	80.23
K34	65063	48315	74.26	48259	48182	74.05	48182	74.05
K35	68676	55246	80.44	55191	55082	80.21	55082	80.21
K36	62190	44685	71.85	44561	44114	70.93	44074	70.87
K37	76000	72962	96	72833	72734	95.7	72734	95.7
Q10	73475	66565	90.6	66237	64671	88.02	64345	87.57
Q11	73136	68224	93.28	67946	67566	92.38	67531	92.34
Q12	70287	59344	84.43	59269	58898	83.8	58898	83.8
Q13	66740	53804	80.62	53700	53303	79.87	53303	79.87
Q14	69642	58904	84.58	58792	58378	83.83	58378	83.83
Q15	70286	60068	85.46	59982	59808	85.09	59803	85.09
Q16	70008	59352	84.78	59322	59291	84.69	59291	84.69
Q17	76214	72686	95.37	72570	72411	95.01	72411	95.01
Q20	73126	65053	88.96	64775	64157	87.73	64062	87.6
Q21	74156	64716	87.27	64510	63994	86.3	63887	86.15
Q22	69885	58044	83.06	58007	57965	82.94	57965	82.94
Q23	67631	51138	75.61	51007	50682	74.94	50682	74.94
Q24	69891	58220	83.3	58154	58057	83.07	58057	83.07
Q25	63173	45696	72.33	45534	45167	71.5	45167	71.5
Q26	69650	57686	82.82	57665	57647	82.77	57647	82.77
Q27	76389	72617	95.06	72497	72440	94.83	72440	94.83
Q30	72318	64824	89.64	64554	63443	87.73	63249	87.46
Q31	71786	60751	84.63	60569	59255	82.54	59172	82.43
Q32	69774	57185	81.96	57086	56762	81.35	56743	81.32
Q33	68374	55485	81.15	55444	55364	80.97	55364	80.97
Q34	70447	59143	83.95	59056	58753	83.4	58753	83.4
Q35	67902	56433	83.11	56360	56144	82.68	56144	82.68
Q36	69607	57732	82.94	57673	57554	82.68	57509	82.62
Q37	76383	72971	95.53	72750	72570	95.01	72570	95.01

Table S2: Relative abundance of Bacterial (16S) and Fungal (ITS) sequences for samples at different timepoints. Information for Phylum (L2) and Genus (L6) are displayed.

Available at:

https://docs.google.com/spreadsheets/d/1_pXft9e4pzVA0AkSJsgBb9cTYtqX9xCh/edit?usp=drive_link&ouid=112012021882731286283&rtpof=true&sd=true

Table S3: Relative abundance of Firmicutes and *Bacillus* species for control plots and plots treated with one of three BCAs at timepoints T0-T7.

	Control		Kos culture filtrate		Kos cells		QST-713	
Firmicutes	Average	SD	Average	SD	Average	SD	Average	SD
TO	25.9316165	5.38086978	24.6886533	4.01519905	21.8295225	8.63911701	25.5393144	1.81365697
T1	12.1848115	1.01359757	16.9547758	2.08686659	14.256531	3.01683692	12.5969442	0.8987556
T2	7.72114117	0.62292937	9.12590908	0.56254724	11.5133267	3.67113464	8.23141282	0.49201094
T3	8.41813293	3.68200244	7.60496432	5.1421299	6.79047212	1.81813096	6.53816963	2.47902778
T4	9.53579409	3.29049307	4.76936022	1.43219331	6.9286236	2.40105452	6.62887143	2.03809801
T5	5.99792929	2.21488143	4.93584976	0.14440705	4.12718679	2.08104687	4.78276753	1.4990311
T6	2.94924054	0.49317876	3.32969738	1.09489217	3.91333234	1.37894041	3.49340117	0.95386202
T7	4.37103436	1.48207679	4.18058972	2.28465863	3.4932842	0.64626613	5.44947522	1.70340856

	C		Kos culture filtrate		Kos cells		QST-713	
Bacillus	Average	SD	Average	SD	Average	SD	Average	SD
T0	0.84848827	0.09863284	0.93786837	0.48093167	0.82147487	0.11433174	0.73013997	0.11132206
T1	0.42331337	0.10252591	0.38355003	0.01116908	0.45228573	0.09199143	0.43510787	0.03991908
T2	0.4309957	0.0506068	0.65342923	0.29749727	0.5205295	0.34100871	0.29044523	0.08007803
T3	0.69611207	0.49337671	0.27302203	0.27509517	0.37630913	0.11890171	0.35284297	0.05463086
T4	0.6043237	0.53328329	0.30177173	0.22639134	0.39388857	0.19056183	0.3854057	0.26427321
T5	0.24992197	0.06676216	0.22074607	0.06252	0.3486448	0.23369295	0.29619357	0.1433859
T6	0.16629217	0.07871209	0.141025	0.02650697	0.2360599	0.12721422	0.16315223	0.03578721
T7	0.48791797	0.25505975	0.2137558	0.21437278	0.1866542	0.06454065	0.46666573	0.41685746

Chapter 7
General Discussion

7.1 General discussion

Traditionally, fungicide products such as benzimidazoles, chlorothalonil, prochloraz and metrafenone have been used in the mushroom industry to control fungal diseases on *A. bisporus* crops. These fungicide products have been an important tool to mushroom growers who were dealing with extremely difficult to control diseases. Fungicide products can be highly effective at inhibiting the growth of fungal pathogens such as *Lecanicillium fungicola*, *Cladobotryum* spp., *Trichoderma* spp. and *Mycogyne perniciosa* (Van Zaayen & Van Adrichem, 1982; Fletcher *et al.*, 1983; Carrasco *et al.*, 2017; Altaf *et al.*, 2022; Navarro *et al.*, 2023). Unfortunately, it has now become apparent that there are several negative consequences linked to the use of these fungicide products. Fungicide chemical residues can be harmful and have toxic effects on non-target organisms within the environment and may also be damaging to human health (Kim *et al.*, 2017; Brauer *et al.*, 2019; Zubrod *et al.*, 2019; Gupta, 2022). An over-dependence on a limited number of fungicide classes within the mushroom industry has also caused many issues with fungicide resistance development (Grogan & Gaze, 2000; Grogan, 2006; Grogan, 2008; Gea *et al.*, 2021). This has contributed to the loss of approval for many fungicide products once used in the mushroom industry Currently, metrafenone is the only fungicide product which is approved for use on mushrooms crops in the European Union (EU) and its current approval status is listed as 'ongoing extension of approval period' (Marchand, 2023a). At the same time there has been a drop off in the number of new chemical active substances being approved in the EU (Marchand, 2023b). This has left mushroom growers in a very difficult position with extremely limited treatment options available to them. This apprehension within the industry has driven a huge interest in finding alternative treatments which may be employed as substitutes.

Biocontrol treatments and BCAs are a promising area which have received a lot of attention in previous years (Fira *et al.*, 2018; Sarrocco, 2023). There has been considerable growth in the number of BCA approvals in recent years in the EU, which provides some reassurance that approved products will be available into the future (Marchand 2023b). Biocontrol treatments would represent a more sustainable and environmentally friendly treatment option for growers and have reduced risk of resistance development (Jaiswal *et al.*, 2022). The antifungal capabilities and success

of biocontrol strains have been documented (Stiling & Cornelissen, 2005; Collinge *et al.*, 2022; Etesami *et al.*, 2023). In an ideal world, biocontrol would be a perfect solution to solve the lack of disease treatment options, which is currently one of the key issues the mushroom industry is facing. The primary aim of this thesis was to investigate the potential of biocontrol treatments and integrated pest management strategies for the treatment of cobweb disease and dry bubble disease.

In Chapters 2, 3, 4 and 5, the inhibition abilities of the *B. velezensis* Kos strain was described, primarily using the culture filtrate (CF) from the bacteria rather than the bacterial cells themselves. This was first displayed in **Chapter 2** and **Chapter 4**, which discussed the *in vitro* inhibition of *C. mycophilum* (cobweb disease) and *L. fungicola* (dry bubble disease). Results from both chapters 2 (**section 2.3.1**) and 4 (**section 4.3.1**) illustrated that the *B. velezensis* Kos cell suspensions could inhibit the growth of both *C. mycophilum* and *L. fungicola* on plate cultures. However, in chapter 2 (**section 2.3.1**) it was noted that on plate cultures, the CF of *B. velezensis* Kos could also inhibit *C. mycophilum*. This indicated that the CF of *B. velezensis* Kos contained anti-fungal compounds which may be contributing to its antagonistic potential. The identification of the inhibitory component within the *B. velezensis* Kos CF was then investigated. The *B. velezensis* Kos CF was fractionated by size and polarity. After which, the >3 kDa fractions were identified as inhibitory towards *C. mycophilum*, while <3 kDa were not. Both polar and nonpolar fractions were able to produce zones of inhibition against *C. mycophilum* but the zone from the >3 kDa, polar fraction was largest, so this was analysed using qualitative QE LC/MS. Several proteins were identified within this fraction, many of which were lytic enzymes, including subtilisin and other peptidases. As the *B. velezensis* Kos isolate was originally isolated by Kosanovic *et al*., (2021) and subsequently revived from long term storage for this work, DNA from the isolate was extracted and sent for Illumina NovaSeq analysis by Novogene Co. Ltd to confirm its identity (**Appendix 9.4**). Results of this work confirmed that the strain was in fact a *Bacillus velezensis* isolate, but the analysis also identified genomic clusters which were responsible for the biosynthesis of antimicrobial secondary metabolites genes which encoded for surfactin, subtilin, bacillibactin, bacilysin, fengycin, bacillaene and macrolactin **(Appendix 9.4)** (Clarke *et al*., 2024). The success of *B. velezensis* as a biocontrol strain has often been attributed to its ability to produce antimicrobial compounds (Fazle Rabbee & Baek, 2020; Li *et al.*, 2021; Baptista *et al.*, 2022; Barale

et al., 2022; Yu *et al.*, 2022; Hammad *et al.*, 2023; Li *et al.*, 2023; Wockenfuss *et al.*, 2024). Therefore, the ability of this strain to produce a range of lytic enzymes in its CF and produce several important antifungal secondary metabolites is hypothesized to contribute to the inhibitory mode of action for the novel *B. velezensis* Kos strain.

In Chapter 4 (s**ection 4.3.1**), the CF from *B. velezensis* Kos was demonstrated to be unable to inhibit the growth of *L. fungicola* on plate cultures. Similarly, *B. velezensis* Kos CF could not inhibit *T. aggressivum* in plate inhibition assays (Kosanovic *et al.*, 2021). Perhaps the agar medium used in plate cultures was not suitable for the CF to work against *L. fungicola* as the CF from *B. velezensis* Kos was successful in inhibiting the growth of both *C. mycophilum* and *L. fungicola* when it was used in flask inhibition assays. In liquid cultures, the fungal mycelium is completely surrounded by the liquid medium which means the CF can access and interact easily. This may be more difficult on agar plates where the CF was added to wells and may have partially diffused into the agar. *B. velezensis* Kos CF significantly reduced *C. mycophilum* growth by 57% ($p < 0.0002$) and *L. fungicola* growth by 45% ($P < 0.0002$). Fluorescent (Chapter 2 **section 2.3.2** and Chapter 4 **section 4.3.2**) and scanning electron microscopy (Chapter 4 **section 4.3.2**) was then used to visualise the fungal hyphae which were grown in the presence of *B. velezensis* Kos for 24 hr. The hyphae of both *C. mycophilum* and *L. fungicola* appeared to be damaged and irregular compared to healthy control treatments. This suggests that the *B. velezensis* Kos CF causes structural damage to the fungal hyphae which may contribute to the reduction in growth which was observed during the flask inhibition assays. Exposure to the *B. velezensis* Kos CF was also shown in Chapters 2 and 4 to prompt proteomic alterations in both *C. mycophilum* and *L. fungicola*. The PCAs from the Perseus analysis clusters samples based on the proteomic similarities, i.e. samples which contain a similar proteome will be placed closer together on the PCA than samples with dissimilar proteomes. The PCA's from both analyses placed the untreated control fungal treatments away from those treated with the *B. velezensis* Kos CF. This high degree of separation between control and *B. velezensis* Kos CF treated samples suggest that the CF is altering the activities of both fungal pathogens. This was backed up with the heat maps from both analyses which placed the untreated control samples on a separate lineage to the *B. velezensis* Kos CF treated samples. The pattern of the heat map itself also suggested an inverse relationship between control and *B. velezensis* Kos CF treated samples. SSDA proteins

which were significantly increased in the untreated control samples were highlighted as being significantly reduced in the treated samples (and vice versa). Volcano plots were used to investigate the specific proteomic changes for both *C. mycophilum* and *L. fungicola*. When the top SSDAs of the volcano plots which were either increased or decreased in abundance were investigated, a similar trend was seen for both *C. mycophilum* and *L. fungicola*. In both analyses, SSDA proteins which were related to stress appeared to be increased when the fungal pathogens were exposed to the *B. velezensis* Kos CF and SSDA proteins related to growth appeared to be decreased in relative abundance. A similar response was seen when this strain was studied with *T. aggressivum* which causes green mould disease (Kosanovic *et al.*, 2021).

The *in vitro* inhibition abilities of the commercially available biocontrol strain *B. velezensis* QST 713 was also investigated in Chapter 4. It was important to include this strain in this work, as it is already being investigated and is in use for the treatment of green mould disease in France (Pandin *et al.*, 2018). It is also approved for use on many different crops such as strawberries, tomatoes, apples and carrots within Europe (Källqvist *et al.*, 2016; Punja *et al.*, 2016; Ayer *et al.*, 2021; Volodin *et al.*, 2023). The human and environmental health risks of this strain have already been studied (Källqvist *et al.*, 2016). If this strain showed evidence of inhibition against the likes of cobweb and dry bubble disease, the process of getting it approved for these diseases would be relatively quick and would provide growers with another treatment option. The *B. velezensis* Kos strain on the other hand is a novel isolate which would require many years of product development and safety testing before it could potentially be used on *A. bisporus* crops. During chapter 4, the CF from *B. velezensis* QST 713 was shown to be highly inhibitory towards *L. fungicola*, even to a greater extent than *B. velezensis* Kos. Many of the same SSDA proteins were increased/decreased in relative abundance in *L. fungicola* in response to both *B. velezensis* QST 713 and *B. velezensis* Kos. This would suggest that both the biocontrol strains were working in a similar way to inhibit *L. fungicola*.

Overall, the results from Chapters 2 and 4 clearly show that the *B. velezensis* Kos is capable of significant levels of growth inhibition to both *C. mycophilum* and *L. fungicola*. *B. velezensis* QST 713 was only studied *in vitro* in the context of dry bubble disease as the QST 713 isolate was not available during *the C. mycophilum* work

during chapter 2. Regardless, *B. velezensis* QST 713 was able to significantly inhibit the growth of the dry bubble disease pathogen *in vitro*. Previous studies have also shown a similar *in vitro* response from *T. aggressivum* (Kosanovic *et al*., 2021), which causes green mould disease on *A. bisporus* crops following exposure to *B. velezensis* Kos. This means that the *B. velezensis* Kos CF has been successful against three of the four most important pathogens of *A. bisporus*. This really highlighted the potential of this strain for disease treatment within the mushroom industry. When investigating potential biocontrol strains, it is common for hundreds of bacterial isolates to be extracted from the environment where they are intended to work. *In vitro* experiments are important for narrowing down the selection process of candidate strains which show the best potential against the target pathogen. However, this does not guarantee that the selected biocontrol strains will be effective in a crop environment. In fact, many believe that solely relying only on the *in vitro* selection processes may have led to the loss of promising potential BCAs (Collinge *et al.*, 2022). Biocontrol strains have often shown strong antagonist potential against pathogens *in vitro* but have failed to replicate the same levels of antagonism when brought forward to a large field trial setting (Besset-Manzoni *et al.*, 2019; Clough *et al.*, 2022). It is accepted that during *in vitro* experiments, the very complex microbial community which is present within the *A. bisporus* compost and casing is not replicated. Rather the biocontrol strain and pathogen are investigated in isolation and are provided with the optimum conditions and nutrients for the microorganism to be able to thrive (Hatab & Gaugler, 1999; Blackburn *et al.*, 2016). Therefore, the aims of chapters 3 and 5 were to test the two biocontrol strains at a crop level to get an accurate interpretation of the antagonistic potential against pathogens within a mushroom crop environment.

Both biocontrol strains were investigated at a crop level during chapters 3 and 5. **Chapter 3** focused on the treatment of cobweb disease with both biocontrol and conventional fungicide treatments. Prior to this, it was discovered that a *C. mycophilum* isolate had been isolated from Ireland which appeared to be highly tolerant to the fungicide product metrafenone. This was confirmed at an *in vitro* level when isolate 1546 grew significantly better than any other *C. mycophilum* strains tested at metrafenone concentrations as high as 100 mg kg^{-1}. It was also further confirmed during *in vivo*, crop trials when metrafenone failed to inhibit the growth of *C. mycophilum* 1546 and could not prevent disease progression. This is a very

concerning result as metrafenone is the only approved fungicide for use on *A. bisporus* crops in the EU. As such, it is anticipated that further resistance to this fungicide may become more widespread. *C. mycophilum* 1546 was isolated from an infected mushroom crop in 2019, which was after the introduction of metrafenone to the mushroom industry. There has been anecdotal evidence of the product not being as effective as it was initially amongst the mushroom industry, but this work confirms that metrafenone may struggle to control cobweb disease in the future. Conversely, *C. mycophilum* 618 which was included in both *in vitro* and crop trial studies, responded extremely well to metrafenone. This strain was isolated prior to the introduction of metrafenone and therefore would not have been previously exposed to the fungicide. Another fungicide, Prochloraz, was very effective at controlling all the *Cladobotryum* isolates tested *in vitro* and the two *C. mycophilum* isolates brought forward to the field trial. It performed the best out of all treatments included during this trial, preventing significant yield reductions and having efficacy values of 73% and 70% for isolate 1546 and 618 respectively. It also performed very well during the second replicate crop trial achieving efficacy values of 69% against *C. mycophilum* isolate 1546 at an inoculation rate of $1x10^6$cfu/m^2 and 73% for the same isolate at $5x10^5$cfu/m^2. Unfortunately, these results will be of little comfort to the mushroom growers as shortly after this work was completed, the approval for prochloraz on mushroom crops was revoked, in part due to its high toxicity to the environment.

The results presented in **Chapter 5** (**section 5.3.1**) showed that the tested *L. fungicola* isolates were moderately sensitive to metrafenone at high concentrations, whereas they were completely inhibited at high concentrations of prochloraz fungicide. It was found that the growth of *L. fungicola* reduced in an incremental fashion as the concentration of metrafenone increased up until a metrafenone concentration of 500 kg/mg^{-1} was used. At this concentration, the level of inhibition was less than the inhibition achieved using 1 kg/mg^{-1} metrafenone. This suggest that the effectiveness of metrafenone may be dose dependent. Over a certain concentration of metrafenone, there appears to be stimulation of *L. fungicola* growth. This will be important to keep in mind as we continue to use metrafenone on *A. bisporus* crops. Correct and stringent application of approved metrafenone rates must be adhered to, to ensure metrafenone effectiveness. Contrary to the significant growth recorded *in vitro* for *L. fungicola* in the presence of metrafenone, the metrafenone fungicide was shown to be very effective at preventing

significant yield reductions and disease development caused by *L. fungicola* isolate 1722 at crop level as described in Chapter 5 (**section 5.3.3 & 5.3.4**). This suggests that metrafenone will continue to be an important tool for controlling dry bubble disease, following the removal of prochloraz approval.

The core focus of this thesis was to compare the efficacy of conventional fungicide products to novel biocontrol treatments. In Chapter 5 an appropriate inoculation or infection rate to use during the crop trial experiments was investigated to mimic on-farm disease conditions. This idea stemmed from the results in Chapter 3, where a high inoculation rate of $1x10^6$cfu/m^2 was applied. This rate was used as it was a standard inoculation rate to include when testing fungicide products at a crop scale. However, results from the first crop trial showed that the biocontrol products were struggling to compete with the fungicide products. *B. velezensis* QST 713 failed to prevent significant yield reductions caused by *C. mycophilum* (isolate 1546) and could not reduce disease progression. An efficacy rate of 30 % was reported at the end of the crop for *B. velezensis* Kos and the treatment prevented significant yield loss compared to the uninoculated control. The *B. velezensis* Kos treatment did perform better than *B. velezensis* QST 713 during this crop trial, but still provided sub-optimal results, especially in comparison to the fungicide treatments. The practical applications of how these biocontrol treatments would be used on mushroom farms was considered. They would not be able to perform as well as the fungicides when they come up against extremely high levels of disease, but are these extremely high disease rates the norm for a standard mushroom farm? To gain more insight, mushrooms farms in Ireland were visited and an effort was made to connect to mushrooms growers to discuss their experience when dealing with disease. Based on these conversations, it was determined that most growers would not experience the extremely high levels of disease that were tested in the first crop trial especially if they had excellent hygiene standards and good disease prevention protocols firmly in place. From that point, lower inoculation rates were included to test the biocontrol treatments so that a rate could be identified at which the biocontrol was effective. This will allow growers to get the most out of the biocontrol products and apply them practically to the crops. During the second crop trial when the inoculation levels were lowered to $5x10^6$cfu/m^2, the *B. velezensis* Kos treatment efficacy improved to 46% compared to an efficacy of 38% for the $1x10^6$cfu/m^2. Disease was also significantly reduced ($P < 0.05$) by *B. velezensis*

Kos treatment at the $5x10^6$cfu/m^2 inoculation. The efficacy of *B. velezensis* QST 713 did not improve against the lower inoculation rate. This suggest that *B. velezensis* QST 713 will not be an appropriate product to use for the treatment of cobweb disease caused by *C. mycophilum* isolate 1546. An inoculation rate of $5x10^6$cfu/m^2 is quite high and further trials at lower levels of inoculation are needed to determine if the biocontrol products have improved efficacy against cobweb, when disease levels are lower.

During chapter 5 and the investigation of the biocontrol strain's ability to treat dry bubble disease, a range of inoculation rates were included, high ($1x10^6$cfu/m^2), medium ($1x10^4$cfu/m^2) and low ($1x10^2$cfu/m^2). During this work, we concluded that the $1x10^6$cfu/m^2, similar to the results presented in chapter 3, was too high to accurately reflect dry bubble disease conditions on the farm. Not even the tested fungicide, prochloraz could prevent significant yield reduction or disease development. Conversely, $1x10^2$cfu/m^2 appeared to be too low to reliably induce dry bubble disease expression. Disease often did not develop on plots inoculated with this low rate and if it did appear, it was not until late into the third flush. Therefore, we propose that researchers who are interested in testing biocontrol treatments at a crop level should include inoculation rates of between $1x10^4$cfu/m^2 to $1x10^5$cfu/m^2. At this range, sufficient disease development will occur which can test the biocontrol treatments, but it will remain within a realistic disease level that will reflect farm conditions more closely. When the biocontrol treatments were tested against $1x10^4$cfu/m^2 *L. fungicola* inoculation rates, both *B. velezensis* QST 713 and *B. velezensis* Kos were able to significantly reduce disease development.

In Chapter 5, after crop trial 1 was complete, it was noticed that disease levels on the $1x10^4$cfu/m^2 and $1x10^2$cfu/m^2 plots were a lot lower than anticipated, especially on the control inoculated plots. During crop trial 1, the practice of 'salting' was carried out to limit cross contamination between plots which were arranged on the shelves using a randomised block design. The plots were also very closely monitored over the course of the trial to identify and treat any signs of disease development. It was hypothesized that the early detection and salting which was carried out during this first dry bubble crop trial could have been acting as an unintentional treatment, which was causing the reduced disease levels. To test this hypothesis a second crop trial was carried out where

early detection and salting was not done routinely, except for one specific 'salting' treatment. For the remaining treatments, disease was allowed to develop and was only removed at the end of each flush, without salting. The level of disease development on these early detection/salting plots was significantly reduced compared to the inoculated unsalted control plots. This result was subsequently replicated during a third crop trial (**section 5.3.4**). The average number of bubble mushrooms which developed on plots inoculated at $1x10^6$cfu/m^2 without any salt was 88, however in crop trial 1, when salt was applied to $1x10^6$cfu/m^2 plots, only 31 bubble mushrooms developed on average. This provides proof that relatively simple and cheap IPM strategies can make a significant impact on disease levels.

Chapter 6 of this thesis investigated the impact of biocontrol treatment application on the native microbial population dynamics within the *A. bisporus* cultivation environment. It has been well established in the literature that specific microbial species play key roles during *A. bisporus* development and contribute to the success of the crop (Mcgee, 2018; Carrasco & Preston, 2020). In fact, conditioning is a key step during substrate preparation which will ensure the substrate favours not only the growth of *A. bisporus*, but also the microbes which promote *A. bisporus* development (Vieira & Pecchia, 2018). With this chapter, the aim was to confirm that the application of living biocontrol agents did not negatively impact any of these key species which in turn could negatively impact *A. bisporus* cultivation. The most highly abundant fungal and bacterial amplicon sequence variants (ASVs) were identified in the control, untreated casing samples from several timepoints, over the course of the *A. bisporus* cultivation cycle. These ASVs were compared to the ASVs reported from casing samples which had been treated with either *B. velezensis* QST 713, *B. velezensis* Kos (CF) or *B. velezensis* Kos (cells). It was determined that there was no significant difference in the microbial community composition between control and biocontrol treated casing samples. This suggests that the application of the biocontrol strains was not impacting casing population dynamics and hence would not negatively impact *A. bisporus* cultivation. Further analysis also indicated that *B. velezensis* could not be identified within the data set, even in the timepoints which were taken 2 days after biocontrol application. This suggest that the biocontrol strains are not persisting in the casing layer after their application enough to make any impact on the sequencing results. It should be noted that as *Bacillus* species produce spores, DNA extraction and

cell lysis may be more challenging to carry out. This may make finding *B. velezensis* DNA within the dataset more challenging. Our aim with this work is to find a more sustainable and environmentally friendly alternative to the chemical fungicides. The results presented in Chapter 2-5 show that these biocontrol strains have significant activity against the pathogen and can control moderate levels of disease on the crop. The results in Chapter 6 suggest that they can achieve this, without persisting and having long term impacts on the soil or its inhabitants. This work supports the idea that biocontrol is a gentle but effective method to employ in the future. However, this result may also point to drawbacks of biocontrol treatment. The results in Chapters 2 and 4 showed that both biocontrol strains display antagonism towards both *C. mycophilum* and *L. fungicola* and that highly significant and drastic inhibition was displayed. Yet when taken forward to the crop trial in chapters 3 and 5, the inhibition levels achieved in the *in vitro* studies, were not replicated. There were signs of antagonism and inhibition, yet it was not as pronounced and effective as what was seen with the *in vitro* studies. It is possible that the microorganisms within the *A. bisporus* casing and compost are outcompeting the *B. velezensis* biocontrol strains and hence are preventing the biocontrol strains from reaching their full antagonist potential during crop trial experiments. Perhaps there is a fine balance when selecting a biocontrol strains. The biocontrol strains should be competitive enough that they could persist in the environment to some degree but perhaps not be able to significantly impact the microorganisms which play critical roles during *A. bisporus* development.

Future work and concluding remarks

The following are areas of this thesis which could potentially be further developed in the future. It was shown in chapters 3 and 5 that the Serenade® biocontrol product (*B. velezensis* QST 713) displayed potential to treat dry bubble disease but failed to work against the *C. mycophilum* isolate 1546 which causes cobweb disease, although inoculation rates were different in both trials. Future work should further investigate the efficacy of *B. velezensis* QST 713 at lower inoculation rates and against other *Cladobotryum* spp. This would determine whether the lack of efficacy is associated with this particular 1546 isolate or *Cladobotryum* spp. as a whole. The inoculation used during the cobweb trials was extremely high. Perhaps *B. velezensis* QST 713 would perform better against lower inoculations, like the ones used for the dry bubble

trials. This would be important for future disease management planning. If it was the case that *B. velezensis* QST 713 was effective against some *Cladobotryum* isolates, identification of the *Cladobotryum* strains causing disease would need to be carried out prior to treatment. Due to the *B. velezensis* QST 713 strain being unavailable at the time, the relationship between *C. mycophilum* and *B. velezensis* QST 713 was not investigated at an *in vitro* level. The results presented in Chapter 3 showed that *B. velezensis* QST 713 was not effective at inhibiting disease development caused by *C. mycophilum*. It would be interesting to see if this would be reflected in *in vitro* studies. This could support the idea that *in vitro* analysis is important for identifying candidate strains which have the best chance of being successful at a crop level.

Another interesting research area which could be investigated in the future is incorporating artificial intelligence (AI) and machine learning into disease treatment protocols on mushroom farms. Labour shortages are a very big issue for mushroom growers currently. Because of this, there has been an increase in research and investment into automatic harvesting systems (Pagliarani *et al*., 2024). Robotic pickers are attached to the side of the shelves within the mushroom growing room and move down the beds to select mushrooms which are at the ideal stage for picking (Reed *et al*., 2001). Automatic harvesters often incorporate hyperspectral imaging (HSI) technology. HIS will take an image the crop and investigate the molecular reflections and absorptions emitted using a range of wavelengths (Wieme *et al*., 2022; Ram *et al*., 2024). This unique spectral signature can be used to provide information on the health of the crop, including whether symptoms of disease are present. This technology can provide information in real-time to the growers (Ram *et al*., 2024). This could allow the response time for dealing with disease to be much faster and therefore more likely to be effective. Another idea would be to incorporate automatic salting as a function of the robotic pickers installed in mushroom farms. Although it is anticipated the HSI will be an important tool for agriculture in the future, it is still in its initial stages of development and further research into how this technology could be adapted for the mushroom industry specifically is required.

Investigation of potential biocontrol treatments often requires determining the strain's ability to reduce pathogen growth in both small scale, laboratory experiments and large-scale *in vivo* trial experiments. *B. velezensis* QST 713 was shown to be able to

significantly inhibit *Botrytis cinerea* colonization *in vitro*, but inhibition was less evident and more variable in an *in vivo* model (Tut *et al.*, 2021). *Bacillus* strains, including *B. velezensis* QST 713 were shown to be effective against Citrus Mal Secco disease in both *in vitro* studies and *in vivo* studies (Aiello *et al.*, 2022). However, the *in vivo* studies were '*in planta*' and were not carried out at a crop scale. This thesis delivers a lot of important information on the use of biocontrol to treat mushroom disease, looking at both *in vitro* and *in vivo* studies. The results of Chapters 2 and 4 are supportive for the use of biocontrol treatments and highlight their antagonist potential against *C. mycophilum* and *L. fungicola in vitro*. Chapter 3 and 5 show that when these biocontrol treatments are tested at a crop trial level, the level of inhibition is limited and not as high as the *in vitro* results. The results presented in Chapter 6 look at the persistence of the biocontrol treatments within the environment and possibly explains why there is a disconnect between *in vitro* and field trial studies.

The level of inoculation or disease pressure used during crop trial experiments can impact the success of biocontrol treatments. *B. velezensis* QST 713 could significantly reduce disease symptoms of yellow rust (*Puccina striiformis*) on wheat crops when disease levels were recorded as moderate but was less effective against high disease pressure (Reiss & Jørgensen, 2017). Similarly, *B. velezensis* QST 713 was only found to be effective against low-moderate disease levels of powdery mildew disease during wheat crop trial studies (Matzen *et al.*, 2019). The biocontrol treatments in this work were not effective against high disease pressure in field trial studies but performed better against moderate disease conditions, especially *against L. fungicola*. If biocontrol treatments are used on mushroom farms in the future, maintaining excellent hygiene standards and disease prevention practices will be critical to avoid extreme disease conditions.

Biocontrol does show potential as a treatment option for mushroom disease in the future. As the biocontrol strains are already ubiquitous within the environment, the risk of the treatment being toxic to the non-target organisms if disposed of inappropriately is low. However, *B. velezensis* QST 713 did cause sublethal effects in adult, winter honeybees causing changes to immune gene expression (Sabo *et al.*, 2020). Human health risks associated with the use of biocontrol is also considerably lower that fungicide use. However, it is important to note that a link between the serine

protease subtilisin, and hypersensitive reactions in animal model studies have been investigated (Thorne *et al.*, 1986; Xue *et al.*, 2005; Florsheim *et al.*, 2015). Subtilisin was identified within the CF of *B. velezensis* Kos, which means further investigation into any health risks associated with this biocontrol strain is warranted. The risk of resistance developing against the biocontrol treatments is a lot lower due to its broad antimicrobial abilities. Currently, only one approved fungicide is available for mushroom growers to use within the European Union. Resistance developing among mushroom pathogens towards this fungicide is highly likely if it continues to be the only treatment method employed by mushrooms farms. In theory, biocontrol could be considered as very suitable alternative to fungicide products. However, a more realistic view of biocontrol capabilities is required if it is to be accepted by the mushroom growers. This thesis has shown that biocontrol treatments cannot provide the same level of protection that growers are used to gaining with chemical fungicides. However, the use of these fungicides is no longer justifiable and has become extremely restricted. Soon growers may no longer have a choice as all approved fungicide products could be removed within the next few years. The future of mushroom disease control will have to be a multifactorial approach. Multiple areas of integrated pest management will need to be used in combination. This could include the use of biocontrol treatments with salting, excellent hygiene standards, disease prevention protocols, as well as educating mushroom pickers to be able to monitor and detect mushroom disease symptoms early. The hope is that all these areas combined, will help mushrooms growers to control disease on their farms, without having to rely on the use of chemical fungicides in the future.

Chapter 8

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8.1 Bibliography for literature cited in Introduction (Chapter 1) and Discussion (Chapter 7)

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Chapter 9
Appendix

Appendix 9.1: Detailed protocol for protein extractions from fungal mycelium and mass spectrometry sample preparation.

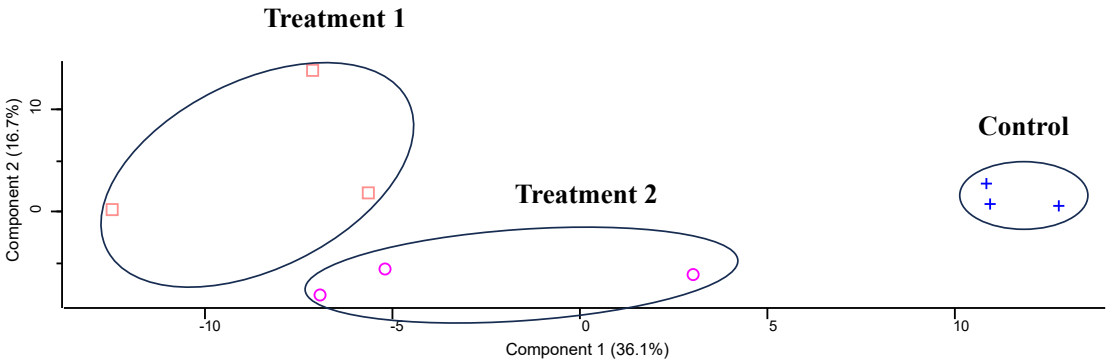
Liquid cultures of fungal species were grown in conditions described in Chapter 3 (section 2.2.7) and Chapter 4 (section 4.2.5). Fungal tissue was separated from liquid medium by passing the contents of the flask through Miracloth (Merk) and squeezing tissue to remove excess liquid. The fungal tissue was then washed with PBS and once again passed through Miracloth to collect fungal tissue for protein extraction. Fungal tissue was transferred to a sterile pestle and mortar.

Liquid nitrogen was poured directly into the mortar to snap freeze the tissue. The tissue was ground to a fine powder using the pestle. Lysis buffer, (8 M urea, 2 M thiourea and 0.1 M Tris-HCl, pH 8.0) supplemented with protease inhibitors (leupeptin, pepstatin A (10 µg/ml) and PMSF (1 mM/ml)) was added to the crushed hyphae to create a paste-like suspension. This suspension was transferred to fresh 1.5 ml Eppendorf's using a Pasteur pipette. Suspensions were sonicated (40% power, cycle 3, for 10 seconds) to disrupt cellular membranes and release the fungal cell contents. Suspensions were centrifuged (14500 x g for 10 min) to pellet cellular debris. The supernatant (cell lysate) was transferred to a fresh tube and quantified using the Bradford protein assay. The Bio-Rad protein assay dye (1ml) was diluted in ddH_2O (4ml). 980 µl of this Bio-Rad solution was mixed with 20 µl protein lysate sample and transferred to a 1 ml plastic cuvette. After a 5 min incubation, the protein concentration of the samples were read using spectrophotometry (Eppendorf Biophotometer) at 595 nm. Ice-cold acetone (100 %) was added to100 µg quantified protein samples in a ratio of five parts acetone to one part protein sample, to concentrate the protein. Acetone precipitation was carried out overnight at -20 ºC.

Precipitated proteins were pelleted by centrifugated (14500 x g for 10 min). Acetone was removed from the samples and air dried. Protein pellets were resuspended in 25 µl resuspension buffer (8 M urea, 2 M thiourea and 0.1 M Tris-HCl, pH 8.0). Two µl

of this solution was used to determine protein quantification using a Qubit™ quantification kit (Invitrogen) (following manufacturers guidelines). Twenty five µl of the solution was used for trypsin digest of protein samples. Ammonium Bicarbonate (105 µl, 50 mM) was added to this 25 µl of protein sample. One µl DTT (0.5 M) was applied to the samples followed by a 20 min incubation at 56ºC to reduce samples. Samples were alkylated with 2.7 µl IAA (0.55 M) and incubated in the dark for 15 min. One µl ProteaseMAX™ Surfactant Trypsin Enhancer stock (Promega) (1%, w/v stock) and 1 µl sequencing grade trypsin (Promega) (0.5 µg/µl) was added to each protein sample which were incubated at 37°C overnight.

After 18 hr, the trypsin digest was inhibited with the addition of 1 µl Trifluoroacetic acid (TFA) and incubation at room temperature for 5 min. Samples were centrifuged (14500 x g for 10 min) and purified peptides were obtained using C-18 spin columns (Pierce). C-18 spin columns were activated with 50% v/v acetonitrile (ACN) and equilibrated with 5% v/v ACN, 0.5% v/v TFA. Peptide samples were bound to the C-18 resin bed. Resin was washed with 5% v/v ACN, 0.5% v/v TFA to remove contaminants. Finally, purified peptides were eluted with 70% v/v ACN and dried at 38°C for 2-3 h in a SpeedVac concentrator (Thermo Scientific Savant DNA 120). Dried, purified peptide pellets were resuspended with 2% v/v ACN and 0.5% v/v TFA prior to mass spectrometry loading.



Appendix 9.2 Principal component analysis (PCA) of control *C. mycophilum* treatment, and *C. mycophilum* treated with either 12.5% v/v (treatment 1) or 25% v/v (treatment 2) *B. velezensis* Kos 96 hr CF, pre ANOVA significance tests.

Appendix 9.3: QIIME2 commands for the amplicon analysis

The following workflow is for the analysis of amplicon sequencing. It is modified from a number of sources including https://docs.qiime2.org/2024.5/tutorials/overview/ and https://github.com/Marylou8/Metataxonic-analysis-using-Qiime2-workflow. Lines preceded with a dash "-" are the actual commands inputted into the Linux bash shell. Output files are shown in bold text. These commands generate all files for taxonomical analysis.

- conda activate qiime2-2023.7
- export TMPDIR=/home/dfitzpatrick-a/ZTMP

#STEP1:IMPORT SEQUENCES (1_): Forward and reverse FASTQ files of each sample must be in Folder (BAC_16S)

- qiime tools import --type 'SampleData [PairedEndSequencesWithQuality]' --input-path BAC_16S --input-format CasavaOneEightSingleLanePerSampleDirFmt --output-path **1_demux-paired.qza**
- qiime demux summarize --i-data 1_demux-paired.qza --o-visualization **1_demux-paired.qzv**

#STEP2: Run DADA2 (2_): This trims the sequences

- qiime dada2 denoise-paired --i-demultiplexed-seqs **1_demux-paired.qza** --p-trunc-len-f 270 --p-trunc-len-r 180 --p-n-threads 64 --output-dir **dada2out**

#Generate .qzv files so we can visualise DADA2 RESULTS (2_)

- qiime metadata tabulate --m-input-file dada2out/denoising_stats.qza --o-visualization **2_stats-dada2.qzv**
- qiime feature-table summarize --i-table dada2out/table.qza --m-sample-metadata-file metadata.tsv --o-visualization **2_table-dada_metadata.qzv**
- qiime feature-table tabulate-seqs --i-data dada2out/representative_sequences.qza --o-visualization **2_rep-seq-dada2.qzv**

#STEP3: Then we annotate our sequences with the premade silva database: (3_)

- qiime feature-classifier classify-sklearn --i-classifier silva-138-99-nb-classifier.qza --i-reads dada2out/representative_sequences.qza --o-classification **3_taxonomy.qza**

#STEP4: Filtering mitochondria and chloroplast from the table and sequences: (4_)

- qiime taxa filter-table --i-table dada2out/table.qza --i-taxonomy 3_taxonomy.qza --p-exclude mitochondria,chloroplast --o-filtered-table **4_table_nomitclo.qza**
- qiime taxa filter-seqs --i-sequences dada2out/representative_sequences.qza --i-taxonomy 3_taxonomy.qza --p-exclude mitochondria,chloroplast --o-filtered-sequences **4_rep_seqs_nomitclo.qza**

#STEP5:we re-annotate our filtered sequences again We could also filter out those features which do not sum at least 3 sequences among all samples and those that only appear in one sample: (5_)

- qiime feature-classifier classify-sklearn --i-classifier silva-138-99-nb-classifier.qza --i-reads 4_rep_seqs_nomitclo.qza --o-classification **5_taxonomy_nomitclo.qza**

- qiime feature-table filter-features --i-table 4_table_nomitclo.qza --p-min-frequency 3 --p-min-samples 2 --o-filtered-table **5_table_nomitclo_10.qza**

#---

#STEP6:CREATE A BARPLOT BY RANKS AND METADATA GROUPS (6_)

- qiime taxa barplot --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_ALL_taxa_barplot.qzv**

#Can create barplots for particular time points (6_)

- qiime feature-table filter-samples --i-table 5_table_nomitclo_10.qza --m-metadata-file TIME_POINTS_TSV/time0.tsv --o-filtered-table **6_time0_table.qza**
- qiime taxa barplot --i-table 6_time0_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_time0-barplot.qzv**
- qiime feature-table filter-samples --i-table 5_table_nomitclo_10.qza --m-metadata-file TIME_POINTS_TSV/time1.tsv --o-filtered-table **6_time1_table.qza**
- qiime taxa barplot --i-table 6_time1_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_time1-barplot.qzv**
- qiime feature-table filter-samples --i-table 5_table_nomitclo_10.qza --m-metadata-file TIME_POINTS_TSV/time2.tsv --o-filtered-table **6_time2_table.qza**
- qiime taxa barplot --i-table 6_time2_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_time2-barplot.qzv**
- qiime feature-table filter-samples --i-table 5_table_nomitclo_10.qza --m-metadata-file TIME_POINTS_TSV/time3.tsv --o-filtered-table **6_time3_table.qza**
- qiime taxa barplot --i-table 6_time3_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_time3-barplot.qzv**

- qiime feature-table filter-samples --i-table 5_table_nomitclo_10.qza --m-metadata-file TIME_POINTS_TSV/time4.tsv --o-filtered-table **6_time4_table.qza**
- qiime taxa barplot --i-table 6_time4_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_time4-barplot.qzv**
- qiime feature-table filter-samples --i-table 5_table_nomitclo_10.qza --m-metadata-file TIME_POINTS_TSV/time5.tsv --o-filtered-table **6_time5_table.qza**
- qiime taxa barplot --i-table 6_time5_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_time5-barplot.qzv**
- qiime feature-table filter-samples --i-table 5_table_nomitclo_10.qza --m-metadata-file TIME_POINTS_TSV/time6.tsv --o-filtered-table **6_time6_table.qza**
- qiime taxa barplot --i-table 6_time6_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_time6-barplot.qzv**
- qiime feature-table filter-samples –i-table 5_table_nomitclo_10.qza –m-metadata-file TIME_POINTS_TSV/time7.tsv –o-filtered-table **6_time7_table.qza**
- qiime taxa barplot –i-table 6_time7_table.qza –i-taxonomy 5_taxonomy_nomitclo.qza –m-metadata-file metadata.tsv –o-visualization **6_time7-barplot.qzv**

#plots specifically for control samples

- qiime feature-table filter-samples --i-table 5_table_nomitclo_10.qza --m-metadata-file control.tsv --o-filtered-table **6_control_table.qza**
- qiime taxa barplot --i-table **6_control_table.qza** --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata.tsv --o-visualization **6_control-barplot.qzv**
-

#Grouped plots for all samples showing top 11 PHYLA

- qiime taxa filter-table --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-include 'p__Campilobacterota,p__Desulfobacterota,p__Gemmatimonadota,p__Bdellovibrionota,p__Acidobacteriota,p__Chloroflexi,p__Patescibacteria,p__Firmicutes,p__Actinobacteriota,p__Bacteroidota,p__Proteobacteria' --o-filtered-table **Top11_filtered-table.qza**
- qiime feature-table group --i-table Top11_filtered-table.qza --p-axis 'sample' --m-metadata-file metadata.tsv --m-metadata-column "group" --p-mode 'mean-ceiling' --o-grouped-table **6_TOP11_table_grouped.qza**
- qiime taxa barplot --i-table 6_TOP11_table_grouped.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata_grouped.tsv --o-visualization **6_TOP_11_taxa-barplot_grouped.qzv**

#Can group replicates together and then plot them on one boxplot. Note additional metadata file for groups (6_)

- qiime feature-table group --i-table 5_table_nomitclo_10.qza --p-axis 'sample' --m-metadata-file metadata.tsv --m-metadata-column "group" --p-mode 'mean-ceiling' --o-grouped-table **6_table_grouped.qza**

- qiime taxa barplot --i-table 6_table_grouped.qza --i-taxonomy 5_taxonomy_nomitclo.qza --m-metadata-file metadata_grouped.tsv --o-visualization **6_taxa-barplot_grouped.qzv**

#CREATE BIOM AND TSV FILES WITH THE TAXONOMY RESULTS (6_). These give EXCEL readable tables with raw data for breakdown of phyla, genus and species depending on level selected. Export at different level (level 2-7)

- qiime taxa collapse --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 2 --o-collapsed-table **6_phyla-table.qza**
- qiime feature-table relative-frequency --i-table 6_phyla-table.qza --o-relative-frequency-table **6_rel-phyla-table.qza**
- qiime tools export --input-path 6_rel-phyla-table.qza --output-path **6_rel-table_2**
- biom convert -i 6_rel-table_2/feature-table.biom -o **6_relative_2.csv** --to-tsv

#Export at level 3

- qiime taxa collapse --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 3 --o-collapsed-table **6_phyla_3-table.qza**
- qiime feature-table relative-frequency --i-table 6_phyla_3-table.qza --o-relative-frequency-table **6_rel-phyla_3-table.qza**
- qiime tools export --input-path 6_rel-phyla_3-table.qza --output-path **6_rel-table_3**
- biom convert -i 6_rel-table_3/feature-table.biom -o **6_relative_3.csv** --to-tsv
-

#Export at level 4

- qiime taxa collapse --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 4 --o-collapsed-table **6_phyla_4-table.qza**
- qiime feature-table relative-frequency --i-table 6_phyla_4-table.qza --o-relative-frequency-table **6_rel-phyla_4-table.qza**
- qiime tools export --input-path 6_rel-phyla_4-table.qza --output-path **6_rel-table_4**
- biom convert -i 6_rel-table_4/feature-table.biom -o **6_relative_4.csv --to-tsv**

#Export at 5

- qiime taxa collapse --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 5 --o-collapsed-table **6_phyla_5-table.qza**
- qiime feature-table relative-frequency --i-table 6_phyla_5-table.qza --o-relative-frequency-table **6_rel-phyla_5-table.qza**
- qiime tools export --input-path 6_rel-phyla_5-table.qza --output-path **6_rel-table_5**
- biom convert -i 6_rel-table_5/feature-table.biom -o **6_relative_5.csv** --to-tsv

#Export at level 6

- qiime taxa collapse --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 6 --o-collapsed-table **6_phyla_6-table.qza**
- qiime feature-table relative-frequency --i-table 6_phyla_6-table.qza --o-relative-frequency-table **6_rel-phyla_6-table.qza**
- qiime tools export --input-path 6_rel-phyla_6-table.qza --output-path **6_rel-table_6**
- biom convert -i 6_rel-table_6/feature-table.biom -o **6_relative_6.csv** --to-tsv

#Export at species level 7

- qiime taxa collapse --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 7 --o-collapsed-table **6_phyla_7-table.qza**
- qiime feature-table relative-frequency --i-table 6_phyla_7-table.qza --o-relative-frequency-table **6_rel-phyla_7-table.qza**
- qiime tools export --input-path 6_rel-phyla_7-table.qza --output-path **6_rel-table_7**
- biom convert -i 6_rel-table_7/feature-table.biom -o **6_relative_7.csv** --to-tsv

#----------

#ALIGNMENT AND PHYLOGENETIC RECONSTRUCTION (7_). Needed for Diversity analyses below

- qiime alignment mafft --i-sequences 4_rep_seqs_nomitclo.qza --o-alignment **7_alignen_rep_seqs_nomiclo.qza**
- qiime alignment mask --i-alignment 7_alignen_rep_seqs_nomiclo.qza --o-masked-alignment **7_masked_alig_rep_seqs_nomitclo.qza**
- qiime phylogeny fasttree --i-alignment 7_masked_alig_rep_seqs_nomitclo.qza --o-tree **7_unrooted_tree_nomitclo.qza**
- qiime phylogeny midpoint-root --i-tree 7_unrooted_tree_nomitclo.qza --o-rooted-tree **7_rooted_tree_nomitclo.qza**

#----------

#Alpha rarefaction. Ensures that sequencing depth is adequate. A flat curve signifies this (8_)

- qiime diversity alpha-rarefaction --i-phylogeny 7_rooted_tree_nomitclo.qza --i-table 5_table_nomitclo_10.qza --p-max-depth 10000 --o-visualization **8_alpha_rare.qzv**

#DIVERSITY plots(8_)

#Applies a collection of diversity metrics (both phylogenetic and non-phylogenetic) to a feature table.

#The parameter "sampling-depth" is taken from the 2_table-dada_metadata.qzv file:

- qiime diversity core-metrics-phylogenetic --i-phylogeny 7_rooted_tree_nomitclo.qza --i-table 5_table_nomitclo_10.qza --p-sampling-depth 10000 --m-metadata-file metadata.tsv --output-dir **8_core_metrics_phylogenetic**
- qiime diversity core-metrics-phylogenetic --i-phylogeny 7_rooted_tree_nomitclo.qza --i-table 6_control_table.qza --p-sampling-depth 10000 --m-metadata-file metadata.tsv --output-dir **8_control_core_metrics_phylogenetic**

#----------

#Calculate group significance (9_)

#From the results in "core-metrics-phylogenetic" we can visualized different diversity methods.

#Alpha diversity methods: Shannon's diversity index, Observed OTUs, Faith's Phylogenetic Diversity and Evenness.

#Beta diversity methods: Jaccard distance, Bray-Curtis distance, unweighted UniFrac distance and weighted UniFrac distance.

- qiime diversity alpha-group-significance --i-alpha-diversity 8_control_core_metrics_phylogenetic/faith_pd_vector.qza --m-metadata-file metadata.tsv --o-visualization 8_control_core_metrics_phylogenetic/**9_faith_pd_group_significance.qzv**
- qiime diversity alpha-group-significance --i-alpha-diversity 8_control_core_metrics_phylogenetic/evenness_vector.qza --m-metadata-file metadata.tsv --o-visualization 8_control_core_metrics_phylogenetic/**9_evenness_group_significance.qzv**
- qiime diversity alpha-group-significance --i-alpha-diversity 8_control_core_metrics_phylogenetic/shannon_vector.qza --m-metadata-file metadata.tsv --o-visualization 8_control_core_metrics_phylogenetic/**9_shannon_group_significance.qzv**
- qiime diversity beta-group-significance --i-distance-matrix 8_control_core_metrics_phylogenetic/unweighted_unifrac_distance_matrix.qza --m-metadata-file metadata.tsv --m-metadata-column group --o-visualization 8_control_core_metrics_phylogenetic/**9_unweighted_unifranc_group_significance_PW.qzv** --p-pairwise
- qiime diversity beta-group-significance --i-distance-matrix 8_control_core_metrics_phylogenetic/bray_curtis_distance_matrix.qza --m-metadata-file metadata.tsv --m-metadata-column group --o-visualization 8_control_core_metrics_phylogenetic/**9_bray_curtis_significance_PW.qzv** --p-pairwise

#----------

#ANCOM: Analysis of composition of microbiomes (10_)

#We can choose the taxonomy level with the parameter "p-level". For example genus level=6

- qiime taxa collapse --i-table 5_table_nomitclo_10.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 3 --o-collapsed-table **10_ALL_table_L3.qza**
- qiime composition add-pseudocount --i-table 10_ALL_table_L3.qza --o-composition-table **10_ALL_comp_table_L3.qza**
- qiime composition ancom --i-table 10_ALL_comp_table_L3.qza --m-metadata-file metadata.tsv --m-metadata-column group --o-visualization **10_ALL_ancom_L3.qzv**
- qiime taxa collapse --i-table 5_control_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 4 --o-collapsed-table **10_control_table_L4.qza**
- qiime composition add-pseudocount --i-table 10_control_table_L4.qza --o-composition-table **10_control_comp_table_L4.qza**
- qiime composition ancom --i-table 10_control_comp_table_L4.qza --m-metadata-file metadata.tsv --m-metadata-column group --o-visualization **10_control_ancom_L4.qzv**
- qiime taxa collapse --i-table 6_time0_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 5 --o-collapsed-table **10_T0_table_L5.qza**
- qiime composition add-pseudocount --i-table 10_T0_table_L5.qza --o-composition-table **10_T0_comp_table_L5.qza**
- qiime composition ancom --i-table 10_T0_comp_table_L5.qza --m-metadata-file metadata.tsv --m-metadata-column group --o-visualization **10_T0_ancom_L5.qzv**
- qiime taxa collapse --i-table 6_time1_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 6 --o-collapsed-table **10_T1_table_L6.qza**
- qiime composition add-pseudocount --i-table 10_T1_table_L6.qza --o-composition-table **10_T1_comp_table_L6.qza**
- qiime composition ancom --i-table 10_T1_comp_table_L6.qza --m-metadata-file metadata.tsv --m-metadata-column group --o-visualization **10_T1_ancom_L6.qzv**
- qiime taxa collapse --i-table 6_time2_table.qza --i-taxonomy 5_taxonomy_nomitclo.qza --p-level 7 --o-collapsed-table **10_T2_table_L7.qza**
- qiime composition add-pseudocount --i-table 10_T2_table_L7.qza --o-composition-table **10_T2_comp_table_L7.qza**

Appendix 9.4: Genome announcement for the novel biocontrol strain, *Bacillus velezensis* Kos, investigated during this study

Genome sequence of *Bacillus velezensis* Kos

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Abstract

Here, we report the draft genome sequence of *Bacillus velezensis* strain Kos, isolated from casing soil used during *Agaricus bisporus* cultivation in Dublin, Ireland. *B. velezensis* Kos exhibits a suppressive ability towards *Cladobotryum mycophilum*, *Trichoderma aggressivum* and *Lecanicillium fungicola* which are common threats to *A. bisporus* production, cultivation, and quality.

Announcement

Several fungal pathogens pose a significant threat to the commercially important white mushroom, *Agaricus bisporus* (1). Historically, the use of chemical fungicides has been used to prevent yield reductions and disease outbreaks. Due to environmental/health considerations, there is now pressure to reduce fungicide use (2). The future of mushroom disease treatment will depend upon integrated pest management, including the use of biological control agents (BCAs) (3). Here we report

the draft genome sequence of the novel strain, *Bacillus velezensis* Kos, which has been shown to have potential as a BCA for mushroom disease (4-6).

The Kos strain was originally isolated during *A. bisporus* cultivation in Dublin, Ireland (global positioning system coordinates 53.38 N 6.33 W") and taxonomical identification showed that Kos is *Bacillus velezensis* (6) which we further confirmed using the average nucleotide identity (see below). *B. velezensis* Kos plate cultures were grown on nutrient agar (Thermo Scientific™ Oxoid™) at 30°C for 24 hr. A loopful of culture from plate cultures was added to 50 ml nutrient broth (Thermo Scientific™ Oxoid™) and grown for 24 hr at 30°C, 120 rpm. Genomic DNA was extracted using the quick-DNA fungal/bacterial miniprep kit (Zymo Research). The genome was sequenced by Novogene Co. Ltd., with the DNA library prepared using the Novogene NGS DNA library prep set in which the DNA was randomly sheared, end repaired, A-tailed, and then ligated with Illumina adaptors. These sequences were amplified using PCR, and DNA of 350 bp was selected, purified, and sequenced using 150-bp Illumina paired-end sequencing on the Illumina NovaSeq platform. Reads with adapters and low quality were trimmed using Skewer (v0.2.2) (7).

In total 12,619,076 high quality paired end reads were obtained and initially assembled and then annotated using NCBI's Read Assembly and Annotation Pipeline Tool with the default settings (rapt-45639894). RAPT utilizes the SKESA (v2.5.1) genome assembler (8), the average nucleotide identity (ANI) tool (9) to assign taxonomy and the Prokaryotic Genome Annotation Pipeline (build6771) (10) to functionally annotate the assembly. Genome quality and potential contamination is also assessed using CheckM (ver2015-01-16) (11). In total the assembly size is 4,194,762 nucleotides in length with a GC content of 45.8 %. The N50 and L50 score are 573,424 and 3 respectively. CheckM showed 98.82% genome completeness and 0% contamination. The longest contig is 1,085,863 nucleotides and there are 30 contigs in total. The ANI with its closest strain, *B. velezensis* NRRL B-41580 (12), was 98.264%. A total of 4,248 genes were predicted, including 4,066 protein-coding genes, 99 RNA genes (12 rRNAs, 82 tRNA & 5 noncoding RNA genes), and 83 pseudogenes. The potential production of secondary metabolites by Kos was analysed using the antiSMASH tool (ver 7.0) with the default setting (13). Genomic clusters with the potential for the biosynthesis of antimicrobial secondary metabolites were predicted. These clusters

involve genes encoding surfactin, subtilin, bacillibactin, bacilysin, fengycin, bacillaene and macrolactin.

The genome sequence of *B. velezensis* Kos will help uncover the molecular mechanisms of pathogen suppression and increase its applications in the mushroom industry.

Data availability statement:

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JBDOQF000000000. The version described in this paper is version JBDOQF010000000. The raw Illumina reads are available at ENA/SRA under the accession number SRX24592991.

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