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Control of the lettuce anthracnose (Microdochium panattonianum Berl.) using Trichoderma spp. and their secondary metabolites

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Control of the lettuce anthracnose (*Microdochium panattonianum* Berl.) using *Trichoderma* spp. and their secondary metabolites

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This thesis comprises only my original work towards the Ph.D. and no material for other degree in any other University.

To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person except when due reference is made in the text, and

This thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies, and appendices.

Acknowledgements

During these three years I have left my comfort zone so many times that now I do not know what discomfort is. Perhaps that is the main ingredient every researcher needs to face before discovering science.

Science, for me is the truth, the truth of how things work, it is unique. It has already been created not invented; science is in the things that surround us. We humans do not do science, we discover it at the end of every experiment. Tiny pieces of the universal truth come along and coexist with us but also, they may be refuted, of course, reminding us how incipient is our knowledge.

My path to find this little piece of science was not only through laboratories and fields but also washing dishes and cleaning toilets and I sincerely thank to every person who supported me when I was at the bottom.

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Summary

The main objectives of this project were to: 1) show that lettuce anthracnose propagules that persist in soil from a previous lettuce crop may be controlled by a cold tolerant strain of Trichoderma inoculated just before the pathogen becomes active and virulent, reducing incidence and severity, and, 2) determine if liquid cultures containing Trichoderma secondary metabolites (TSMs) can be sprayed for maximum leaf coverage to reduce the incidence and severity of the disease when condition for disease are ideal.

Lettuce anthracnose *Microdochium panattonianum* (MP) is a key winter disease in Victoria, Australia, and other temperate regions in the world. Cultural methods to reduce the severity of this disease are not practical nor economically feasible. While chemical control can be achieved it is not flawless and can cause health issues.

Propagules of MP persist in soil sheltered in leaf debris and germinate with the onset of cold and wet conditions in the subsequent winter. Unfortunately, this pathogen has received little scientific attention but as winters become wetter this disease is causing greater loss and this body of work offers a more sustainable management tool.

Trichoderma species are common saprophytic fungi in soils rich in organic matter. They have a proven ability to control crop diseases with a multiplicity of strategies including parasitism, competition, antibiosis and, at the same time, stimulating plant defences and growth. However, these natural processes vary with species and strains of Trichoderma.

This research project showed that the incidence and severity of the lettuce anthracnose can

be reduced by applying Trichoderma cold tolerant strains to the soil. Secondly, it showed that spraying Trichoderma liquid filtrates containing metabolites to the leaves also reduced and managed lettuce anthracnose.

From a pool of 27 Trichoderma isolates, 8 grew at 10°C. These 8 isolates were characterised morphologically *in vitro* under different culturing conditions of temperature, pH and media.

Some isolates were identified by molecular techniques to species while others still require more investigation.

Tests *in vitro* on solid media prepared with liquid filtrates confirmed that liquid filtrates from one isolate related to *T. viride* and one from *T. composticola* affected the morphology and growth of MP. These filtrates contained 6 pentyl-alpha-pyrone (6PP,) a volatile metabolite with a characteristic coconut smell.

Other Trichoderma liquid filtrates containing metabolites such as cytosporone S and 6PP, produced by some strains of *T. aureoviride*, *T. atroviride* and *Trichoderma* sp., killed the pathogen.

Fourteen isolates produced 6PP, a well-documented Trichoderma secondary metabolite (TSM). Of these, some have been identified as T. *viride*, *T. composticola*, *T. atroviride*, *T paratroviride*, *T. neokoningii* and *T. asperelloides*. Others are yet to be fully identified to species. Isolates of *T. harzianum*, *T. polysporum*. *T aureoviride, T. longibrachiatum* and *T. spirale* did not produce this metabolite.

Isolates of Trichoderma, TC1, CAROL, 71558 and 1536 require further identification because they were outliers on the phylogenetic tree. None of these isolates produced 6PP.

Challenged isolates produced higher quantities of 6PP than did the individual liquid cultures. *Trichoderma atroviride* (PMF) from New Zealand produced 137 mg/l of 6PP, but when challenged by *Trichoderma paratroviride* (NSW) the 6PP concentration was 160 mg/l.

Incidence and severity of MP were reduced significantly in a field trial using the cold tolerant isolate 356 related to *T. composticola.*

TSMs applied as foliar sprays have the potential to control many foliar diseases in crops and is a new field of research.

The use of Trichoderma cold tolerant strains for control of winter active pathogens is a new strategy for the use of Trichoderma products.

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6PP:	6-pentyl-alpha-pyrone
ABC:	Australian broadcasting corporation
Acl1:	ATP-citrate synthase sub-unit 1
BCA:	Biocontrol agent
CWDE:	Cell wall degrading enzyme
CZA:	Czapek dox agar
DAD:	Diode array detector
DAI:	Days after inoculation
ESI:	Electro spray ionisation
HA:	Harzianic acid
ISR:	Induced systemic resistance
JAS:	Jasmonic acid
LA:	Lettuce anthracnose
LC-q-*tof*- MS:	Liqui chromatography, quadrapole, time of flight, mass spectrometry
MP:	*Microdochium panattonianum*
MRL:	Maximum residue level
PDA:	Potato dextrose agar
PDB:	Potato dextrose broth
Rpb2:	RNA polymerase II
SAR:	Systemic acquired resistance
SARDI:	South Australia research and development institute
ET:	Ethylene
Tef:	Translation elongation factor
TSM:	Trichoderma secondary metabolite
VCG:	Vegetative compatibility group
VIT:	Vegetative incompatibility test

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Chapter 1

GENERAL INTRODUCTION

Like many other human activities, agricultural practices around the world are becoming non-sustainable because they consider only maximum profits and not a balance between productivity and land protection. Many farmers and agricultural scientists have lost understanding of the complexity of interactions between soil, water and air and their impact in sustainable agricultural production. In some situations, contamination has passed the point where soil biology can repair the damage in a reasonable time frame (Lal et al. 1997).

Natural methods or at least more sustainable ways to prepare and fertilise soils and manage pests, weeds, and diseases are available already. These contrast with the high input system that for decades has focused on maximum yields but actually created serious problems for productivity and led to an ecological crisis (Rosset and Altieri 1997).

Farmers and the whole horticulture industry are under pressure as retail distributors demand "perfect" fruits and vegetables while convincing consumers that visual perfection is the best and safest option. This is not always correct! Retailers have strict specifications for size, minimum disease damage and insect infestation and these conditions produce unnecessary food waste as explained by Craig Reucassel (2017) in his ABC TV series "war on waste".

During more than 25 years of experience as a field production agronomist, I have witnessed many batches of wasted fresh, almost perfect product, rejected from market. Produce is returned to the farms to be destroyed or destroyed by supermarkets at farmers' cost. This situation pushes farmers to increase chemical use to minimise those "imperfections" so that

they protect their investment.

For many years, it was thought that health problems related to agrochemical exposure were related only to farm workers or farmers (World Health Organisation 1990), but the sad fact is that currently there is not a human group that is completely unexposed to pesticides (Kim 2017).

Risks implicated with pesticide residues in fruits and vegetables are well documented. Cancer (Caron-Beaudon et al. 2016), leukemia, diabetes, Parkinson's disease and asthma (Kim 2017) and even mental disorders such as ADHD (Attention deficit hyperactivity disorder) (Wagner-Schuman et al. 2015) and reduced cognitive performance in children and adults (Viel et al. 2015). Exceptionally low levels of exposure have more dramatic effects at early human development stages making children more susceptible than adults (Mascarelli 2013).

Among the vegetable crops in Australia, lettuce was the sixth largest in 2009 with 7411 ha, increasing to 8000 ha in 2015. Victoria grows 50% of the Australian lettuce production (Australian Bureau of Statistics 2014-15).

Lettuce anthracnose (MP) *Microdochium panattonianum* (Berl), is a winter plant pathogen that has received minimal research efforts. This organism produces a foliar disease commonly known as "shot hole" that can cause extensive losses in wet and cold conditions in both head and loose-leaf lettuce. It is found in most of the lettuce cropping areas around the world. In Australian temperate regions it is noticeably more destructive since 2009 when winters turned from relatively dry to very wet. The unusually cold and wet conditions during

2010-2011 winter-spring period in south eastern Australia made it nearly impossible for growers of outdoor lettuce to avoid severe losses due to anthracnose. (Rogers and Kimptom 2012).

Development of tolerant varieties of lettuce is an option but has not yet been developed for this pathogen. Seed companies still do not consider “shot hole” sufficiently important for cost effective investment. However, change in global weather conditions will make it necessary to find alternatives to chemical sprays for control of this and other crop diseases.

This disease can be controlled effectively only with the chemical, Prochloraz (Rogers and Kimptom 2012), a fungicide from the imidazole group that has been found to feminise offspring of male rats after perinatal exposure by acting as an antiandrogenic (Vingaard et al. 2006) and causing cryptorchidism - one or two of the testicles not descending from abdomen to scrotum when in foetus stage - in sons of Danish female gardeners exposed to this chemical (Weidner et al. 1998).

It is estimated that an average crop cycle of a winter lettuce in Victoria is 12 weeks from transplant to harvest and during this period, plants are exposed to ideal conditions for expression of the disease; i.e. temperatures below 15°C and wet leaves for at least 8 hours. As a result, farmers spray Prochloraz several times for a successful crop; some of them, desperate to protect their investment, spray to the limit of the withholding period established on label of 7 days.

Maximum residue levels (MRL) of this chemical for closed head and loose leaf lettuce are 2 and 3 mg/kg respectively, according to the Australian Agricultural and Veterinary

Chemicals (APVMA) code instrument N° 4 (MRL) standard 2012 (last compilation 24th May 2019), which means that it is legal to commercialise lettuce with residues as high as these values, not necessarily nil. Meanwhile, over a million Australians regularly purchase organic fruit and vegetables and beverages to reduce the risks implicated. According to the Australian Bureau of Statistics, 2075 organic certified producers were reported in 2017, and by 2019, the total value of organic production reached AU$ 2.6 billion (Australian Organic Market Report 2019). Most of this produce is exported to China, the USA, Japan, South Korea, and Singapore.

The solution is in the soil, but how can soil help?

Soils have four essential functions: soils sustain biomass production and biodiversity by a huge pool of genes; regulate water and air quality; preserve archaeological, geological, and astronomical records; and support of socioeconomic infrastructure. The first two of these are crucial for environmental and agricultural processes (Lal et al. 1997). Soils provide physical and nutritional support for crops and animal production. They are also environmental purifiers, detoxifying and neutralising poisons, complexing heavy metals, filtering water, air and themselves, and finally, providing a genetic bank of information in the form of microbial diversity that has the potential to solve many agricultural problems (Lal et al. 1997).

Many of those soil organisms that contribute to the genetic pool are identified as biological agents to control plant diseases. They use several mechanisms to reduce crop disease incidence and severity. One of these strategies is the production and release of secondary metabolites, biological compounds that are a fundamental part of their relationship with

other organisms. Some examples of the use of these metabolites include Penicillin, a group of antibiotics isolated from soil fungi (Gaynes 2017); the insecticide, Spinosad, employed broadly in horticulture against caterpillars, isolated from a bacterial species, *Saccharospolyspora spinosa*, and Abamectin, a powerful miticide and insecticide, produced from fermented broths of the actinobacterium, *Streptomyces avermitis* (Mujica et al. 1999).

Research into these important natural substances demands standardisation of techniques, including inoculum preparation and size, nature of the growth media, incubation, and culturing conditions and, finally, modern instrumentation (Balouiri et al. 2015). Scientific instrumentation has evolved and produced sophisticated equipment and techniques that facilitate the identification of these compounds to molecular levels opening a vast area of applied research with potential practical uses in agriculture.

Using soil biota and their metabolites as part of a strategy to control diseases in crops is a new paradigm. Additionally, selective production and use of these active compounds demand understanding that a single strain of organism is unable to solve all crop health problems all the time under all conditions. In every agricultural environment, a meticulous selection of strains must be performed prior to their application in the field.

Biological control in plant pathology is the purposeful utilisation of living organisms, other than crop resistant varieties, to supress the activity and population of one or more plant pathogens (Pal et al. 2006). In a simpler way is the use of microorganisms to control plant pathogens (Parnell et al. 2016). Trichoderma species are one of the most studied biological control agents; they are widespread opportunistic soil inhabitants, feeding on decomposed organic matter (Brožová 2004) and parasitising and killing other microorganisms

(Druzhinina et al. 2011). They can adapt effectively and occupy a foreign ecological niche and produce secondary metabolites, small molecules that are not involved directly in growth but are important in signalling, developing, and establishing relationships with other organisms. Antibiotics and enzymes with antimicrobial properties are included in this list (Hoffmeister and Keller 2007; Mukherjee et al. 2012). However, not all the species and strains of Trichoderma are adapted to all conditions of temperature, soil pH, soil moisture where they exert control capacity. The efficacy of Trichoderma propagules to survive, germinate and colonise leaves and control crop foliar diseases, for instance, lettuce anthracnose, depends additionally on many other factors such as leaf exudates, relative humidity, free water, light, wind, pollution and sprayed chemicals all of which are dynamic variables (Elad and Kirshner 1992). Using effective secondary metabolites that are not affected by environmental conditions to be sprayed on leaves can be a significant contribution in the strategies to control foliar diseases.

Trichoderma spp. and their secondary metabolites are proposed in this thesis because they constitute a novel strategy to control diseases in crops rather than only applying their reproductive structures such as conidia and chlamydospores on soil. The combined strategy deserves more attention.

This project had two hypotheses:

1. Living microbial control agents (such as Trichoderma) can be used for the control of lettuce anthracnose (LA) inoculum in soil.

2. Trichoderma secondary metabolites (TSMs) produced in liquid cultures can be used as a preventative/curative foliar spray to reduce LA incidence and severity on lettuce leaves

These hypotheses will be validated by:

- Molecular identification resulting in a phylogenetic tree.
- Practical applications evaluated in pots and field experiments

With reference to hypothesis one, the aims are:

- Gathering Trichoderma isolates from native sources and commercial formulations;
- Characterising isolates in different culturing conditions providing a broader idea of how the environment can alter their morphology and behaviour.

With reference to hypothesis two, the aims are:

- To test incompatibility of Trichoderma isolates in couplets to predict antagonistic activity and maximum TSM production;
- Culturing incompatible couplets from vegetative incompatibility test, filtering, identifying and quantifying TSMs by LC-q-*tof*- MS technique.

This research project started with the isolation of Trichoderma species across Australia taking advantage of the diverse climate and soil conditions. A collection was established based on single conidia cultures. The isolates were characterised observing morphological

characteristics and their responses to different culturing temperatures, pH and media composition. Finally, the isolates were identified molecularly and located in a phylogenetic tree. Cold tolerant isolates were selected successfully and tested in vitro, pots and field experiments. In pots and field, two application techniques were tested, the standard conidial spray, and the use of compost as carrier of mycelia. At the same time, vegetative compatibility tests provided evidence that several isolates cannot share the same substrate; these couplets were cultured in liquid media to determine if that incompatibility allowed them to produce more TSMs. Some of these liquid cultures were tested *in vitro*, and one of them in an actual commercial lettuce field comparing it with copper sulphate, the standard practice to control LA in this farm.

In general, the overall aim of this research is to validate an integrated strategy to control lettuce anthracnose using Trichoderma isolates and their metabolites.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

There is little research to find alternatives for control of lettuce anthracnose, *Microdochium panattonianum* (Berg.) in Australia. Control of this pathogen even using chemicals is a challenge because of the architecture of lettuce plants, especially the closed head types like Romaine. Tight heads protect pathogenic conidia in the centre of the plants that are transported from soil by rain splash before lettuce heads close. They incubate and start infection protected from the environmental conditions and chemical spray. As the plants mature the infection moves from the core to the outer leaves where conidia are released to the environment and infect surrounding plants in a relatively short time (Moline and Polack 1975).

No reports are available of biological control agents or cultural practices being used to control MP other than removing sick plants before incorporation into soil post-harvest. Resistant varieties are not available yet because this disease is considered lower priority than resistance to, for example, berry-aphid, *Nasonovia ribisnigri*, or downy mildew (Rogers and Kimpton 2012). However, some lettuce cultivars like Lotus (RZ) and Zazu (Terranova) appear more tolerant than others to anthracnose. Spraying "soft" compounds such as copper sulphate, lime sulphur and other sulphur formulations are common practices among the farmers, especially the organic farmers to reduce the disease impact in the winter lettuce crops. The use of compost as a potential soil amendment to control soil borne diseases is not fully understood by commercial farmers; those who apply compost are focused mainly in the improvement of the physical and chemical properties of soil but not

in the suppressive diseases properties of composts.

2.2 The Pathogen

Lettuce anthracnose (LA), *Microdochium panattonianum* (Berl) previously, *Marssoninia panattoniana* (Berl). Sutton, Galea and Price (Galea et al. 1986) is a leaf pathogen congeneric with *Microdochium nivale*, *Microdochium. oryzae* and *Microdochium stoveri,* that causes extensive damage in temperate regions of Australia, New Zealand, Europe, and Asia in winter seasons (Rogers and Kimpton 2012).

After an incubatory period of at least 8 hours at 15° C or less, and wet leaves, conidia (Fig. 2.1) germinate, produce appressoria and penetrate the leaf tissue causing white spots that become pink when sporulating in midribs (Paterson and Grogan 1991) or round yellow spots on leaf blades; both finally turn brown with white edges (Galea and Price 1988a) (Fig. 2.2). Damage is initially more evident on the lower leaves (Paterson and Grogan 1991). Finally, as a mechanism of plant defence, wound edges could be healed; dead tissue is dropped leaving a hole which gives the disease common name “shot hole” (Moline and Pollack 1975).

In field, the disease commonly starts in randomly distributed foci of one to several plants (Paterson and Grogan 1991). In post-harvest, lettuce heads can also develop anthracnose and in cold storage and cause disease affecting the shelf life of the product (Moline and Pollack 1975).

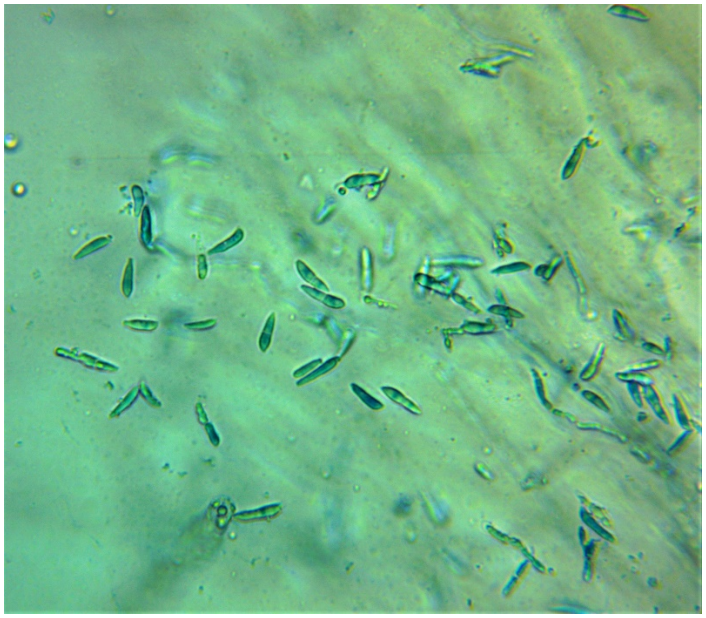


Fig. 2.1 *M. panattonianum* bicelled conidia (X400)

Fig. 2.2 Lettuce plant with typical symptoms of anthracnose including the “shot hole”

In spring as air and soil temperatures rise, this pathogen becomes inactive and its propagules such as conidia and microsclerotia lie dormant in soil. Microsclerotia have been found in cells of lettuce plants and remain in debris until they are released after the tissues become necrotic (Paterson and Grogan 1991). The microsclerotia can survive for several years in the soil (Krnjaja and Ivanovic 1996). However, conidial life span is short, approximately 20 weeks (Rogers and Kimpton 2012). A common lettuce farming practice involves the incorporation of sick plant residues into the soil by ploughing, thereby removing the need to dispose of residue off-site and saving money but spreading the microsclerotia inoculum in the soil for the next season (Paterson and Grogan 1991).

Longevity of MP inoculum depends on soil conditions such as temperature and moisture and the presence of lettuce crop debris. Unidentified fungistatic substances in soil that are not present after soil pasteurisation were found to stop MP conidial germination (Galea and Price 1988b). These authors suggested that more work to identify these substances and microorganisms producing them is needed.

Disease life cycle of MP shows there are several critical points where integrated practices can be implemented to reduce the MP disease incidence and severity. Control practices may include removing residues of infected crop, avoiding soils where this disease has been particularly virulent the year before, use of more tolerant or faster maturing varieties (Rogers and Kimpton 2012), avoiding August - September cropping and employing biological control agents (BCAs).

2.3 The biological control agents

Of many fungal species used as biocontrol agents (BCAs), Trichoderma species are the most researched and widely used in commercial practice. Since their first application in the 1930's, *Trichoderma* spp. have become popular BCAs to protect plants against diseases all over the world (Ha 2010). They occur naturally in contrasting environmental conditions such as dry and hot soils in Libya (Abadi 2008), wet and cold tundras in the Himalayas (Ghildiyal and Pandey 2008), in the amazon rainforest soils (Delabona et al. 2012) and in soils in Australia (Wong et al. 2002). Recently, Trichoderma species have been isolated from marine conditions (Mukherjee et al. 2013). They are found colonising rhizospheres (rhizosphere competence) (Ahmad and Baker 1988) and growing in plant roots in symptomless infection (endophytes) (Druzhinina et al. 2011). Apparently, the rhizosphere-competent Trichoderma strains are more effective for suppressing plant pathogens in a wider spectrum of environmental conditions than the rhizosphere-incompetent strains (Brožová et al. 2004). In a co-evolutionary process (Mukherjee et al. 2013), plants allow Trichoderma to colonise their roots by detecting specific compounds (Woo and Lorito 2006) such as auxin-like and proteinaceous metabolites that allow fungi to penetrate plant tissues (Djonovic et al. 2006). *Trichoderma* spp. are also found growing, to a lesser extent, on aerial parts of plants (phyllosphere competence) (Elad and Kirshner 1992).

Trichoderma spp. are characterised *in vitro* by fast growing colonies with white, green, or yellow cushions (Fig. 2.3). In soil, they are usually found at 10^1-10^3 colony forming units (cfu) per gram of soil (Abadi 2008; Asha et al. 2013).

Scientific investigation of *Trichoderma* spp. has established their link to a variety of

mechanisms to reduce incidence and/or severity of soil borne diseases such as *Rhizoctonia solani* (Anees et al. 2010); *Pythium aphanidermatum*, (Jeyaseelan et al. 2012); *Sclerotinia sclerotiorum,* (Geraldine et al. 2013, Elias et al. 2017) and *Fusarium solani* (Koutecka and Druskova 1998). *M. nivale* was controlled by a cold tolerant *Trichoderma atroviride* in its natural cold environment (McBeath 2002).

Fig 2.3 Sporulating *Trichoderma longibrachiatum* colony cultured on PDA media. Notice the yellow pigmentation in the media produce by the fungus.

The antagonistic ability of *Trichoderma* spp. is not only restricted to the control of fungal crop pathogens. Some strains of *T. harzianum* and *T. longibrachiatum* have been found colonising eggs and juvenile stages of the plant pathogenic nematode, *Meloidogyne incognita* (Herrera-Estrella et al. 2016).

De Paula et al. (2012) stated that Trichoderma applications were not recommended to control the white mould, *S. sclerotiorum*, in beans when average temperatures were below 20°C in the Brazilian fall-winter season. They concluded that the conditions during the

experiment favoured the pathogen more than the antagonist. In a similar experiment, two isolates of Trichoderma were selected for their ability to control sclerotia from *S. sclerotiourum* in the same crop under similar temperature conditions (Morandi et al. 2007). These authors referred to these isolates as 'cold tolerant' Trichoderma isolates.

Because Trichoderma strains vary in their tolerance to cold, isolates must be selected for their activity under specific pathogen, environmental and crop conditions that promote disease (Nelson 1991), the "pathosystem" (Fravel 2005).

In terms of Trichoderma nutrition, Khattabi et al. (2004) looking for alternatives for the effective control of sugar beet root rot, tested nitrogenous compounds such as urea, nitrate, ammonium and horse manure to evaluate the best combination to deplete *Sclerotium rolfsii* growth by enhancing Trichoderma antagonist activity. Urea contributed to pathogen growth inhibition, but nitrate and ammonium stimulated it. Horse manure was the best to promote Trichoderma activity, reducing pathogen growth and controlling the disease.

2.3.1 The beneficial contribution of Trichoderma on plant

It has been demonstrated that some Trichoderma species help to improve plant health by stimulation of the induced systemic resistance (ISR), and plant growth directly or indirectly (Vinale et al. 2008). *T. virens* was reported to induce production of phytoalexins in cotton plants improving their defence metabolism (Hanson and Howell 2004). The ISR is a mechanism not dependent on production of salicylic acid as the systemic acquired resistance (SAR) is, but on another two plant hormones, jasmonic acid (JAS) and ethylene (ET) (Pieterse et al. 2014). ISR is based on stimulation of enhanced sensitivity of tissues to

JAS/ET rather than an increase in their synthesis (Pieterse et al. 2014). SAR and ISR cross communicate enhancing the defence capacity of the plant (Mukherjee et al. 2013) and are produced by different groups of microorganisms (Köhl et al. 2019) including beneficial rhizobacteria (Harman 2000).

Clear evidence of Trichoderma activating ISR was found by Manganiello et al. (2018) when they achieved significant reduction in levels of infection by *R. solani* in tomato seedlings by up-regulation of genes related to ISR. They compared the effect of seed inoculated with conidia and leaves sprayed with harzianic acid, a secondary metabolite produced by *T. harzianum* M10, initially isolated in Western Australia. The level of plant protection was significantly higher in both when leaves were sprayed and comparable with the protection obtained when the seed was pre-inoculated with conidia. The outcome of the resistance mechanism mediated by a BCAs depends on the balance between growing conditions for the BCA, the plant physiological stage and the specific pathogen (Köhl et al. 2019).

Phytohormones such as indole acetic acid (IAA) related compounds are induced by Trichoderma (Contreras-Cornejo et al. 2009). Some strains of Trichoderma induce root branching and higher shoot biomass in response to the synthesis of fungal auxin-like compounds (Contreras-Cornejo et al. 2016). More recently (Lombardi et al. 2018) found that stressed tomato roots attracted Trichoderma by a specific and enhanced chemotropism mechanism not recognisable by pathogens.

Harman (2000) reported higher corn yields and less nitrogen input using seeds treated with *Trichoderma harzianum* T22 compared with untreated seeds. A similar response was obtained in lettuce crops using *T. virens* GV41 and *T. harzianum* T22; increasing yield and

nitrogen uptake (Fiorentino et al. 2018). Altomare et al. (1999) reported improved solubility of nutrients in vitro using both *T. harzianum* T22 culture and media treated with its filtrates. They concluded that the increased bioavailability was based on chelation and reduction processes rather than on increased acidity of media. Cucumber plants cultured in hydroponic conditions treated with *T. harzianum* T203 also showed significantly increased uptake and concentration of copper, manganese, phosphorus, and sodium in roots (Yedidia et al. 2001). Salinity tolerance (Contreras-Cornejo et al. 2014) and drought tolerance (Contreras-Cornejo et al. 2009) were enhanced by using Trichoderma applications.

2.3.2 Mechanisms of Trichoderma biocontrol

Trichoderma spp. are competitors for food sources and space. Mycoparasitism, antibiosis and resistance induction are the main mechanisms Trichoderma species use to exert disease control (Ghisalberti and Sivasithamparam 1991; Vinale et al. 2017). Antibiosis by Trichoderma is a natural process mediated by Trichoderma secondary metabolites (TSMs) synthesis that includes chemically diverse substances and cell wall degrading enzymes (CWDE) (Ghisalberti and Sivasithamparam 1991). These antibiotics and enzymes often work synergistically (Woo et al. 2006) and are linked to specific genes that could not be expressed under laboratory conditions when they are in pure culture (Mukherjee et al. 2012). This is because only a small set of genes producing TSMs are activated *in vitro*. The remaining genes are only activated using specific elicitors (Vinale et al. 2017). Elicitors include the presence of pathogen cell walls in the media as the challenging mechanism. Both hydrolytic enzymes and antibiotics production increase significantly when challenge occurs (Schirmböck et al. 1994).

Efficacy of TSM might be specific, i.e. filtrates of *T. harzianum* and *T. hamatum* inhibited growth of *P. aphanidermatum* by 83% and 8% respectively (Sivan et al. 1984). Abundance and diversity of TSMs are controlled by specific genes and other conditions that trigger their expression (Mukherjee et al. 2012). Initially, in *in vitro* experiments it was thought that TSMs are produced only during active fungal growth by tips of growing hyphae and released in points of contact with the pathogen (Michalikova and Michirina 1997). Further investigation determined that they are also produced but in less concentration in the sporulation phase (Vinale et al. 2008). Trichorzianines, which are partially responsible for the antifungal activity of *T. harzianum*, were isolated from sporulating cultures (Bodo et al. 1985). Therefore, any factor that affects the growth of mycelia and sporulation also affects the production of TSMs. Vinale et al. (2009) found that TSM production *in vitro* is affected not only by the presence of another microbe acting as the elicitor but also by its viability. Some metabolites are produced only when the challenging organism is viable. Therefore, the Trichoderma - pathogen co-culture technique is a good first approach to investigate more diverse production of TSMs (Vinale et al. 2017). It is apparent that productivity of conidia and mycelia for TSM production demands different conditions (Ferrigo et al. 2014). Talla et al. (2015) using a strain of *T. viride* found that maximum mycelia growth was achieved at 37°C but conidiation at 24°C. They suggested selective incubation temperatures for a maximum production of conidia.

2.3.3 Trichoderma in farming practice

Many Trichoderma formulations are available commercially (Woo et al. 2014), but their individual environmental requirements are not taken in consideration and explained to farmers when they are introduced in farming systems (Pers. Comm. Nicholas Huvelle,

2016). Understanding the mode of action of a BCA is essential to achieve optimum disease control (Köhl et al. 2019).

For efficacy in field conditions, formulations based only on conidia or chlamydospores as mechanisms of fungal dispersion and biocontrol of crop diseases, have strong limitations when sprayed on leaves to control foliar diseases (Pascale et al. 2017). However, by contrast, secondary metabolites are not affected by normal environmental conditions and are candidates for foliar disease control (Abadi 2008).

An individual species or strain of Trichoderma is not able to express all these above advantages under all environmental or agricultural conditions. Some strains are efficient producers of cell wall degrading enzymes and/or antibiotics (Vinale et al. 2008), while others are specialised plant growth promoters (Nieto-Jacobo et al. 2017) or chelating agents to enhance plant mineral nutrition (Anke et al. 1991). Alvarado and Rivera (2016) reported differences in the antagonistic capacity of strains within the same species of Trichoderma against *Sclerotium cepivorum* when challenged in *in vitro* conditions. They concluded that control efficacy depends on the isolate. Likewise, different Trichoderma strains have nutritional preferences when they colonise soils or plants and establish relationships with crops (Carreras-Villasenor et al. 2012). Not all isolates show the same rhizosphere or phyllosphere competence (Lo et al. 1997). Therefore, defining a specific set of parameters for each potential BCA for growth and development (Carreras-Villasenor et al. 2012), and factors affecting the relationship between plants, pathogen and other microorganisms, including other Trichoderma species, requires field testing in multiple environments (Vinale et al. 2008). Methods employed to produce, formulate, and apply these organisms in field may affect profoundly their efficacy (Lo et al. 1997).

2.4 Trichoderma morphological characteristics and environmental preferences

Classic morphologically based taxonomy has been the historical foundation of fungal species identification and the starting point for plant pathology experiments to evaluate biological activity under different conditions (Guigón-López et al. 2010). Colony morphology of Trichoderma isolates cultured on the defined medium, Czapek Dox Agar (CZA), that includes nitrate as the only one source of nitrogen, was used as the first level of identification in their classification (Gilman 1957). However, misidentification happened.

Chaverri et al. (2015) tested 4 commercial Trichoderma products labelled as *T. harzianum*, and, after running molecular identification, they concluded that none were *T. harzianum.* Molecular techniques, introduced at the end of the 1990's for Trichoderma species, have the advantage of higher precision and faster results and, therefore, are replacing the morphological processes (Kullnig-Gradiner et al. 2002). However, the molecular instrumentation is not as easily accessible as a microscope. Combining both techniques leads to the better outcome (Castle et al. 1998).

Trichoderma species are classified by some authors as Deuteromycetes or mitosporic fungi because their sexual reproduction and structures are rare, lacking, or unknown (Kullnig-Gradinger et al. 2002; Agrios 2004). They produce single-celled, elliptical phialospore-conidia (Kiffer and Morelet 2000), released apically without an inflated apical cell in conidiophores irregularly branched (Watanabe 2010).

Historically, Trichoderma/Hypocrea are difficult to distinguish morphologically and physiologically (Chaverri et al. 2003; Schuster and Schmoll 2010; Druzhinina et al. 2011),

despite their heterogeneous genomic structure and behaviour (Gomez et al. 1997). Initially all were identified as *T. viride* (Schuster and Schmoll 2010). In 1969 their taxonomy was clarified by Professor Mein Rifai in a major taxonomic review of this genus and species (Verma et al. 2007). Since the taxonomic review, Trichoderma phylogenetic classification resulted in 100 species by 2005 (Druzhinina and Kubicek 2005), greater than 1100 Hypocrea/Trichoderma strains from 75 molecularly defined species by 2011 (Druzhinina et al. 2011) and 292 registered species by 2018 (Kubicek et al. 2019).

A current trend in Trichoderma classification is establishing chemotypes based on the biological chemistry of isolates (Hanson 2005). However, initial isolate identification and characterisation is the fundamental first step before specific and practical applications for biocontrol can be considered (El-Refai et al. 2013). Additionally, it is essential that the classified isolates are included in the phylogenetical lineage grouping system. This allows many biological Trichoderma attributes with practical application such as production of TSMs to be linked usefully (Chaverri et al. 2015).

Most Trichoderma species have their optimum developmental temperature between 25-30°C (Guigón-López et al. 2010b). These authors also determined that Trichoderma strains from warm climate and temperate climate have different temperature requirements for biocontrol; warm climate strains work better for antibiosis than the temperate climate strains. In locations with widely fluctuating weather conditions like southern Australia where hot and dry summers and cold and wet winters alternate, it is possible that cold and warm species of Trichoderma coexist but alternate in activity with the conditions described above. Therefore, the dynamics and interactions are important factors that must be understood (Lo et al. 1997). Among the species of Trichoderma, *T. viride* strains are

restricted to low temperature areas and *T. harzianum* to warm temperatures, while *T. hamatum* and *T. koningii* have diverse climate requirements (Ghildiyal and Pandey 2008). A cold tolerant isolate of *T. atroviride* tested in Alaska succeeded in controlling *M. nivale* (McBeath 2002).

It is known also that fungi such as Trichoderma prefer acidic rather than alkaline conditions with optimal growth in soil at pH between 4 to 6 (Baker 1986; Trushina et al. 2013). *T. viride* and *T. polysporum* are more closely correlated with acidic coniferous forest soils while *T. hamatum* is more correlated with neutral pH deciduous forest soils, even when both substrates developed historically from the same base composition (Widden 1979). Domsch et al. (1980) found that pH 3.7-4.7 is the optimal level for *T. harzianum* for maximum biomass production. Rahman et al. (2009) could not find a conclusive correlation between pH and production of colony form units (cfu.) by Trichoderma species under their experimental conditions.

Conidial production is correlated more closely with low moisture, low organic matter, low pH, exposure to UV-blue light and mycelial physical damage (Mukherjee et al. 2013). Nitrate is not the preferred source of nitrogen for Trichoderma and low phosphorus and potassium content promote conidia formation (Rahman et al. 2011). Dextrose (glucose) rather than sucrose as the sole source of carbon produces five times more mycelial growth in Trichoderma under *in vitro* conditions irrespective of the source of nitrogen used (Rossi-Rodrigues et al. 2009).

For the above reasons Trichoderma *in vitro* research demands media standardisation because it guarantees reproducibility of study conditions. Undefined media that contains

yeast or potato, such as potato dextrose agar (PDA), are variable from batch to batch and even manufacturer to manufacturer (Griffith et al. 2007) when compared with defined media such as CZA in which (Grant and Pramer, 1962) nitrogen is in inorganic form but not in amino acids form as in PDA.

2.5 Trichoderma physiology

Trichoderma species, like all filamentous fungi, are constantly exploring their spatial environment (Carreras-Villasenor et al. 2012). Many genes that encode proteases and oligopeptide transporters are expressed during this process (Druzhinina et al. 2011). For example, cell wall degrading enzymes (CWDE) (Vinale et al. 2008) are continuously released into the microenvironment along with the expanding mycelia and are connected to the nitrogen depletion receptors (Druzhinina et al. 2011) that are linked mostly to their amino acid metabolism system (Seidil et al. 2009). When a suitable food source is detected, Trichoderma mycelia grow in the direction of the nutrient gradient (Benhamou and Chet 1993); synthesising and releasing at the same time a more complex array of metabolites (Vinale et al. 2008). The above attributes can be used in artificial production of these compounds by the appropriate modification of fungal growing conditions (Lorito and Scala 1999).

Cell wall degrading enzymes produced by Trichoderma are not toxic to humans or animals (Abadi 2008). They resist desiccation, are stable up to 60°C and are active over a wide range of pH and temperature in agricultural environments (Abadi 2008). Therefore, they are easier to manipulate than the living propagules in commercial formulations. Nevertheless, these mechanisms and biochemical uses may represent risks for mycorrhizae, bacteria, plants, insects, aquatic and terrestrial animals, and humans. Their effects need to be studied before

they are used in practical applications (Brimner and Boland 2003).

2.6 Vegetative compatibility/incompatibility

Genetically, when two strains of Trichoderma are compatible, their alleles in all vegetative compatibility loci are the same (Malik and Vilgalys 1999, Burguess et al. 2009). They belong to the same vegetative compatibility group (VCG) and produce heterokaryons (Krnjaja et al. 2013). Heterokarions are cells with two or more genetically distinct nuclei that share a common cytoplasm (Malik and Vilgalys 1999; Strom and Bushley 2016). Heterokarions are physiologically active and they can generate independent colonies from the two parent strains (Malik and Vilgalys 1999). According to these authors, heterokarions are associated with more vigour because of the sexual recombination (parasexual reproduction). They are source of future strain variation with biotechnological applications in biocontrol, bioremediation, and production of novel pharmaceuticals (Strom and Bushley 2016). Researchers have created hybrids *in vitro* to use the enzyme producing ability of the parent strains (Strom and Bushley 2016). Hassan (2014) created a hybrid by protoplast fusion between two species of Trichoderma one of which synthesised large quantities of chitinase and the other β-glucanase. Cole (1998) mentioned that because of a natural restriction of the nuclear transference by allelic incompatibility in a vegetative compatibility system within a territory, less virulent strains of plant pathogens can be produced. This may explain the occurrence of new strains of a disease in the field.

On the other hand, if two colonies are not compatible, the cells (heterokarions) that are produced by anastomosis (Burguess et al. 2009) are killed resulting in a visual gap (barrage), between two colonies cultured *in vitro* (Moore et al. 2011), or zone lines that occur when

two genetically different mycelia approach (Smith et al. 2006). The barrage forming isolates can be different species or even strains within species. For example, Gomez et al. (1997) found barrage formation between *T. harzianum* strains. When antagonists become competitors, they protect their own space and food sources and because of this territorial defence mechanism, they can produce a more diverse range of metabolites than they would produce unchallenged. All these biochemicals are released into the barrage area (Vinale et al. 2008). Co-culturing strains of Trichoderma to increase metabolite productivity is a simple process, but every strain in a collection must be challenged with every other strain in that collection (Burguess et al. 2009).

2.7 Trichoderma secondary metabolites (TSMs)

TSMs are small molecules of relatively low molecular weight (Vinale et al. 2014) but their chemical structure is remarkably complex (Ioca et al. 2016). These compounds are not directly involved in growth or internal metabolic pathways but are important in signalling and developing and establishing relationships with other microorganisms and plants (Hoffmeister and Keller 2007). Some of these compounds are antibiotics (Ghisalberti and Sivasithamparam 1991), with antifungal properties targeting hyphal growth or sporulation processes; others are cell wall degrading enzymes and chelating agents (Vinale et al. 2014); and some function like plant hormones enhancing plant growth, stimulating mineral uptake, and therefore increasing mineral content in plant parts (Marra et al. 2019).

Dennis and Webster (1971) classified TSMs as volatile and non-volatile compounds. Volatile TSMs can diffuse into the soil air pores and radiate further than can non-volatile metabolites which only disperse in the soil solution. These authors were the first to describe

how these compounds were effective in reducing plant pathogen fungal hyphal growth and they also stated that TSM production and distribution varies between isolates. More than 100 TSMs were reported by 2008 (Reino et al. 2008) and more than 1000 by 2014 (Hermosa et al. 2014). The list of reported TSMs is continually increasing.

Gliotoxin was the first reported TSM, described by Richard Weindling in 1934, initially from *Trichoderma lignorum* that was re-classified as *Gliocladium virens* (Mukherjee et al. 2012), and finally as *Trichoderma virens* (Vinale et al. 2014). Strains of *T. virens* group 'Q' produce large amounts of gliotoxin in liquid culture and are effective in controlling *R. solani* but not in controlling protistas (oomycetes), such as Pythium or Phytophthora species, which are controlled by another metabolite, gliovirin, produced by *T. virens* strains of the group 'P' (Mukherjee et al. 2012 and Vinale et al. 2014).

Some species of Trichoderma, including *T. harzianum*, *T. viride*, *T. koningii* and *T. atroviride* are profuse producers of a distinctive 'coconut smell' that is attributed to 6-n-pentyl-2H-pyran-2-one (6-pentyl-α-pyrone) or simply 6PP, the most studied volatile TSM (Vinale et al. 2014). This metabolite was discovered by accident by Dennis and Webster (1971) when they were testing antagonistic activity of Trichoderma isolates in dual cultures. They noticed that the most effective isolates against *R. solani* and *G. graminis* var. tritici were characterised by their 'coconut smell' (Hanson 2005). 6PP is effective in controlling sclerotia-forming pathogens and take-all fungus (*Gaeumannomyces graminis*) (Ghisalberti and Sivasithamparam 1991). However, Dewan and Sivasithamparam (1988) already had found that production of this compound was very variable between isolates of *T. harzianum*. They found the isolates that did not produce or produced it in small amounts did not control the pathogens.

6PP is a fungistatic and fungicidal compound (El Hassan et al. 2007); not toxic to mammals at concentrations needed for pathogen control. This metabolite is an efficient inhibitor of *R. solani* (Vinale et al 2006); *Roselinia necatrix* (Arjona-Girona et al 2014), *Botrytis cinerea* and *Alternaria brassicola* (Kottb et al 2015). Arjona-Girona et al. (2014) achieved control of white root rot (*Rosellinia necatrix*) by treating lupin seeds with a solution of 1 mg/l of 6PP. Vinale et al (2008) treated tomato and canola seedlings with 1-10 mg/kg stimulating plant defence molecular triggers. El Hassan and Buchenauer (2009) found a higher dose were required for maize seedlings to control *Fusarium moniliforme* (200 mg/kg). Jeyaseelan et al. (2012) tested two isolates of *T. harzianum* and *T. viride*, synthesizing volatile compounds that produced significant delay in *Pythium aphanidermatum* colony growth *in vitro* after 24 hours of incubation, but not after 48 hours. This loss of efficacy over time was also reported by Hanson (2005) when the same effect was observed with *Botrytis cinerea* colonies. El-Hassan et al. (2007) reported a reduction of 93% of mycelia growth and conidial germination of *Fusarium moniliforme* at 250 µg/ml and 300 µg/ml of 6PP respectively *in vitro*. In nature, this metabolite is degraded by hydroxylation to less bioactive molecules, confirming that it is not persistent in the environment and useful for practical application (Hanson 2005).

Harzianic acid, produced by strains of *T. harzianum* was found to control *S. sclerotiorum*, *R. solani* and *Pythium irregulare* (Vinale et al. 2014). Additionally, it has been identified as a plant growth promotor and chelating agent of soil iron Fe^{+3} (phytosiderophore) making iron bioavailable for plants (Vinale et al. 2013). TSM peptaibols (Howell, 2003; Harman et al. 2004 mentioned by Shi et al. 2012) are peptides which contain an unusual aminoacid, α-aminoisobutyric acid (Aib), a C- terminal hydroxylated (amino alcohol) and a N-terminal-acetylated aminoacid, with many antimicrobial properties which are largely still unknown.

Peptaibol is derived from '*pep*tide', *Aib* and alcoh*ol* (Brito et al. 2014). Shi et al. (2012) mentioned that from 317 reported peptaibols, 190 were synthesised by Trichoderma isolates.

Fusalanipyrone is a rare fungal monoterpenoid metabolite (Abraham and Arfmann 1988) produced by *F. solani* (Abraham et al. 1990) with moderate inhibitory effect on lettuce seedlings growth (Shimizu et al. 2005). Cytosporone S was isolated from a strain of *Trichoderma sp.* FK6626 with antifungal and antibacterial properties (Ishi et al. 2012). Cytosporone A, B and C showed allelopathic activity against lettuce seeds and seedlings (Zamberlan et al 2012) and antibacterial and antifungal activity (Ishi et al. 2012). Harzianic acid (HA) reported as an antifungal, plant growth promoter and phytosiderophore (Vinale et al. 2013). HA is effective in controlling *P. irregulare*, *S. sclerotiorum* and *R. solani* (Vinale et al. 2009). The HA role as a chelating agent selectively protects Fe^{+3} for plant nutrition, depriving pathogens of this important nutrient resulting growth suppression (Vinale et al. 2013).

Enzymatically, *T. harzianum,* the most studied Trichoderma species targets organisms by producing an enzyme that catalyses chitin breakdown, a primary component of fungal cell walls (Zeilinger et al. 1999). Mycoparasitism by coiling and penetrating pathogen hyphae is accompanied by antibiotic production that permeates the perforated hyphae and prevents re-synthesis of the host cell wall (Lorito et al. 1996; Mc Beath 2002).

Formal identification to molecular level must be performed for every single isolate because the TSM synthesis repertoire can vary widely between different isolates of the same species (Vinale et al. 2014). *T. harzianum* isolates were found to produce different chemicals with different levels of activity against 'take all' fungus, thus establishing a classification system

based on ‘chemical types’ (Ghisalberti and Sivasithamparam 1991).

2.8 Trichoderma as a potential BCA for lettuce anthracnose

Trichoderma species are well known for their capacity to colonise soils and therefore reduce pathogen infections on plant parts. They also have an inherent ability to colonise intercellular compartments of roots (Druzhinina et al. 2011) and leaf surfaces, a mechanism called phylloplane competency (Schumann and D’Arcy 2006). However, the efficacy of Trichoderma to colonise a leaf and control a foliar disease directly in field conditions depends on many factors such as leaf exudates, temperature, relative humidity, free water, light, radiation, wind and pollution all of which fluctuate widely (Elad and Kirshner 1992). *Trichoderma* spp. as mentioned before, have efficacy in controlling many pathogens that produce foliar diseases, but unfortunately most of the research has been performed *in vitro* and in optimal conditions for the antagonist (Adedeji 2008; Rahman 2009; Evueh et al. 2011; Pakdaman et al. 2013; Thakur and Harsh 2014; Kabir et al. 2016). Laboratory conditions are radically different from field conditions, making the accuracy of laboratory-based predictions problematic (Elad and Kirshner 1992; Sawant 2014; Woo et al. 2014).TSMs reduced the incidence and severity of foliar diseases in grapes under field conditions (Pascale et al. 2017) and in greenhouse tomato crops (Vinale et al, 2017), but there is no report of MP control using TSMs.

The ability of Trichoderma species to control soil borne diseases is not only associated with external factors as described above, but also on their growing stages. Conidia are the most effective mechanism of survival and dispersal (Carreras-Villasenor et al. 2012) but have the lowest capacity for control, while the actively growing mycelia gives the best control (Bae

and Knudsen 2005). In unfavourable conditions, Trichoderma conidia remain dormant and they will remain inactive until conditions are optimal (Carreras-Villaseñor et al. 2012, Ŝimkoviĉ et al. 2015). Baker (1986) impregnated seeds with *T. harzianum* conidia and sowed them under suboptimal temperatures for the antagonist, finding no control of Pythium or Rhizoctonia. Therefore, commercial formulations based on conidia or chlamydospores sprayed in the field are not necessarily a guarantee of success because the conditions maybe suboptimal for the antagonist (Mendoza-Mendoza et al. 2015). Screening for a successful biocontrol agent involves its isolation from environment based upon the pathosystem of interest (Parnel et al. 2016). Papavizas et al. (1984) found that mycelial fragments were more effective than conidia for supressing soil borne diseases 10-20 days after application. Time is required for an effective control and development of mycelia biomass from conidia (Ŝimkoviĉ et al. 2015). Methods of control that demand use of conidia must be complemented by practices that stimulate their survival and proliferation in soil (Papavizas et al. 1985).

2.9 Practical application of Trichoderma to control pathogens.

There are several ways to increase soil borne disease suppression: (1) applying known biological control agents (BCAs); (2) enhancing the growth and development of pre-existing BCAs in soil and (3) applying a complex population of microorganisms (St. Martin 2015).

Thermal aerobic composts provide opportunities for consistent biological control of crop diseases particularly if they are inoculated with BCAs (Hoitink et al. 1997, Bernal-Vicente et al. 2012). Maximum disease suppression from compost is achieved when the BCAs recolonise the compost in the curing to maturity stages (Hoitink et al. 1997, Bonanomi et al.

2018). Some authors including Parkash and Saikia (2014) consider that Trichoderma species are compost fungal activators (CFAs). Additionally, they can reduce the composting process time when inoculated onto the raw materials. Interestingly, Stone et al. (2003) applied paper mill residues to a sandy soil and succeeded in controlling soil borne diseases such as *Pythium* sp that cause damping off in cucumber and beans and *C. lindemuthianum* which causes anthracnose in cucumber, respectively. They attributed but could not confirm this response to the activation of plant defence mechanisms in the cucumbers and beans.

Since lettuce anthracnose is a soil borne disease that produces survival microsclerotia and causes damage in aerial parts of plants in cold conditions, the mechanisms of control must be used strategically, first to keep the pathogen away from the plants by applying cold tolerant Trichoderma isolates and, secondly, by reducing the damage to leaves using TSMs. Single or mixed combinations of culture liquids produced under challenged conditions containing different metabolites such as CWDEs are suitable alternatives for the control of foliar pathogens. The CWDEs are resistant to desiccation and are stable up to 60°C in a wide pH range (Abadi 2008).

Selective production of TSMs for pathogens may be achieved by modifying culturing conditions which include substrate composition, pH, temperature, and agitation (Lorito and Scala 1999).

Many strains of Trichoderma are reportedly resistant to chemical fungicides (Abadi 2008). *T. atroviride* P1 was selected for its resistance to Benomyl and for its compatibility with *T. harzianum* T22, another strain with well-known commercial formulation. These two strains were investigated for their compatibility with organic products and copper oxychloride

which showed no effect on mycelial growth in concentrations up to 5 mM (Vinale et al. 2004).

Currently, there are no references of biological control of lettuce anthracnose, *Microdochium panattonianum,* using Trichoderma and or its metabolites.

Chapter 3

MATERIALS AND METHODS.

3.1 Introduction

Different approaches were implemented to identify and characterise suitable cold tolerant Trichoderma isolates and identification of TSMs that reduce MP incidence and severity.

3.2 Trichoderma isolates - Collection and isolation of pure cultures

Initially, 23 Trichoderma isolates were gathered. Four more were added just after the first screening. In total twenty-seven isolates of Trichoderma were collected from different sources and conditions (Table 3.1 and Figure 3.1).

South Australia Research and Development Institute (SARDI) provided mixed colony plates from pistachio isolations. New South Wales Herbarium (DAR) provided six isolates. Agpath P/L (AG), Garfield, Victoria, provided eleven samples cultured from soil and plant material. The remaining Trichoderma isolates (7) were commercial formulations.

Fungal material from above cultures were soaked in sterile water and an aliquot of 100 µL from each suspension was spread onto potato dextrose agar (PDA) (Amyl Media, Dandenong) in 90 mm Petri dishes and incubated at room temperature. This method was repeated until pure cultures of possible Trichoderma isolates were obtained.

N	Source	Original Name	Origin
1	Pistachios buds	355	South Australia
2	Culture collection	71558	NSW Plant Pathology Herbarium
3	Culture collection	33681	NSW Plant Pathology Herbarium
4	Commercial formulation	PHY	Metcalf, Tasmania
5	Commercial formulation	NS	Barmac, USA,
6	Lupin roots	LUP	Annuello, VIC
7	Macadamia mulch	MACA	Ballina, NSW
8	Soil sample	NUT	Orange, NSW
9	Commercial formulation	PMF	Agrimm, New Zealand
10	Culture collection	71556	NSW Plant Pathology Herbarium
11	Pistachios buds	356	South Australia
12	Food waste sample	CAROL	Melbourne, VIC
13	Culture collection	45899	NSW Plant Pathology Herbarium
14	Wood sample	VIR	Virginia, SA
15	Culture collection	66079	NSW Plant Pathology Herbarium
16	Soil sample	1535	Twin peaks, WA
17	Soil sample	1536	Twin peaks, WA
18	Commercial formulation	GAU	Metcalf, Tasmania
19	Commercial formulation	ANT	Metcalf, Tasmania
20	Commercial formulation	LM	Agrimm, New Zealand
21	Commercial formulation	PMR	Agrimm, New Zealand
22	Culture collection	59837	NSW Plant Pathology Herbarium
23	Pistachios buds	99	South Australia.
24	Compost sample	TC1	Melbourne, VIC
25	Avocado tree sample	ANAND	Bellthorpe, QLD
26	Compost sample	1567	Mornington Peninsula, VIC
27	Soil sample	BIANCA	Melbourne, VIC

Table 3.1 Trichoderma isolates used in the experiments. Source and location where they were isolated.

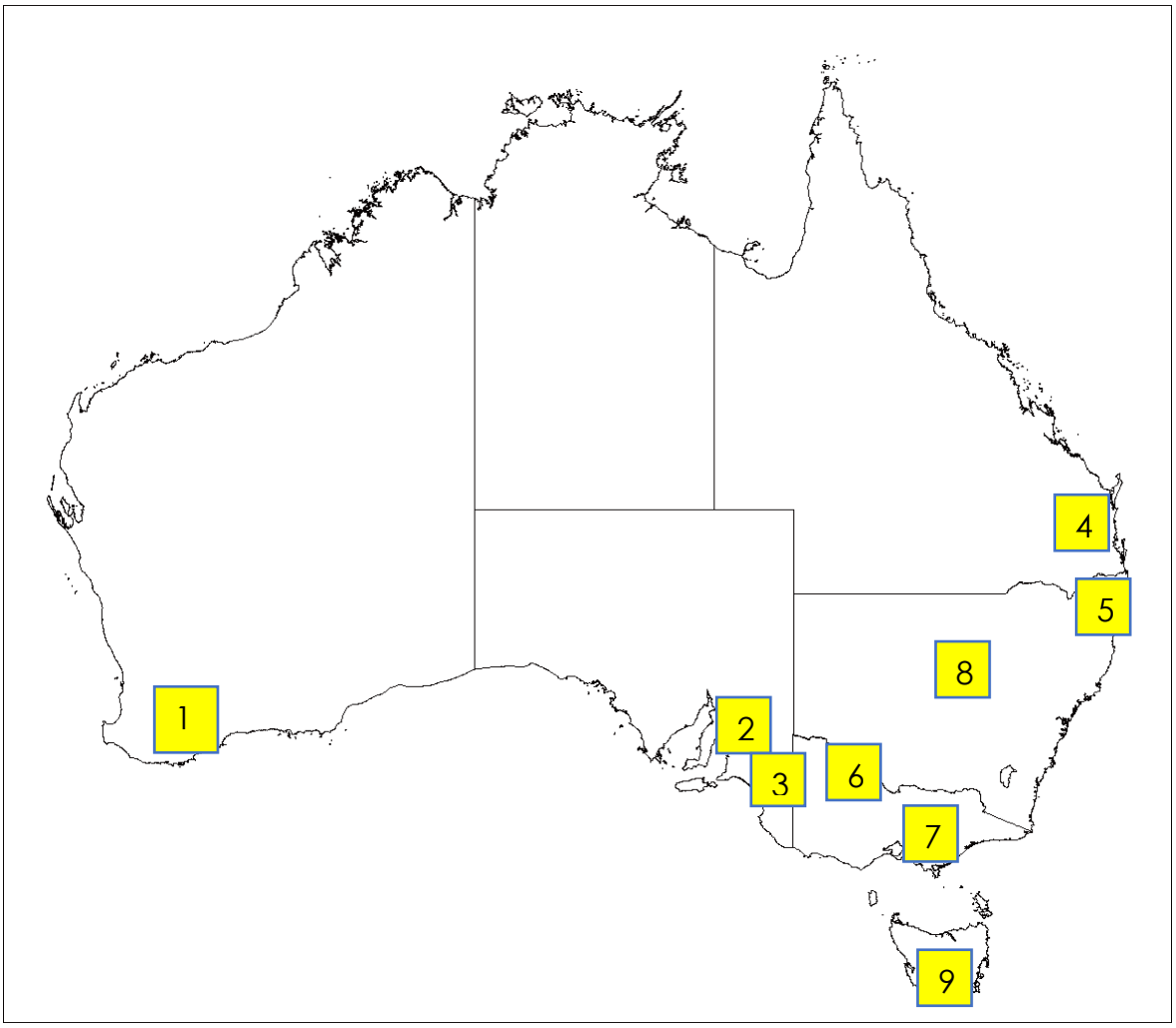


Fig. 3.1 Map of Australia showing approximate geographical origin of Trichoderma isolates: 1 Twin peaks (WA); 2 Virginia (SA); 3 Adelaide Hills (SA); 4 Belthorpe (QLD); 5 Ballina (NSW); 6 Anuello (VIC); 7 Melbourne and Mornington Peninsula (Vic); 8 Orange (NSW) and 9 Molesworth (TAS)

The Trichoderma-like colonies, distinguished by the green spore colour, were observed using an Olympus SZ 30 stereo microscope and conidia were transferred onto fresh PDA twice until colonies appeared uniform. Colonies were observed and identified as Trichoderma under compound microscope Olympus BX 50 using the morphological distinctive Trichoderma phialide as the identifier together with the green conidial mass colour. From these dishes, spores were dislodged and suspended in sterile physiological saline solution (0.85% NaCl). Using a micropipette and sterile tips, aliquots of 100 µL were transferred from the solution to fresh PDA, spread with a sterile hockey stick and incubated

right side up at 22 ± 1° C. After 24 hours, using a stereo microscope, individual conidia were selected and removed carefully by cutting an agar square carrying one germinated conidium and transplanted spore face down onto fresh PDA and incubated at 25°C in a Thermoline incubator until spore germination was observed. This procedure resulted in genetically pure isolates. These single spore isolates became the foundation isolates for all further research for this project.

From the commercial formulations used in these experiments a suspension of 0.1 g of freeze dry formulation was added to 100 ml of sterile water. Aliquots of 100 µL were added to PDA plates and spread with sterile hockey sticks and incubated at room temperature. Single spores or single hyphal tips, in the case of fast germinating spores, were removed and treated as above.

Mother cultures of each Trichoderma isolate were established on PDA slants in triplicate. The established cultures were covered with sterile water to the top of the agar slant to prevent desiccation and were refrigerated at 8°C. This collection is refreshed every 3 months using the same protocol.

3.3 Morphological characterisation in diverse culture conditions

All Trichoderma isolates were cultured *in vitro* under different conditions (pH, temperature and media) (Table 3.2) to determine how these conditions and their combinations affect morphological characteristics such as phialide and conidia dimensions, colony shape, colour and growth speed, and the coconut aroma that is linked to a key volatile TSM.

Trichoderma isolates	pH values (3)	Media (2)	Incubation Temperature (°C) (3)
27	5.0 6.0 6.5	Czapek Dox agar (CZA) Potato dextrose agar (PDA)	10 18 25

Table 3.2 Variables for morphological tests: Isolates, pH, culture media and temperature

Czapek Dox (CZA) and PDA (Amyl Media-Dandenong) were tested as defined and undefined media, respectively, at three incubation temperatures (10, 18 and 25°C) and three media pH values (5.0, 6.0 and 6.5). Three replicates for each combination of the 27 isolates, 3 temperatures, 3 pH media values and 2 substrates were assessed.

For each isolate, 54 Petri dishes were inoculated with a 10µl drop of conidial suspension placed in the centre of each dish and incubated in darkness until 100% of agar surface was covered with mycelia and sporulation was initiated. Morphological characteristics such as mycelia colour and texture and conidia colour were evaluated. As colony growth was reduced by temperature, plates were assessed up to 7, 10 and 30 days for incubation temperatures 25, 18 and 10°C, respectively.

Using an Olympus BX 50 microscope, prepared slides of each isolate were examined. Length and width of 50 phialides and 50 conidia were measured and recorded. Average values were calculated using MS Excel and processed statistically in Minitab 17 (Minitab, LLC).

3.4 Molecular identification of Trichoderma isolates

For formal identification of all Trichoderma isolates, molecular tests were performed. Isolates were cultured on PDA at 25° C for three days until all agar surfaces were covered with mycelia (Alvarado-Marchena and Rivera-Méndez 2016).

Extraction of DNA was performed using a Qiagen DNeasy® plant mini kit following the protocol supplied with the kit. A satisfactory quality and quantity of DNA in tube samples was checked using both a nanodrop spectrophotometer and agarose electrophoresis test (Sambrook et al. 1989). Tubes containing DNA were stored at -20°C until required (Griffin et al. 2002).

For the electrophoresis tests, the medium containing 3 gr of agarose in 200ml of deionized water (1.5%) was boiled in a microwave oven, swirling every 30 seconds, until dissolved completely. Twenty microliters of SYBR Safe DNA stain was then added and mixed well. Sixty ml of gel were poured in the tray; two combs were introduced and smoothly removed after the gel solidified. The tray was introduced into the electrophoresis chamber and covered with 1X borate-EDTA buffer solution. This solution was previously prepared dissolving 242 gr of Tris base in 750 ml of deionized water, 57.1 ml of acetic acid and 100 ml of 100 mM EDTA solution were added, finally the volume was adjusted to 1l by adding deionized water. From this mother stock 1ml was added to 49 ml of deionized water to prepare a 1x solution. Three microliters of loading dye were placed on a piece of Parafilm, one for each sample. Two microliters of each DNA sample were added and mixed well. The mix was placed in the gel wells. The chamber lid was added checking the correct position of cords. The voltage was set to 90 volts for 35 minutes. The gel was removed from the

chamber and placed in a Bio-Rad Gel-EZ-documentation apparatus. An image of bands corresponding to the extracted DNA was obtained.

Based on arguments provided by Jaklitsch and Voglmayr (2015) the markers based on the *ITS* region, were for many years the reference for fungal molecular identification, but now they are considered outdated with no satisfactory resolution for Trichoderma genus. Professor Paul Taylor (pers. comm. January 2019.) suggested using primers for *tef*, *rpb2* and *acl1* based on this information.

Polymerase chain reactions were performed according to protocols published by Carbone and Kohn (1999) using the *tef1*, *rpb2* and *acl1* primers (Table 3.3). Amplicons were tested using the electrophoresis technique (explained above) confirming they were satisfactorily pure.

Gene	Primer	Sequence	Reference
tef 1	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn (1999)
tef 1	TEF1LLErev	AACTTGCAGGCAATGTGG	Jaklitsch et al (2005)
rpbII	frpb2-5f	GA(T/C)GA((T/C)(A/C)G(A/T)GATCA(T/C)TT(T/C)GG	Liu et al. (1999)
rpbII	frpb2-7cr	CCCAT(A/G)GCTTG(T/C)TT(A/G)CCCAT	Liu et al. (1999)
acl1	acl1-230up	AGCCCGATCAGCTCATCAAG	Gräfenhan et al. 2011
acl1	acl1-1220low	CCTGGCAGCAAGATCVAGGAAGT	Gräfenhan et al. 2011

Table 3.3 Genes, primers and their sequences used in Trichoderma isolates identification

Master mixes were performed using for *tef1* gen (in µl).

EF1-728F	TEF1LLErev	dNTP's	$MgCl_2$	Taq	buffer	Isolate DNA	sterile water.
1	1	0.4	0.8	0.1	4.0	2 (at 10 ng/µl)	10.7

Protocol for sequencing reactions was as follows:
95° C for 8 minutes,
35 cycles at 95° C for 15 s, 55° C for 20 s, 72° C for 60 s and
72° C for 5 minutes.

Master mixes were performed using for *rbp2* and *acl1*:

frpb2-5f	frpb2-7cr	dNTP's	$MgCl_2$	Taq	buffer	Isolate DNA	sterile water.
2	2	2	0.75	0.2	5.0	2 (at 10 ng/µl)	11.05

acl1-230up	acl1-1220low	dNTP's	$MgCl_2$	Taq	buffer	Isolate DNA	sterile water.
2	2	2	0.75	0.2	5.0	2 (at 10 ng/µl)	11.05

Applying the following protocol:

95° C for 3 min,
5 cycles of 95° C for 45 min, 64°C for 45 min and 72° C for 2 min;
5 cycles of 95° C for 45 min, 62°C for 45 min and 72° C for 2 min;
30 cycles of 95° C for 45 min, 56°C for 45 min and 72° C for 2 min;
and 72° C for 8 min.

Samples were sent to Macrogen, South Korea, for sequencing. Sequences of genes were processed and compared with the reference GenBank accessions using nucleotide algorithm analysis BLAST (blast.ncbi.nlm.nih.gov). Phylogenetic trees were developed using the Molecular Evolutionary Genetic Analysis (MEGA-X) following protocols by Jaklitsch and Voglmayr (2015).

3.5 Selection of putative biocontrol Trichoderma

Trichoderma isolates were tested for their ability to grow at optimal pathogen temperatures. This established the foundation of the selection process. All the work described below was carried out under sterile conditions.

3.5.1 Direct plate confrontations

Pilot dual culture experiments were performed *in vitro* at 25±3°C to evaluate the interaction between the isolates and the pathogen. Preliminary results failed because the optimal incubation temperature for the pathogen was different from that of the antagonist. All future experiments involving MP were incubated at 10°C.

3.5.2 Production of culture filtrates containing Trichoderma secondary metabolites

An *in vitro* experiment was carried out to evaluate the capacity of the Trichoderma isolates to produce metabolites that are effective in reducing or at least interfering with the normal growth of the pathogen. To assess the Trichoderma ability to produce more metabolites when challenged, pathogen propagules were added the to the liquid cultures. All experiments were incubated at 10°C.

3.5.2.1 Liquid culture filtrates

This experiment was carried out to obtain the full set of filtrates containing secondary metabolites produced by Trichoderma isolates without presence of MP.

For each Trichoderma isolate, duplicate PDA plates were inoculated with a 10µl conidial suspension of 1 x 10^4 cfu/ml. and incubated at 25°C. After three days, using a sterile circular 9mm cutter, 4 discs were cut from the culture prior to sporulation and placed in 150 ml sterile potato dextrose broth (PDB) (Amyl Media, Dandenong) and shaken at 200 rpm for 10 minutes daily for 7 days at room temperature (Fig. 3.2). Containers were left loose lidded for air exchange.

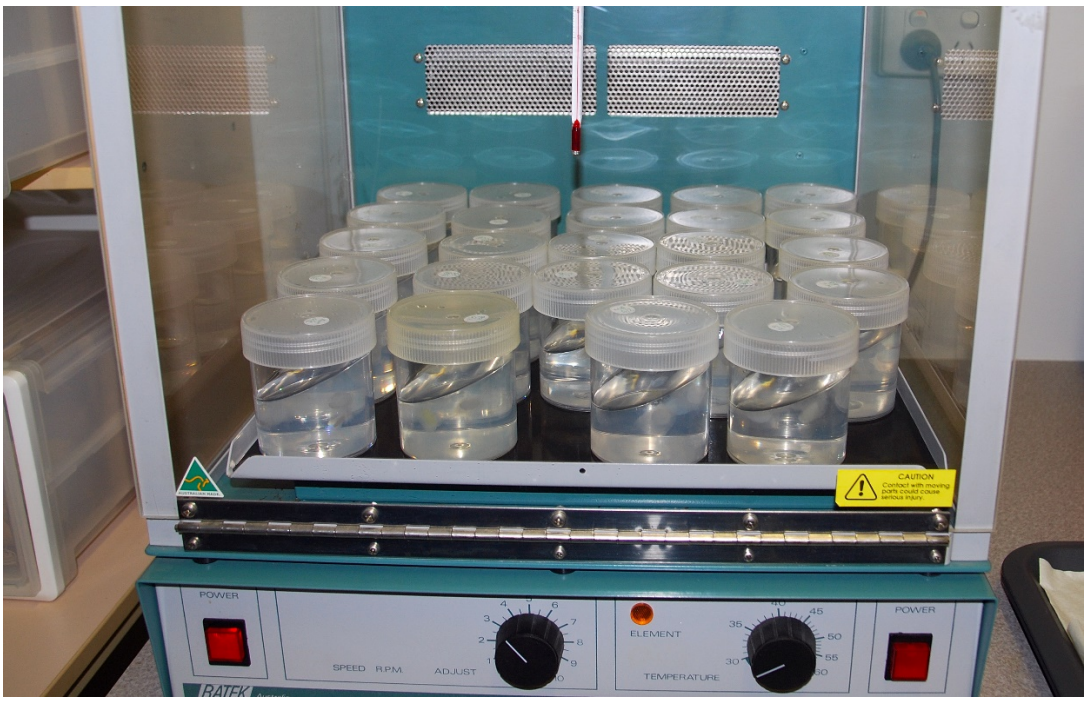


Fig. 3.2 Liquid cultures containing Trichoderma isolates in PDB shaken at 200 rpm.

After this incubation period, 15 ml of solution were taken from each culture and filtered using Minisart Sartorius 0.22µm filters. The filtrates were placed in sterile identified tightly lidded 20 ml containers (Fig. 3.3) and refrigerated at 4°C.

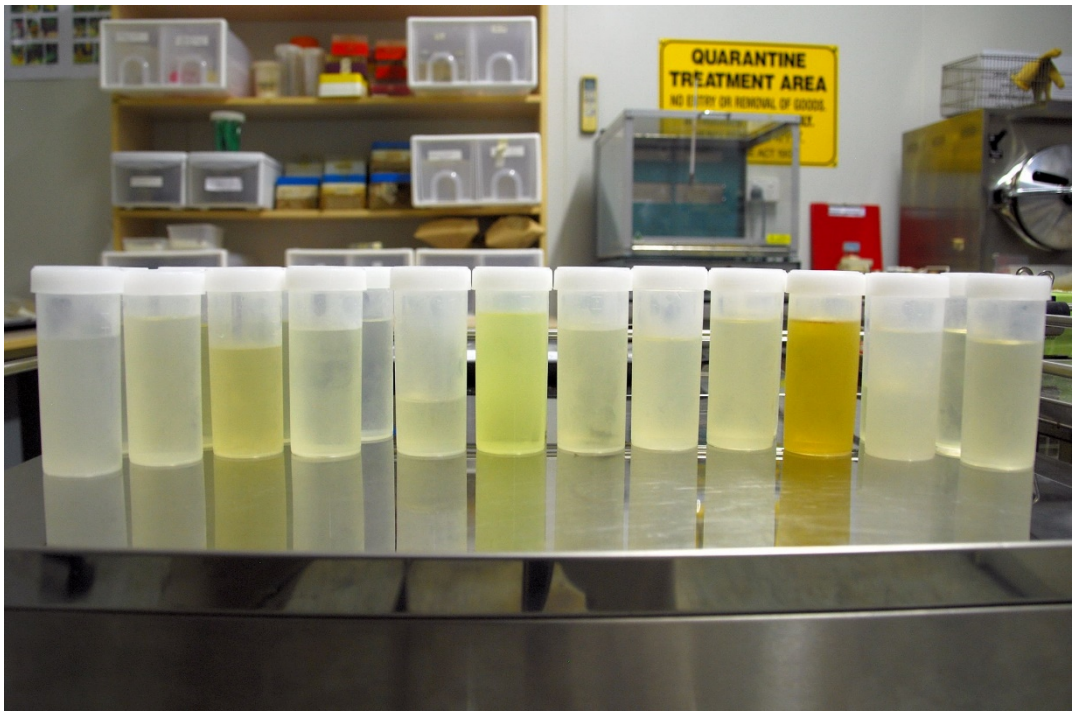


Fig. 3.3 Filtrates from Trichoderma isolates liquid cultures showing different natural pigmentation

The culturing process continued with the remaining liquid for a further 7 days, after which another 15 ml sample was filtered as above. The remaining culture was filtered and refrigerated at 4°C for later trials.

3.5.2.2 Liquid culture filtrates from MP induction

Filtrates in this experiment used the same protocol described in 3.5.2.1, however in this case, discs containing the sporulating pathogen were added at the same time as the Trichoderma mycelia discs. MP colonies were cultured on PDA and incubated at 10°C for 7 days. From these plates, two 9 mm diameter discs with growing colonies were introduced into each of the liquid cultures. Containers were agitated for 10 minutes daily for 7 and 14 days as described above in 3.5.2.1

3.5.3 Bioassays of culture filtrates (CFs) on MP

In these experiments, liquid cultures previously obtained in 3.5.2.1 and 3.5.2.2 were tested for their capacity to inhibit or alter pathogen growth under the well technique. Also, MP disks inoculated as challengers in the filtrates were assessed.

3.5.3.1 Well tests of CFs in MP plate cultures

Efficacy of each liquid culture from each isolate was tested in a 4 treatment by 5 replicate design as follows.

Treatment 1: 7 days of incubation without pathogen challenge

Treatment 2: 14 days of incubation without pathogen challenge

Treatment 3: 7 days of incubation with pathogen challenge

Treatment 4: 14 days of incubation with pathogen challenge

PDA dishes were inoculated with a conidial suspension of the pathogen prepared from 1ml of conidial suspension of 10^5 cfu/ml from the collection tube diluted in 99 ml of sterile water (1:100 dilution) resulting in 10^3 cfu/ml. From this suspension, 200 µl were transferred to each plate and spread evenly with a sterile disposable plastic hockey stick, incubated at 8-10° C for two weeks, checked every three days and considered ready for the wells experiment when colonies were 3-4 mm diameter. Using sterile 9 mm disc cutters, four agar discs were cut in a square pattern and removed from each plate to form wells (Fig. 3.4 and 3.5). Hundred microliters of each Trichoderma filtrate were introduced aseptically into each well daily for 7 days and incubated at 10°C. An untreated control was prepared as above using sterile water in the wells.

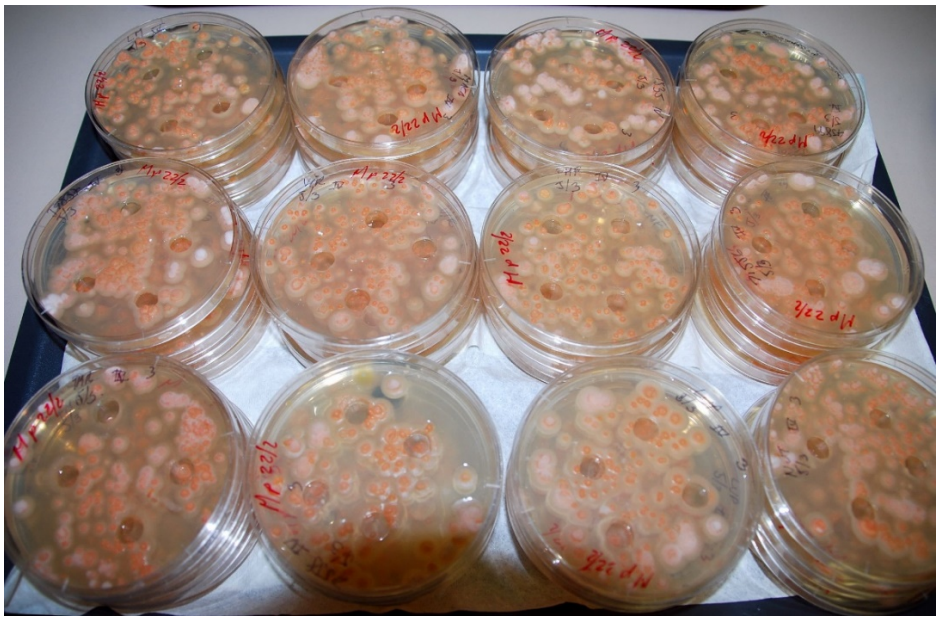


Fig. 3.4 *Microdochium panattonianum* colonies incubated in cold conditions. Wells were cut in square pattern

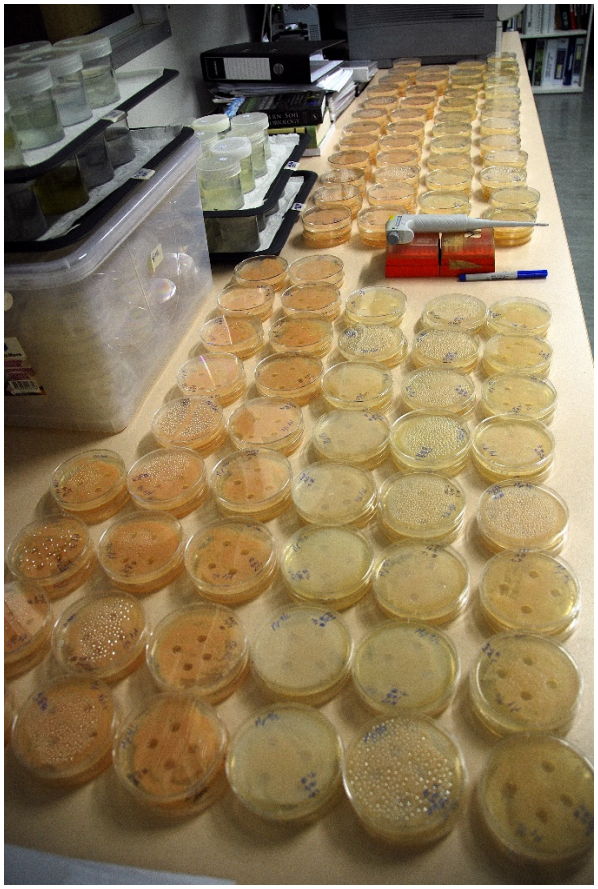


Fig. 3.5 Layout of complete wells experiment. 4 sets of plates. Orange pigmentation denotes older colonies that corresponded to the 7 days CF. Pale colonies corresponded to the 14 days CF.

Observations of morphological characteristics of MP growth at the edge of the wells were performed for 7 days using the stereo microscope.

3.5.3.2 Tests of pathogen discs immersed in CFs

In aseptic conditions the MP discs were removed carefully from the challenged liquid cultures (3.5.2.2) (Fig. 3.6). Using a stereo microscope, residual mycelia were detached gently from discs. These were then rinsed with sterile water three times in 10 ml sterile vials. Finally, discs were divided in four equal pieces and each piece placed centrally on a fresh 9 cm diameter PDA plate and incubated at 10° C.

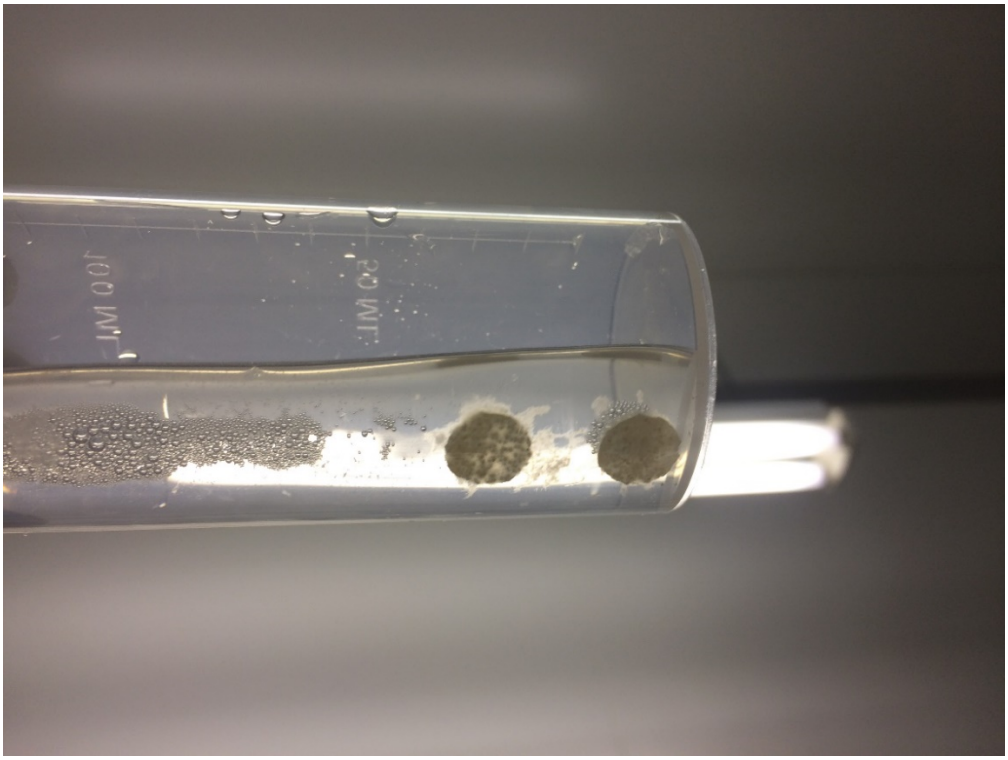


Fig. 3.6 Challenging *Microdochium panattonianum* discs cultured in presence of Trichoderma.

Colony diameter and growth were measured every two days from day 6 to day 18 after inoculation. Colour, colony diameter and sporulation time were recorded.

3.5.3.3 Test of MP on substrates containing CFs

To test the efficacy of the compounds in the liquid filtrates against MP using the previously stored liquid cultures (3.5.2.2), a trial was set up to evaluate the ability to inhibit the pathogen growth *in vitro* and to determine if they are heat stable. Only filtrates from isolates 356 and 1535 were selected for this pilot experiment because they are wild types and the best cold tolerant isolates.

Agar modified as described above was prepared using 60 ml of the filtrate and 2.3 g of PDA in two 200 ml Erlenmeyer flasks plugged with cotton, sealed with aluminium foil and sterilised in a Siltex HC2 (MK-1-94) autoclave. An untreated control was prepared with water replacing the filtrate. Treated and untreated agar plates were inoculated with a conidial suspension of 10^5 cfu/ml of MP and spread with sterile hockey sticks; incubated at 10°C for 10 days and checked every two days using a stereo microscope to detect differences in the hyphal morphology and colony growth.

3.6 Effect of temperature and pH to select cold tolerant Trichoderma strains

Following the pathogen disc assessment (3.5.3.2), it became obvious that not all Trichoderma isolates had the same tolerance to low temperatures.

An experiment was set up to evaluate the performance of the selected cold tolerant isolates at different media pH. This trial might help to assess potential performance of Trichoderma isolates in different soil pHs that reflect most agricultural natural soil pH conditions. Three pH values were considered initially: 5.5, 6.5 and 7.5. Potato dextrose agar made at label rate in three 500 ml Pyrex glass bottles was gently swirled until all powder was in suspension,

then pHed using 1N Na (OH). However, after sterilisation, the pH value dropped to 5.0, 6.0 and 6.5, respectively. These pH values were considered the reference pH media values for this experiment.

Conidial suspensions of 10^5 cfu/ml were prepared for each of the seven most cold tolerant isolates (59837, 45899, LUP, 356, LM, PMF and PMR). Using three replicates per isolate and pH, a 10µL drop of suspension was placed in the centre of each relevant PDA plate. Plates were incubated at 10° C, checked each two days and colony diameters were measured at 6, 8, 10, 14 and 16 days after inoculation.

3.7 Vegetative incompatibility tests (VIT) among Trichoderma strains

Two Trichoderma isolates can interact in different ways when they grow in the same substrate. Their colonies can overlap or stop growing before physical contact forming a barrage. A barrage which is a gap between two colonies is the expression of the inhibition of growth mediated by metabolites from either or both isolates.

In this experiment all isolates were challenged against one another to observe their compatibility/incompatibility and to predict if a more diverse collection of metabolites is produced that maybe useful in controlling MP.

For each of the 27 Trichoderma isolates, 2 PDA plates were inoculated using a 1 x 10^5 cfu/ml and incubated at 25°C until mycelia were produced. From prepared plates above, 9 mm agar discs containing mycelia were cut and placed onto PDA plates using the template of 6 sectors and one central point (fig. 3.7). The isolate being tested was placed in the central

position, and in one sector 6. Other isolates were placed in the remaining sectors at 2 cm separation. Plates were incubated at 25°C and checked daily. Plates were evaluated for barrage formation. This trial was triplicated. Information from this experiment formed the foundation for all the TSM experiments in 3.9. No microscopic observations regarding heterokarion formation or dead hyphae were performed in this experiment. The barrage formation was considered the only assessment parameter.

3.8 TSM production in co-cultures

Presence of a competitor triggers the expression of genes to produce to a more diverse TSM production compared with a single isolate. Trichoderma isolates were cultured in couplets according to the incompatibility chart (Table 4.7).

3.8.1 Liquid culture phase

PDA plates were prepared, inoculated with 27 isolates in triplicate and incubated at 25°C for 3 days as before (3.3). At the same time, 20 ml of sterile PDB were transferred into 163 x 50 ml plastic Falcon conical tubes. Discs of mycelia only were cut and introduced into the Falcon tubes in couplets (two different isolates) according to the vegetative incompatibility chart (See Table 4.7). Additionally, 27 single isolate filtrates were prepared by introducing two discs in the corresponding tubes to determine if there is a difference in TSM production between challenged and unchallenged conditions. Falcon tubes were incubated at 25°C and shaken for 5 minutes at 120 rpm daily for 7 days. Five ml from each culture were filtered using sterile 20 ml Terumo® syringes and Merck Millex-GP 0.22 µm polyether sulphone gamma sterilised syringe filters and placed in Technoplast 5 ml screw capped gamma sterile

tubes (Fig. 3.8), packed and sent to the Faculty of Agricultural Sciences of the University of Naples for TSM testing. The remaining liquid culture was stored at 8°C.

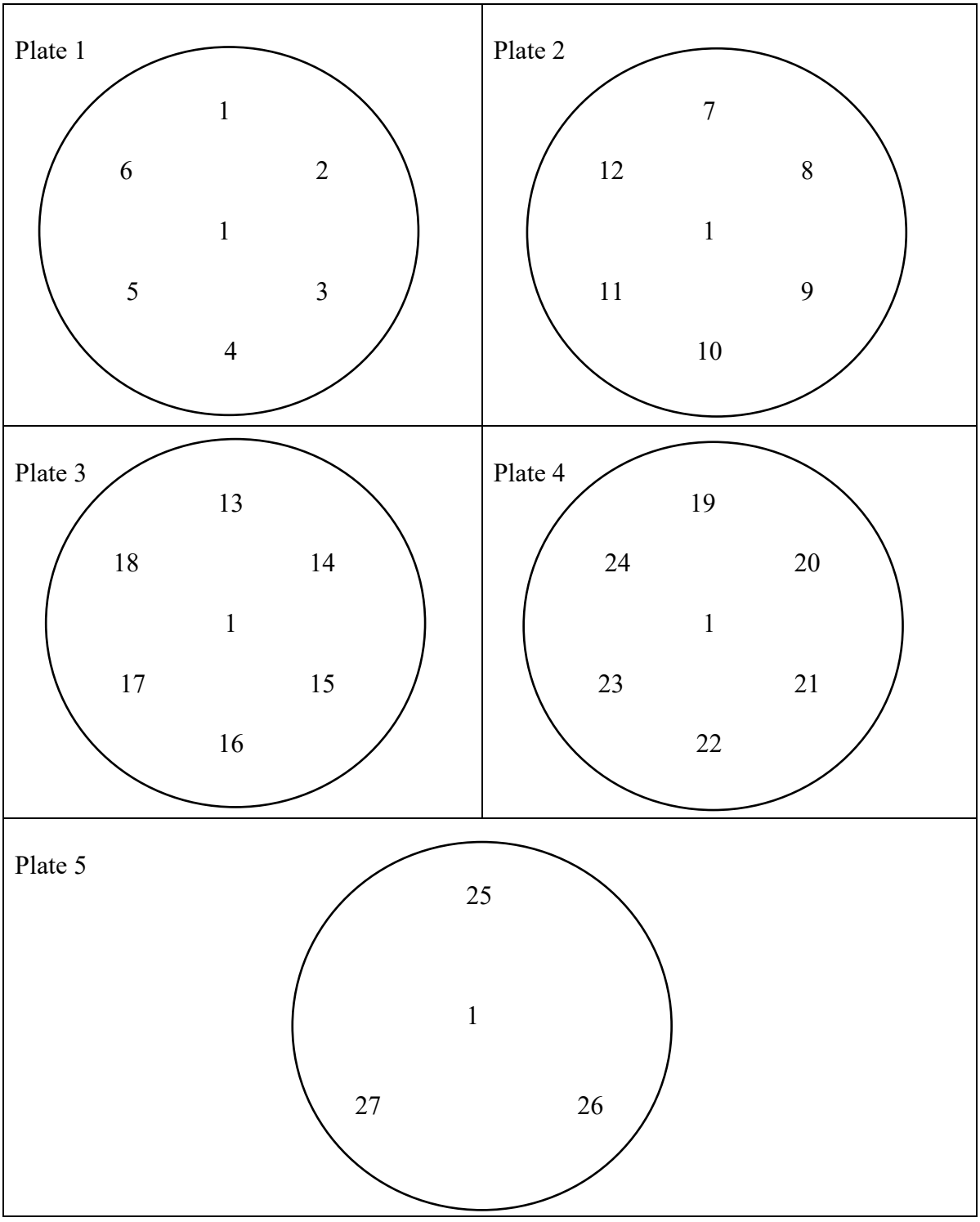


Fig. 3.7 Trial layout for isolate 1 placed in centre. Same layout was applied in sets of 5 plates for all 27 isolates changing only the central disc

Fig. 3.8 Filtration of Trichoderma cultures using sterile syringes and Minisart 0.22µ filters

3.8.2 Biochemical characterisation of CFs

Samples were processed using liquid chromatography mass spectrophotometry, quadrupole time of flight mass spectrophotometry (LC-MS/MS Q-TOF) analysis. All analyses were carried out on an Agilent HP 1260 Infinity Series liquid chromatograph equipped with a Diode Array Detector (DAD) system (Agilent Technologies, Santa Clara, CA, USA) coupled to a Q-TOF mass spectrometer model G6540B (Agilent Technologies) with a Dual ESI (electro-spray ionization) source. Separations were performed on an Ascentis ® Express C18 (2.7 µm, 50 mm x 3.0 mm i.d., Supelco ©, Bellefonte, PA, USA) column (Manganiello et al. 2019). The analyses were performed at a constant temperature of 37 °C and using a linear gradient system composed of A: 0.1% (v/v) formic acid in water, and B: 0.1% (v/v) formic acid in acetonitrile. The flow was 0.4 ml/min, 95% A graduating to 100% B in 6 min, 100% B 6-8 min, 95% A 8-9 min and equilibrating 95% A 9-14 min.

The UV spectra were collected by DAD every 0.4 s from 190 to 750 nm with a resolution of 2 nm. The MS system was equipped with an ESI source and operated with Agilent MassHunter Data Acquisition Software, rev. B.05.01 in the positive and negative mode. Mass spectra were recorded in the range m/z 100-1700 as centroid spectra, with 3 scans per second. Two reference mass compounds were used to perform the real-time lock mass correction, purine (C5H4N4 at m/z 121.050873, 10 mol/L^{-1}) and hexakis (1H,1H, 3H-tetrafluoropentoxy)-phosphazene (C18H18O6N3P3F24 at m/z 922.009798, 2 mmol L^{-1}). The capillary was maintained at 2000 V, fragmenter voltage at 180 V, cone 1 (skimmer 1) at 45 V, Oct RFV at 750 V. Gas temperature was 350 °C during the run at 11 L/min, and the nebulizer was set at 45 psi. The injected sample volume was 7 ml.

Data analysis were evaluated using MassHunter Qualitative Analysis Software B.06.00 and compared with known compounds included in an in-house database. The database contains information of about 4000 known secondary metabolites isolated from more than 80 different fungal genera, and recorded according to their name, molecular formula, monoisotopic mass and producing organism. Positive identifications of fungal metabolites were reported if the compound was detected with a mass error below 10 ppm and with enough score.

3.9 Field and Pot experiments

Three pot and two field experiments were carried out to evaluate efficacy of Trichoderma isolates to reduce incidence and severity of MP.

3.9.1 Trial with Trichoderma conidia and compost

A pilot trial was set up to evaluate the effect of one cold tolerant Trichoderma isolate 356 on incidence and severity of MP on lettuce plants growing in trays. This isolate performed best in *in vitro* trials but the colonies complete Petri dish cover in shorter time when the initial inoculum is mycelia rather than conidia. Compost was found a suitable material to work as a carrier to deliver mycelia to the trays. Plastic trays (40 x 30 x 10 cm) were cleaned, disinfected, and filled with soil from a field with high anthracnose severity from the previous year.

Five treatments were tested as in Table 3.4.

Treatment	Description
T1	Compost pre-inoculated with Trichoderma isolate 356
T2	Compost
T3	Trichoderma 356 (Conidia) + Humic acid (4g/l)
T4	Trichoderma (conidia)
T5	T5. Untreated control (UTC)

Table 3.4 Description of treatments tested in the tray experiment

Compost was incubated previously with 356 conidia as described above and scattered on top of the soil at a rate of 12 g per tray and incorporated into the soil. Treatment 2 received compost at the same rate. Humax dry powder by Zadco® was mixed at a rate of 4 g/l of water and 250 ml. of this suspension were applied in each tray for treatment 3. Finally, treatment 4 contained the conidial suspension in water. Ten grams of the colonised oat/sugar cane mulch in 1 litre of water was mixed, filtered, and poured evenly over each tray.

Four Romaine lettuce plants were transplanted per tray two days after inoculation (Fig 3.9). Plants were irrigated in such a way as to cause splash onto the leaves to promote infection. Amino acids were used as fertiliser weekly and hand weeding occurred several times during the growing period. Plants in the trays were assessed for size, incidence and severity of MP. Other winter diseases such as *Sclerotinia minor* and *Botrytis cinerea* were assessed at the same time.

Fig. 3.9 Lettuce seedlings transplanted in trays under open conditions in Garfield, Victoria, winter 2017

3.9.2 Controlled environmental chamber trials

Trials were set up to evaluate the efficacy of Trichoderma liquid filtrates *in vivo* and the minimal population of the pathogen to develop disease. As it was summer and conditions unsuitable for another field trial, plants were grown in an environmental incubator.

3.9.2.1 Assessment of MP propagule concentration to produce disease

This trial was set up to evaluate the minimal concentration of pathogen conidia to produce disease in lettuce seedlings. This is an important parameter to predict infection for future investigation.

Anthracnose conidia from the culture collection were inoculated onto 2 PDA plates and incubated at 10°C for 7 days. Two colonies were dislodged from the agar and placed in 10 ml of sterile water and shaken gently for 1 minute to release conidia. Presence of conidia was confirmed using a compound microscope and counted using a haemocytometer. The conidial suspension was diluted with sterile water to provide the following treatment concentrations (Table 3.5).

Treatment	Description
T1	Untreated Control (sterile water)
T2	MP 10^2 cfu/ml
T3	MP 10^3 cfu/ml
T4	MP 10^4 cfu/ml
T5	MP 10^5 cfu/ml

Table 3.5 Concentrations of MP propagules tested in lettuce plants to promote disease expression under refrigerated incubation.

Conidial suspensions were placed in individual 30 ml sanitised spraying bottles. Treatments were sprayed using ten pulses (2 ml), covering all surfaces of each plant. Integrity of spore concentration was maintained by applying the lower concentrated first treatment and

disinfecting the bench top between applications. This trial was replicated following the above protocol. White Romaine lettuce plants were purchased in trays from a commercial nursery (Fig 3.10 and 3.11).

Fig. 3.10 Plants of lettuce previously inoculated with pathogen and incubated in refrigerated conditions

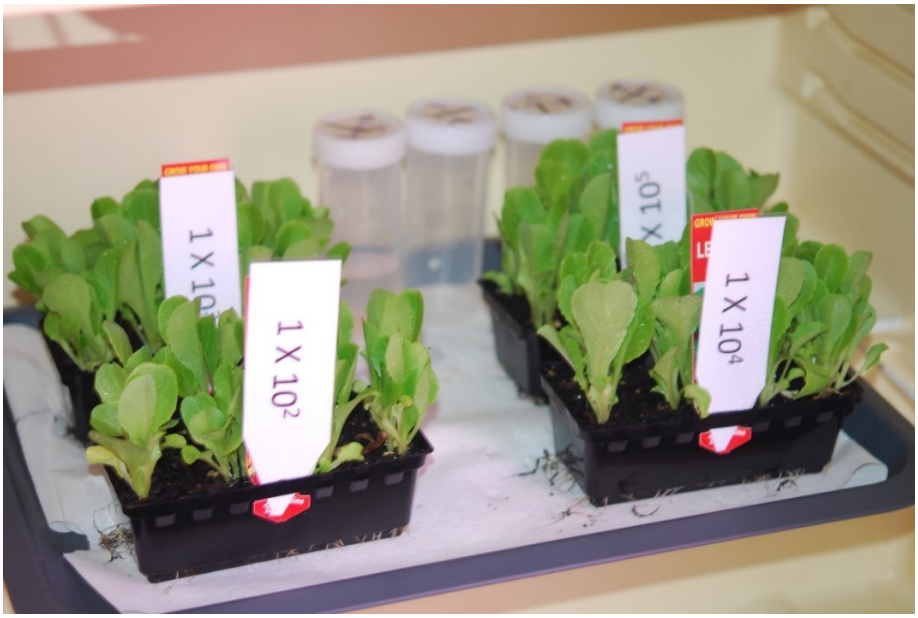


Fig. 3.11 Replicate of second pot trial in refrigerated incubator to evaluate minimum pathogen concentration to promote disease.

The number of plants per container varied from 11-15 and 17-23 in first and second trials, respectively. The Thermoline® refrigerated incubator was set up for 12 hours light for both trials and 10°C and 14-15°C for first and second respectively. One day after the treatments, sterile water was applied to wet the leaves to promote pathogen conidial germination. Leaves were wetted every 60 minutes for 8 hours. Sixty ml of water was applied every two days to the lettuce plants wetting only the substrate but not plants. Leaf wetness was maintained by spraying water on plants every two days. Additionally, plastic containers filled with 200 ml water were placed in the incubator to promote high humidity. Plants were observed during the experiments but were assessed finally for anthracnose damage 45 days after inoculation for the first trial and 27 days for the second trial.

3.9.2.2 Post infection control tests with CFs

This trial was installed to evaluate the efficacy of the liquid filtrates to control MP. Filtrates from single and challenged 1535, originating from Western Australia, proved one of the fastest growing cold tolerant isolates. This was tested on Gradara lettuce plants. Availability of lettuce varieties from commercial nurseries dictated what was available at experimental time. Trials were carried out in the same refrigerated incubator as described in 3.10.2.1. to simulate the winter season (10°C and daylength 12 hours). Two months old Gradara lettuce seedlings were collected from a commercial nursery. All plants showed evidence of anthracnose infection and were transplanted to 5 x 5 x 15cm. plastic pots filled with compost in a rack for 50 pots (Fig 3.12).

Fig. 3.12 MP infected lettuce plants sprayed with Trichoderma liquid cultures to evaluate control of the disease. Plants were incubated in refrigeration to promote disease

MP was sprayed with challenged liquid filtrates of 1535 to determine if there was an improvement in efficacy when compared with unchallenged liquid filtrates of 1535 (Table 3.6).

Treatment	Description
T1	Filtrate 1535 vs 1536
T2	Filtrate: 1535 vs GAU
T3	Filtrate 1535 vs ANT
T4	Filtrate 1535 vs LM
T5	Filtrate 1535 vs ANAND
T6	Filtrate 1535 vs BIANCA
T7	Filtrate 1535 vs TC1
T8	Filtrate 1535
T9	Untreated control (sterile water)

Table 3.6 Description of treatments sprayed on lettuce plants to control MP under refrigerated conditions

Liquid filtrates were produced following the same protocol for secondary metabolite analysis experiments (3.8.1).

Five plants (replicates) and 9 treatments (45 plants) were tested. Plants were sprayed separately with the treatment filtrates with enough liquid to cover all leaves using a new 30 ml atomiser. Both bottle and spray were rinsed in tap water and sterile water between applications. Plants in pots were set up in a random block design and returned to the incubator. Plants were sprayed with water daily to maintain leaf wetness and encourage the expression of the disease. Soil moisture was maintained by placing the tray in a sink and applying the same volume of 10 ml of water per pot each two days, drained and returned to the incubator. Once a week plants were watered with a solution of amino acids 6% N at a 1% rate. Treatment filtrates were sprayed on 100% of each leaf surface 3 times at days 0, 5, 10 after transplanting.

MP spots were assessed on new leaves only because, at planting, the seedlings had only 4 fully extended leaves all infected with MP. New leaves with spots were removed and the number of spots per leaf was recorded using a stereo microscope. For each plant, six 0.5 x 0.5 cm squares of tissue containing a spot were cut from the leaves and placed in 30 ml sterile McCartney bottles for surface sterilisation. The sterilisation procedure used a 1% chlorine solution (1:4 v/v commercial bleach 4% Cl: sterile water) gently shaken by a full arm extension for one minute. The chlorine solution was drained, replaced with 10 ml of sterile water and shaken for one minute as above, drained and rinsed three more times. Using a laminar flow cabinet, the now sterile tissue pieces were removed and dried on sterile paper towel to minimise wetness reducing potential contamination risks. The dry squares were embedded in PDA leaving the infection spot just touching the surface of the agar and

incubated at 10° C for three weeks. MP colony formation was recorded.

3.9.3 Field Trial 1 – Experimental design and treatments

Eight cold tolerant isolates previously selected under *in vitro* tests (3.6) were tested in an experimental plot within a commercial lettuce crop at Corrigan Produce Farms, Clyde North (South East Melbourne) in winter.

Soil was ploughed using a disc plough incorporating residues of a previous conventional (chemical fertilisers and pesticides used) celery crop. Two days later, a rotary plough and a bed former completed the soil preparation. No chemical fertilisers, herbicides or fungicides were applied to this plot at any stage of the research work. The experimental plot was isolated using obvious exclusion signs to prevent any accidental spray applications. Physical evidence of possible accidental spray application was identified by looking for tractor tracks in the furrows. The experimental plot was planted in winter. The time of planting was selected so that the most sensitive crop stage was aligned with the MP maximum virulence period. Mid-sized Romaine lettuce seedlings of variety “Subie” by Nunhems® recognised as a winter variety with exceptional vigour were obtained from a Berwick nursery, as a scheduled farmer’s requirement. Plants were inspected carefully for any symptoms of MP expression. A semi-automatic lettuce planter was used to plant the crop.

Eight Trichoderma cold tolerant isolates were tested using two inoculation methods: 1) conidia sprayed on top the the soil surface, and 2) conidia pre-inoculated and incubated in compost in Garfield, Victoria, and left to colonise for 7 days before being scattered on top the soil surface. Bays of 6 beds of 6 x 1.8 m were orientated north to south and bordered by

overhead sprinkler rows (Fig 3.13). As the winter season was wet enough, irrigation was not needed.

Fig. 3.13 First field trial showing details of the *Trichoderma* application technique in compost as a carrier

Eighteen treatments were tested with 4 replicates per treatment which corresponded to 72 experimental plots. To avoid the edge effect, 5 meters of each bed at either end were also planted. Total experimental area was 864 m^2. (Table 3.7 and Fig 3.14).

Lettuce density was 140 plants per experimental plot with 4 replicates: 560 plants per treatment on a grid of 20 x 20 cm. The crop was hand weeded three times when weeds were 2-3 cm tall. The tool was cleaned and disinfected with 10% methanol spray before moving to the next plot. For crop fertilisation, amino acids (Aminofeed by Suboneyo®, India), Biomin Manganese and Biomin Boron by Zadco® were sprayed three times at a rate of 50 l/ha, 1kg/ha and 1kg/ha respectively, as a source of nutrients for the crop. Finally, the assessment parameters are explained in table 3.8.

Treatments applied were as follows:

Treatment	Trichoderma Isolate	Inoculation Technique
T1	356	Compost pre-colonised
T2	PMF	Compost pre-colonised
T3	PMR	Compost pre-colonised
T4	LM	Compost pre-colonised
T5	LUP	Compost pre-colonised
T6	59837	Compost pre-colonised
T7	1535	Compost pre-colonised
T8	45899	Compost pre-colonised
T9	356	Conidia sprayed on ground
T10	PMF	Conidia sprayed on ground
T11	PMR	Conidia sprayed on ground
T12	LM	Conidia sprayed on ground
T13	LUP	Conidia sprayed on ground
T14	59837	Conidia sprayed on ground
T15	1535	Conidia sprayed on ground
T16	45899	Conidia sprayed on ground
T17	COMPOST	Water sprayed
T18	UNTREATED CONTROL	Water sprayed

Table 3.7 Description of treatments in the first field trial. Eight cold tolerant Trichoderm*a* isolates and two inoculation techniques.

	A	B	C	D	E	F
1	T 1	T 4	T 2	T 3	T 6	T 5
2	T 11	T 14	T 7	T 12	T 15	T 8
3	T 9	T 6	T 4	T 10	T 5	T 13
4	T 17	T 2	T 15	T 18	T 12	T 16
5	T 18	T 9	T 1	T 7	T 17	T 10
6	T 6	T 11	T 14	T 4	T 8	T 18
7	T 10	T 3	T 5	T 2	T 16	T 13
8	T 8	T 15	T 9	T 17	T 7	T 10
9	T 16	T 6	T 11	T 1	T 18	T 12
10	T 3	T 13	T 5	T 14	T 4	T 17
11	T 13	T 1	T 16	T 12	T 2	T 15
12	T 14	T 8	T 9	T 3	T 11	T 7

Fig. 3.14 Random distribution of the treatments in the first field trial

A. Incidence	**Number of plants affected with at least one MP spot**
B. Severity	**Percentage of the leaf or plant surface affected by MP spots**
1%	One or two spots of MP in a plant
5%	Two spots in two leaves or three spots in one leaf
10%	More than three spots in two internal leaves. (maximum damage for a commercial head)
20%	1/5 of total number of leaves is affected with at least one spot
50%	½ lettuce leaves in a head are affected with spots
100 %	All leaves in a head have at least one spot
C.	**Number of commercial lettuce heads per experimental unit**

Table 3.8 Parameters of assessment in the first field trial.

3.9.3.1 Preparation of Trichoderma Inoculum

Trichoderma conidia from 8 cold tolerant isolates were prepared by conidial inoculation into a substrate of 50 g of sterile soaked sugar cane mulch and whole oats 50/50 v/v incubated at room temperature in plastic clamshell punnets and replicated three times.

The clamshells were incubated in a dark confined area to minimise external contamination. After seven days, the substrate was inspected and gently shaken to homogenise the distribution of the fungal propagules in the substrate. The process was repeated 10 days later. The colonised substrate was ready to use after 21 days of incubation.

Ten grams of colonised substrate was placed in a 1l beaker with 500 ml of boiled cold water and stirred to release conidia. Conidia concentration was counted using a hemocytometer

and adjusted to 1 x 10^5 cfu/ml. Two hundred ml of conidial suspension was inoculated into 15 l of mature compost. After three days, the compost was inspected and evaluated for mycelial formation, then watered, turned and stored under cover for 7 more days at 2-14°C. Compost, as a suitable carrier for the Trichoderma propagation structures, was assessed against the standard protocol of freeze-dried conidial formulation sprayed directly onto crop and soil. Compost treatments were spread at a rate of 0.5 kg/m² one day after the lettuce crop was planted.

3.9.3.2 Assessment of MP distribution in experimental plot

MP incidence and severity in field is governed by the inoculum density in the soil. Symptoms of the disease appear in isolated areas in the plots that expand to infest more plants following the wind direction and rain splash. It is important to know the areas with high propagule concentration to correlate these with incidence and severity assessment.

Ten samples of soil from the field trial regardless of the treatment were taken from areas with low, medium, and high disease incidence and severity to assess the minimum pathogen population that causes disease. From each soil sample a 1/10 dilution was prepared, and solids allowed to settle for one minute before a serial dilution was performed to 1/1000. From these 10^{-2} and 10^{-3} suspensions, 200 µl were inoculated onto duplicate fresh PDA and spread using sterile plastic hockey sticks and incubated at 8°C to promote MP colony formation. PDA plates were assessed from day 7 and then daily until MP colonies developed after which cfu/g of soil were calculated.

3.9.4 Field Trial 2 – Experimental design and treatments

Erratic distribution of the pathogen in the soil from field trial 1 resulted in a large error in the subsequent analysis. To reduce experimental error in the second field trial the number of replicates was increased to 12 per treatment and only one Trichoderma cold tolerant isolate (356) was used.

The second field trial was carried out in a commercial organic farm in Pearcedale (Coolibah Herbs) again in winter. In trial two crop density was 14 plants per square meter. (Fig. 3.15). Four preferred midi cos lettuce varieties (Lotus RZ®, Quintus RZ®, Zazu® and Gradara RZ®) were tested. All varieties used in this experiment are cold season varieties. Ideally, both trials should be carried out on the same farm under the same conditions. However, farm 1 was not made available for the second trial. A different farm had to be found.

Fig. 3.15. Second field trial installed in a commercial lettuce crop in an organic certified farm. Pearcedale, Victoria

Use of compost was not included in this trial because, on this second farm, compost is generally applied prior to crop planting. This was different from the conventional farming system in the first trial where no organic matter was used in the cropping system. The standard practice for control of MP on the second farm involved using copper sulphate as this farm is an organic certified farm. This is the reason copper sulphate was included in the research project. Also, this allowed us to evaluate the compatibility of Trichoderma with this copper formulation (Table 3.9).

Treatment	Description
T1	Untreated Control
T2	Trichoderma isolate 356
T3	Copper sulphate
T4	Trichoderma isolate 356 + Copper sulphate

Table 3.9 Description of treatments in field trial 2

Lettuce seedlings were transplanted in winter and treatments were applied three times, on weeks 2, 3 and 4 after planting. The blend of oats and organic sugar cane mulch described earlier was used in this experiment. Similarly, as in field trial 1 (3.9.3.1), isolate 356 conidia were diluted to a 2.2 x 10^6 cfu/ml suspension and used for applications. A 15l backpack sprayer was used to apply treatments. Five hundred millilitres of Trichoderma 356 conidial suspension were diluted in 15l of clean water. Before spraying copper sulphate, the water pH was reduced from pH 7.0 to 5.5 using 0.2 g of citric acid (manufacturer recommendation) and checked using a handheld pH meter. Untreated control experimental plants were sprayed with water only. The assessments were performed in weeks 9, 10 and 11 following the same criteria used in the first trial (3.9.3).

Similarly, as in the first trial, samples of soil were collected to quantify the population of the pathogen and its distribution in the field. Five sub-samples of approximately 2 cm^3 each of soil were collected per experimental plot at 1 cm depth (10 cm^3). Four random samples were taken per treatment (16 samples) following the protocol as in 3.9.3.2.

Chapter 4

RESULTS

4.1 Introduction

This chapter presents a logical sequence of data and information from all the experiments. Starting from the classical morphological characteristics, basic information was gathered about how colonies from different isolates behave under different temperature, pH and media conditions. Molecular identification tests add critical data for the identification of the isolates and location in a phylogenetic tree. Vegetative compatibility/incompatibility trials contributed to the morphological and molecular findings to provide a critical link to the metabolomic assessments. Results from *in vitro*, pots and field experiments, supported the ability of Trichoderma to control MP.

4.2 Effects on Trichoderma morphological characteristics

Large differences in morphological characteristics were found between Trichoderma isolates, at different incubation temperatures, culture media, and media pH.

4.2.1 Temperature

In the experiment described above (3.3), temperature was the major variable that affected Trichoderma activity. Isolates with ability to grow in all experimental temperatures (356. 1535, 71556, CAROL, 59837, LM and LUP) showed significantly faster ($p<0.05$) growth at 25°C and 18°C (Appendices 1-7). Growth was slowed by (47%) at 18°C. (Fig. 4.1).

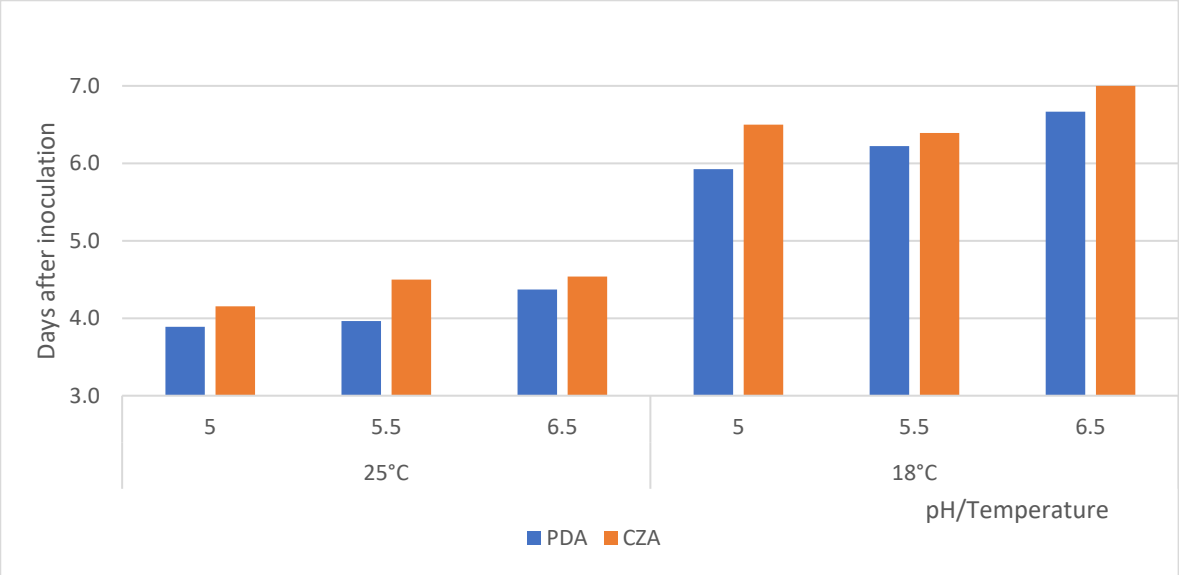


Fig. 4.1 Average number of days for Trichoderma colonies to cover a 90mm Petri plate at 25° and 18°C in two culture media and three pH media values (The shorter the column the faster growing conditions). NB at both temperatures, growth on PDA (undefined) was faster than in CZA (defined)

The fastest growing isolates 3 days after inoculation on PDA at 25°C were ANT, 355, VIR, PMR, 71556, 71558, MACA, CAROL, LUP and ANAND (40% of the isolates tested). The slowest were BIANCA and NUT whose colonies took 7 days to cover dishes (Fig. 4.2).

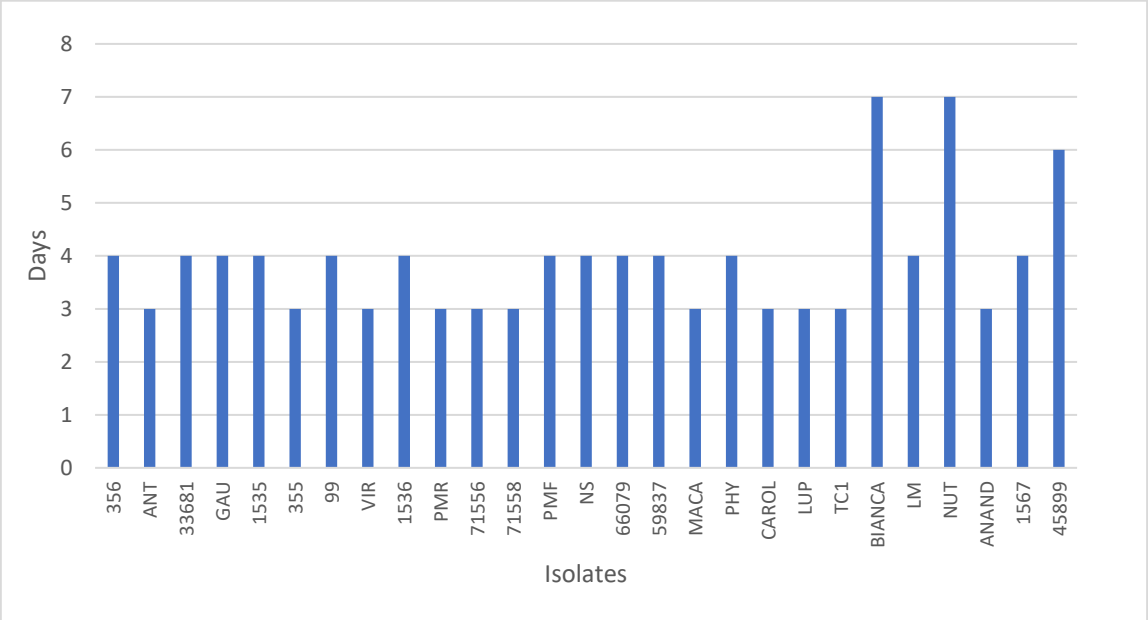


Fig. 4.2 Number of days for Trichoderma isolates to cover a 90mm PDA at 25°C

At 10°C, pH 5.0 in PDA, isolates completed full plate cover according to table 4.1. These were selected as cold tolerant isolates.

Isolate	356	1535	71556	59837	CAROL	LUP	LM	45899
Days	20	11	18	12	21	27	10	21

Table 4.1 Number of days for Trichoderma cold tolerant isolates to cover a 90 mm PDA plate at 10°C

Conidia from isolates 33681, VIR, 66079 and MACA are not cold tolerant. They did not germinate at 10°C after 30 days. Petri dishes with these strains were removed from the fridge and incubated at 25°C to determine conidia viability. Inactive conidia germinated, colonies revived, grew, and covered plates in 4 days as in the test at 25°C. Based on their ability to grow at 10°C, Trichoderma isolates can be classified in three groups (Table 4.2). Fully active, they grow consistently and relatively rapidly; partially active, they grow less rapidly, and inactive, when conidia do not germinate.

Isolates 71556 and LUP grew most rapidly at each of the experimental temperatures (25, 18 and 10°C). Strains 45899 and BIANCA were slow growing under the cold experimental conditions, but their range of temperature tolerance was greater than all other experimental isolates. Of all isolates tested only two out of eight cold tolerant strains (356 and 1535) completed their life cycle, phialide and conidia production in all combinations of media, temperature, and media pH.

Isolate	Fully active	Partially active	Inactive
356	X		
1535	X		
71556	X		
59837	X		
CAROL	X		
LUP	X		
LM	X		
45899	X		
ANT		X	
GAU		X	
355		X	
99		X	
1536		X	
PMR		X	
71558		X	
PMF		X	
NS		X	
PHY		X	
TC1		X	
BIANCA		X	
NUT		X	
ANAND		X	
1567		X	
33681			X
VIR			X
66079			X
MACA			X

Table 4.2 Trichoderma isolates grouped by their viability at 10°C

For all isolates, incubation temperature significantly affected phialide length and width ($p<0.05$) (Appendices 8 and 9). Phialide length was 8.63µ, 7.8µ and 7.3µ at 18°, 25° and 10°C, respectively and width was 2.23µ, 2.16µ, 1.87µ at 18°, 10° and 25°C respectively. By contrast, conidia were significantly longer ($p<0.05$) (Appendix 10) at 10°C and decreased in length with increasing temperature (18°-25°C). Conidia length was 3.51µ, 3.21µ and 2.91µ at 10, 18 and 25°C, respectively.

4.2.2 pH

There is no significant difference in colony diameter on media at any of the 3 experimental pH values. Isolates 355 and BIANCA took two days longer to cover a 90 mm PDA plate at 25°C when pH was raised from 5 to 6.5 (Fig. 4.3).

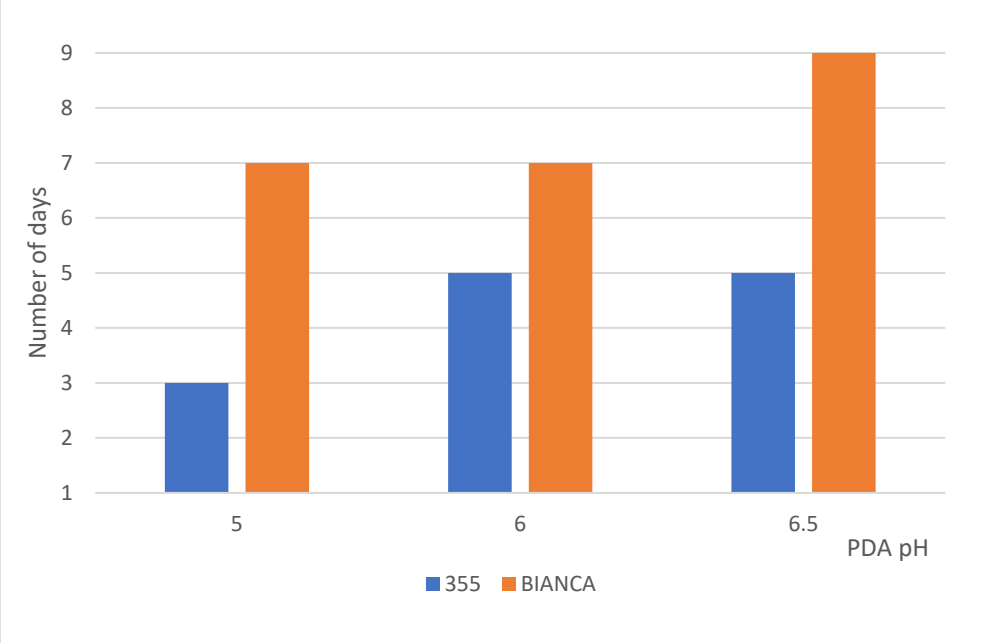


Fig 4.3 Number of days to cover a 90 mm PDA plate at 25°C by isolates 355 and BIANCA at different pH media

However, as the medium became more acidic the mycelia look more abundant, thicker and more felty textured (Fig 4.4).

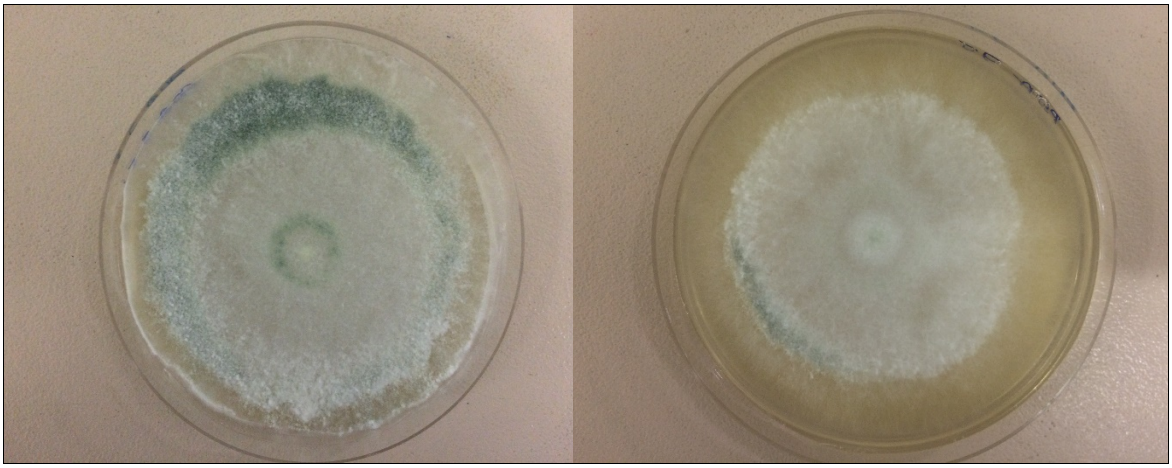


Fig 4.4 *Trichoderma paratroviride* (71556) cultured in PDA at 25°C. Left: pH 5.0, right pH 6.5, 3 days after inoculation

The effect of the media pH on conidiation depended on the isolate as shown in Table 4.3

Isolate	Conidiation at pH **5.0**		Conidiation at pH **6.5**	
	DAI	Characteristics	DAI	Characteristics
356	5	From centre, full circle	5	From centre, full circle
1535	4	From centre, star shape.	5	From centre, star shape.
71558	1	From centre, two rings	2	From centre and one ring at 15 mm.
ANT	1	From centre, starts at 10 mm.	4	From centre, starts at 10 mm.
CAROL	3	From centre in two rings	3	From centre in one ring at 10 mm.
GAU	3	From centre, starts at 10 mm.	4	From centre, starts at 10 mm.
MACA	2	From centre, multiple rings	2	From centre, multiple rings
NS	4	From centre, starts at 15 mm.	4	From centre, starts at 15 mm.
PHY	4	From centre, starts at 10 mm.	4	From centre, starts at 10 mm.
VIR	2	From centre, starts at 10 mm.	2	From centre, starts at 10 mm.

355	5	From plate wall	7	From plate wall
99	3	From plate wall in a ring at 15 mm.	3	From plate wall, starts at 20 mm.
1536	5	From plate wall	6	From plate wall
33681	4	From plate wall in a ring at 15 mm.	4	From plate wall in a ring at 15 mm.
59837	10	From plate wall in a ring at 10 mm.	10	From plate wall
71556	3	From plate wall in a ring at 10 mm.	3	From plate wall in a ring at 20 mm.
ANAND	3	From plate wall	3	From plate wall in a ring at 20 mm.
LM	4	From plate wall in a ring at 10 mm.	4	From plate wall and in a ring at 15 mm
LUP	4	From plate wall	6	From plate wall
TC1	4	From plate wall	3	From centre in a full circle.
NUT	5	From centre in a full circle	5	From centre in a full circle
PMR	4	From plate wall	4	Even distribution throughout agar.

Table 4.3 Characteristics of conidia production on PDA at 25°C and two different media pH. DAI= Days after inoculation.

These data showed that some isolates produce conidia along with hyphal growth while other isolates produce conidia when vegetative growth has reached its maximum, ie. full volume of Petri plate. On average, phialide length remained the same at different media pH, but they were significantly wider at less acidic pH ($p<0.05$). (Fig 4.5) (Appendix 11). Conidia dimensions are affected significantly by media pH ($p<0.05$). Conidia were shorter at pH 6.5 and wider at less acidic media (Table 4.4) (Appendices 12 and 13).

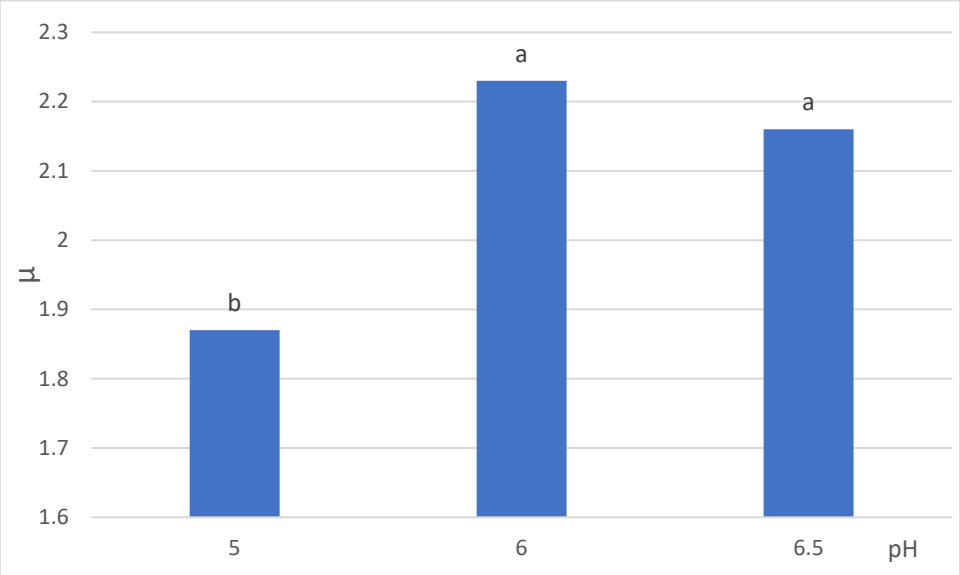


Fig. 4. Effect of the media pH on Trichoderma phialide width (µ) (Appendix 11).

Media pH	Conidia length (µ)	Conidia width (µ)
5.0	3.23a	2.82a
6.0	3.08 b	2.83a
6.5	3.31a	2.70 b

Table 4.4 Effect of the media pH on conidia dimensions

4.2.3 Culture media

Potato dextrose agar produced thick and felty Trichoderma colonies that grew in and on the agar (Fig. 4.5 left). Czapek dox agar produced thin mycelia with small cottony white balls that formed relatively few phialides and conidia (Fig. 4.6 right).

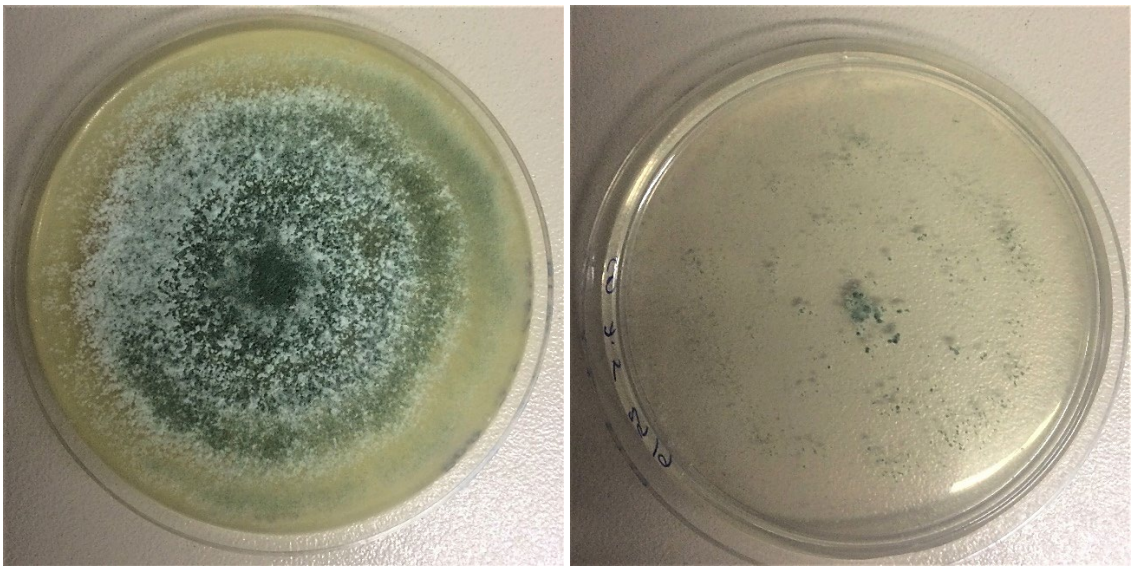


Fig. 4.6 Isolate MACA (*Trichoderma longibrachiatum*) cultured at 18°C and pH 6.5, 7 days after inoculation; left on PDA and right on CZA

Culture media produced significative differences ($P<0.05$) in phialide length and width (Table 4.5) (Appendices 14 and 15). Conidia was significantly ($P<0.05$) longer on PDA than on CZA (Appendix 16).

	PDA	**CZA**
Phialide length (µ)	8.90a	6.95b
Phialide width (µ)	3.0b	3.4a

Table 4.5 Average Trichoderma phialide dimensions by culture media (App.14 and 15).

For partially active isolates, cold temperatures delayed colony growth and development, food resources were depleted, chlamydospores developed while hyphae and phialides began to disintegrate.

4.2.4 Pigmentation and aroma

Isolates CAROL (*Trichoderma* sp.), VIR and 99 (*T. harzianum*), MACA (*T. longibachiatum*) and ANAND (*Trichoderma spirale*) produced yellow pigmentation that stained the PDA at all temperatures at which they grew. This pigmentation was not produced by these isolates on CZA. Similarly, the most documented Trichoderma secondary volatile metabolite, 6PP, developed only when selected isolates were cultured on PDA. Coconut aroma attributed to this metabolite was identified in 11 out of 27 isolates, *T. viride* (1535) *T. neokoningii* (355), *T. composticola* (356), *T. hamatum* (1536), *T. paratroviride* (71556), *Trichoderma* sp. (LUP), *T. asperelloides* (NS), (*T. atroviride*) PMR, PMF, NUT and LM when cultured on PDA but not on CZA. This aroma was also present at 10°C.

4.3 Molecular identification and classification of Trichoderma isolates

After following the protocol described by Jaklitsch and Voglmayr (2015), the isolates were identified as in table 4.6.

After processing sequence data, only 4 isolates produced satisfactorily aligned sequences with the three primers used (*tef, rpb2* and *acl1*). Phylogenetic multigene analysis determined 59837 (*T. viride*), PMF (*T. atroviride*), 66079 (*T. aureoviride*) and LM (*T. atroviride*) (Fig.4.7).

N	Species	Original Name	Clade
1	*T. neokoningii*	355	Green/harzianum
2	*T.* sp	71558	unknown
3	*T. composticola*	33681	Viride
4	*T.* sp	PHY	unknown
5	*T. asperelloides*	NS	Viride
6	*T. atroviride*	LUP	Viride
7	*T. longibrachiatum*	MACA	Longibrachiatum
8	*T. paratroviride*	NUT	Viride
9	*T. atroviride*	PMF	Viride
10	*T paratroviride*	71556	Viride
11	*T. composticola*	356	Viride
12	*T.* sp	CAROL	unknown
13	*T. polysporum*	45899	Polysporum
14	*T. harzianum*	VIR	Green/harzianum
15	*T. aureoviride*	66079	Green
16	*T. viride*	1535	Viride
17	*T.* sp	1536	unknown
18	*T.* sp	GAU	unknown
19	*T.* sp	ANT	unknown
20	*T. atroviride*	LM	Viride
21	*T. atroviride*	PMR	Viride
22	*T. viride*	59837	Viride
23	*T. harzianum*	99	Green/harzianum
24	*T.* sp	TC1	unknown
25	*T. spirale*	ANAND	Green
26	*T. viride*	1567	Viride
27	*T.polysporum*	BIANCA	Polysporum

Table 4.6 Trichoderma isolates used in this study identified by species and clades.

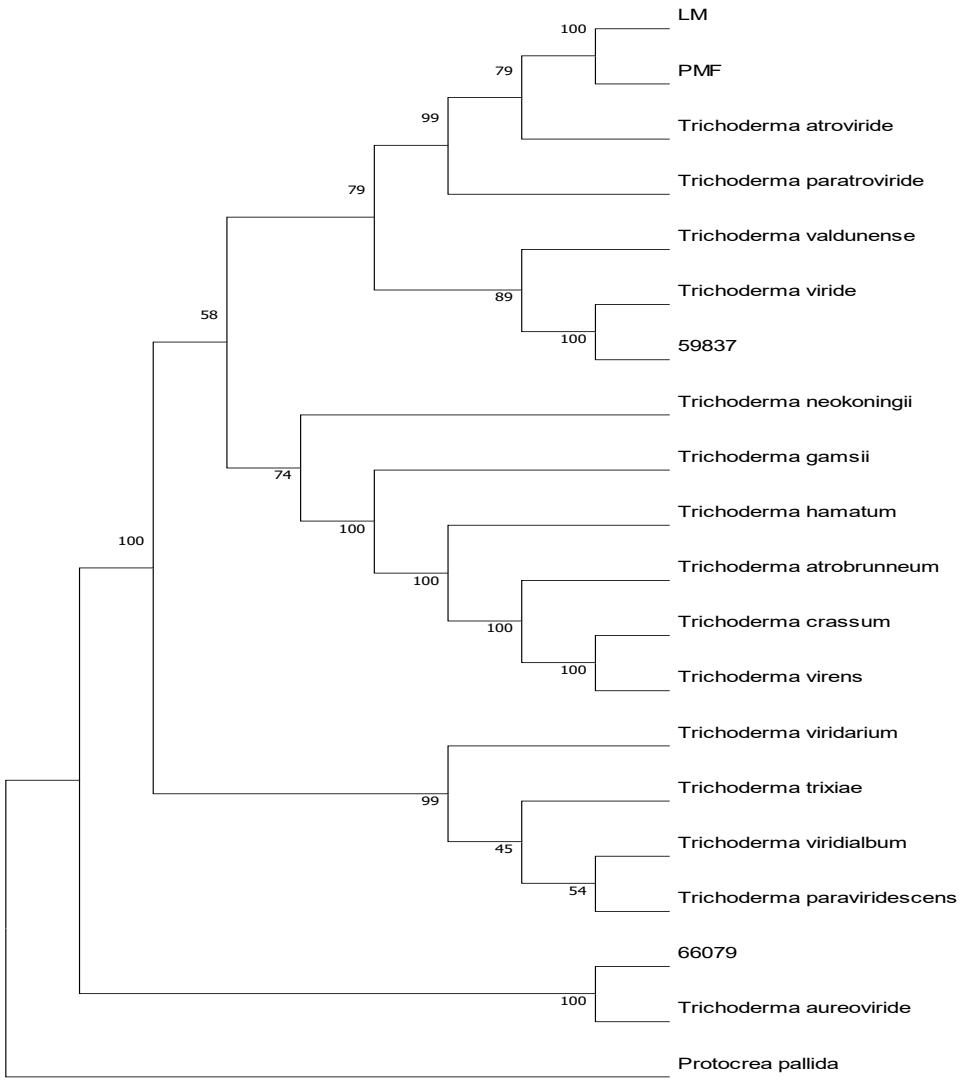


Fig. 4.7 Phylogenetic tree for genes *tef1*, *rpb2* and *acl1*

Sequence analysis of two genes (*tef and rpb2*) identified isolates 355 (*T. neokoningii*), 1567 (*T. viride*) and 71556 (*T. paratroviride*). VIR and 99 are closely related to *T. harzianum* (Fig. 4.8). LUP, LM and PMF formed a clade related to *T. atroviride.* GAU and TC1 are closely related to *T. pyramidale*, but evidence is not conclusive.

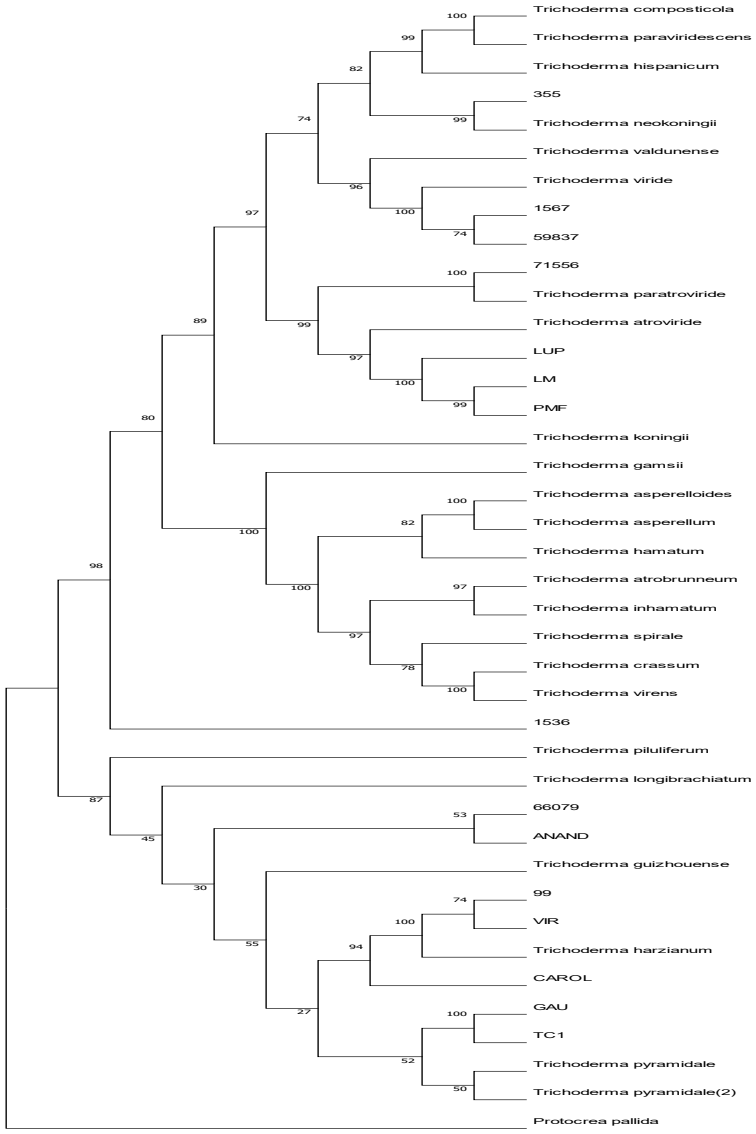


Fig 4.8 Phylogenetic tree for genes *tef* and *rpb2*

The single gene (*tef)* identified isolate MACA as *T. longibrachiatum.* Additional research is needed to identify isolates CAROL, TC1, 1536 and 71558.

4.4 Trichoderma cultured filtrates efficacy *in vitro* in wells

The first evidence of Trichoderma liquid filtrates affecting the MP normal growth was recorded during these experiments using mycelial cultures. Also, it was noticed that filtrates

maintained their biological activity after autoclaving, i.e. they are heat stable.

4.4.1 Liquid filtrates in well experiment

Preparation of the liquid filtrate wells experiment is described in 3.5.3.1. Normal growth of hyphae of MP was not disrupted. When viewed under a stereo microscope it was apparent that none of the 23 Trichoderma liquid filtrates from any of the 4 sets had any influence on hyphal structures of MP (Fig 4.9).

Fig. 4.9 *Microdochium panattonianum* hyphal structures at well edges showing normal growth

4.4.2 Pathogen/Trichoderma discs assessment

After pathogen agar discs were incubated for 14 days under challenged conditions, agar discs containing the pathogen were removed from the liquid cultures. In aseptic conditions they were rinsed, divided into 4, inoculated on fresh PDA and incubated at 10°C to determine survival.

Instead of MP colonies, 19 out of 23 *Trichoderma* isolates grew from the agar pieces. Eight of those (356, PMR, LM, PMF, LUP, 59837, 45899 and 1535) completely covered the agar surface in 10, 12, 12, 11, 15, 15, 17 and 15 days respectively at 10°C (Figures 4.10 and 4.11). Because of the plates were overgrown by the Trichoderma colonies, no observation could be made on the effect of filtrates on MP.

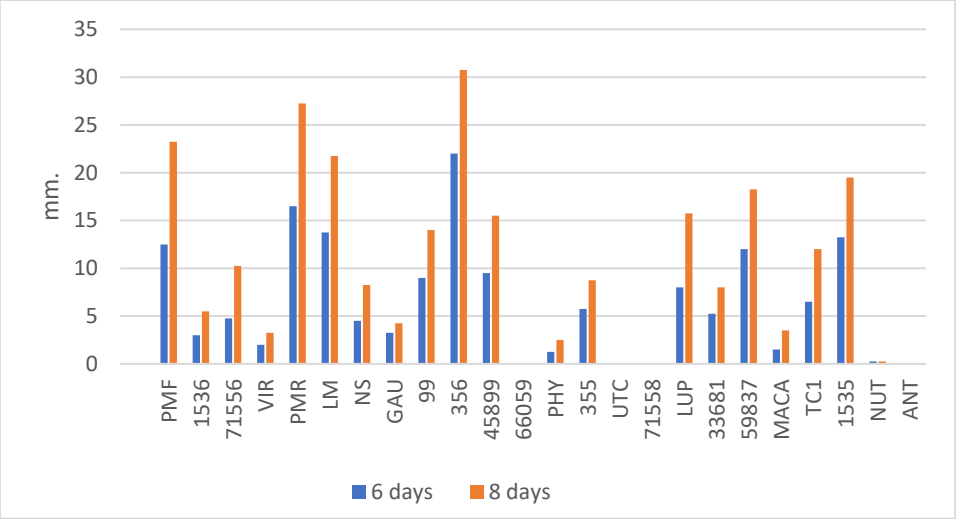


Fig. 4.10 Radius of Trichoderma isolates colonies incubated at 10°C after challenge culturing experiment

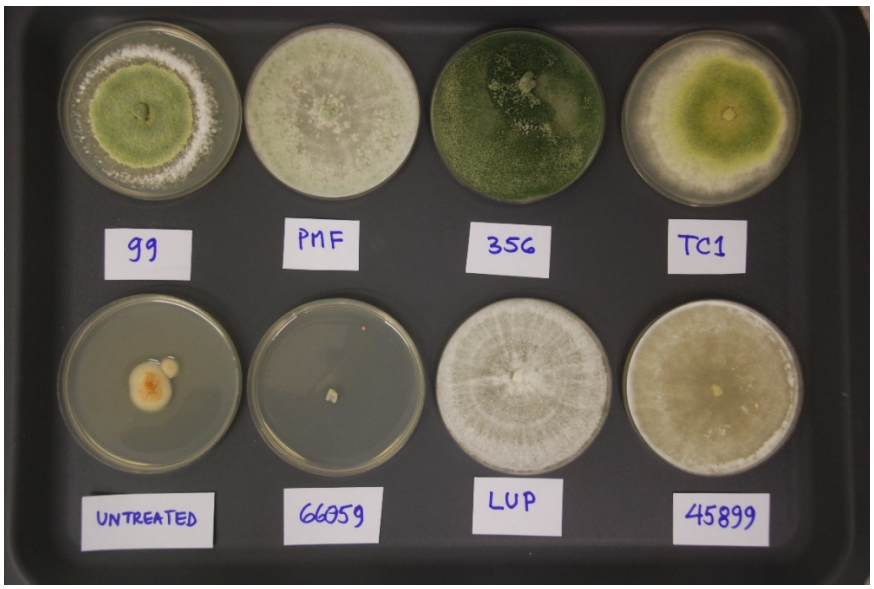


Fig. 4.11 Colonies formed at 10°C incubation temperature. Untreated shows a healthy MP colony

4.5 Growth of selected cold tolerant Trichoderma *in vitro*

After cold tolerant Trichoderma isolates were identified, an additional experiment was set up to confirm their performance. Cold tolerant isolates 356, PMR, LM, PMF, LUP, 59837, 45899 from conidial suspensions produced healthy colonies *in vitro* at cold temperatures in all replications. Statistical significance ($p<0.05$) showed differences in colony growth between the cold tolerant strains at 10°C at 6, 8, 10 (Appendix 17) and 14 (appendix 18) days after inoculation. Isolate 356 was the fastest growing in cold conditions from conidial inoculations (Fig 4.12).

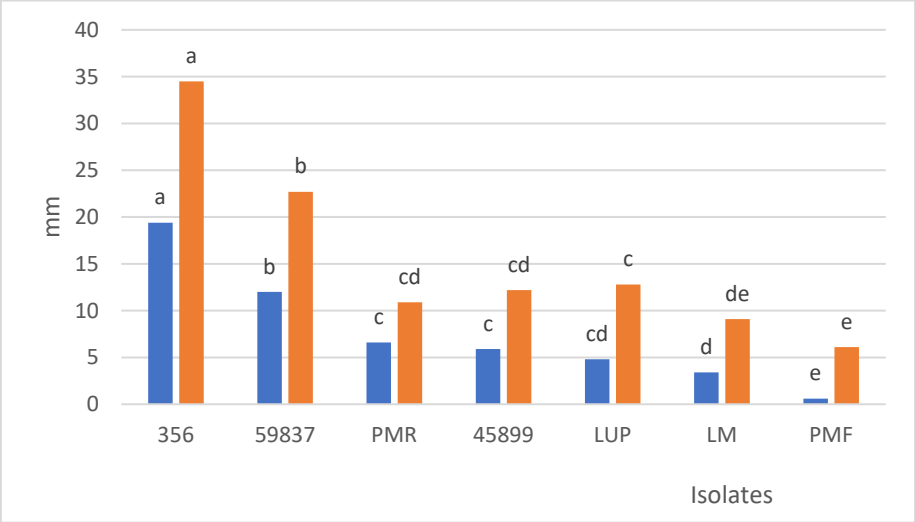


Fig. 4.12 Cold tolerant Trichoderma isolate colony radius, 10 (blue) and 14 (orange) days after inoculation incubated at 10°C from conidial suspension (Appendices 17 and 18)

Average colony radius 14 days after inoculation was 34.5, 22.7, 12.8, 12.2. 10.9, 9.1 and 6.1 mm for isolates 356, 59837, LUP, 45899, PMR, LM and PMF, respectively. When comparing colony radius, growth was faster when Trichoderma was propagated from mycelia on agar plugs than from conidial suspensions at 10°C (Fig. 4.13).

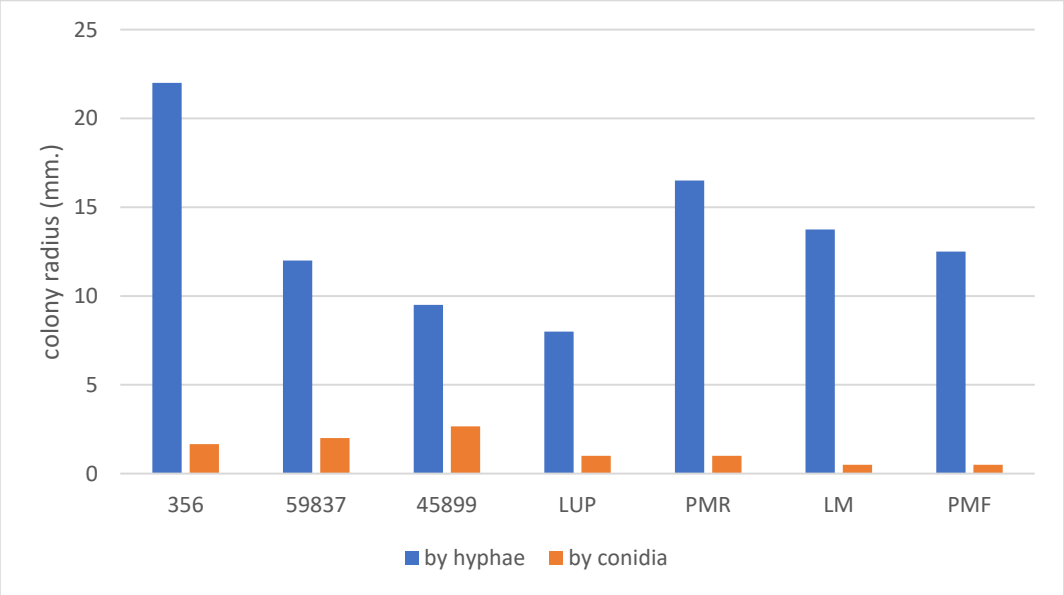


Fig. 4.13 Comparison of colony radius based on the initial inoculum 6 days after inoculation at 10°C

The average colony radius (7 cold tolerant isolates as above) was significantly larger ($p<0.05$) when colonies were cultured on acid pH media. Radii were 18.3, 15.3 and 12.8 mm. when were cultured at pH 5.0, 6.0 and 6.5 respectively, 14 days after inoculation at 10°C (Fig. 4.14) (Appendix 19).

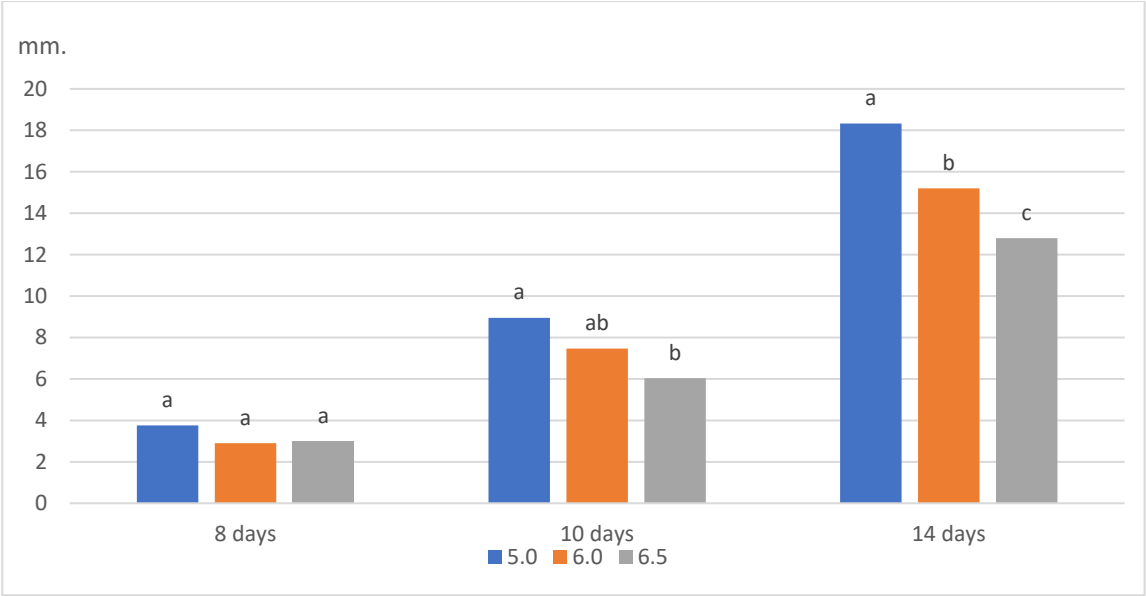


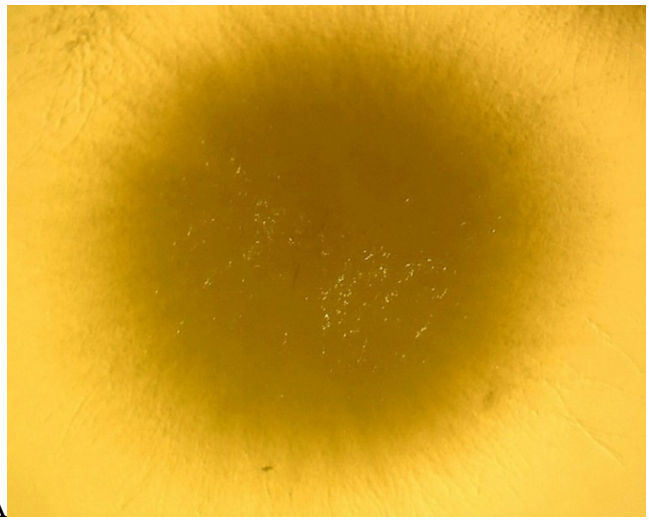
Fig. 4.14 Effect of three pH media on Trichoderma cold tolerant isolate colony growth (App. 19).

4.6 Test of MP on substrates containing liquid filtrates

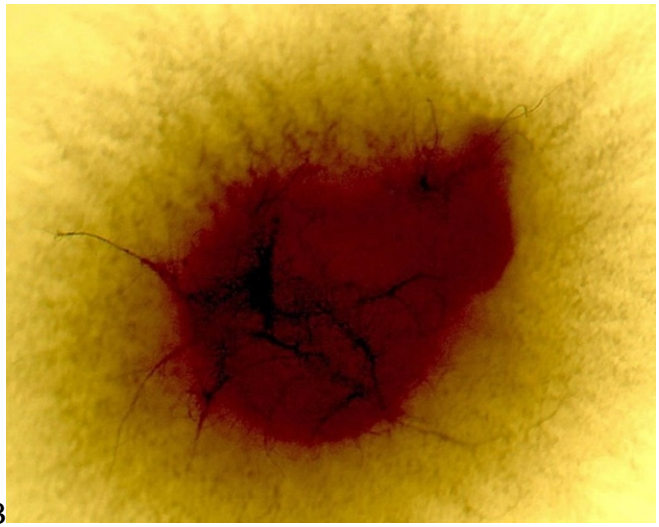
This experiment used filtrates from two cold tolerant isolates, 1535 and 356, selected for further analysis (3.5.2.2). PDA prepared using the liquid filtrates reduced the size and number of colonies and the hyphal growth of MP *in vitro* when compared with the results from the wells experiment (4.4). The culture filtrate from isolate 1535 (*T. viride*) and from isolate 356 (*T. composticola*) had similar effects on the pathogen structures. The pathogen hyphae were twisted, tangled and vertical rather than straight and uniformly radial as seen on the untreated plate. Also, hyphal architecture showed modifications resembling vertical taproots with very thick proximal ends and fine and hairy distal ends rather than the normal thin and homogeneous colony texture (Fig 4.15 A, B and C).

Results from 4.4 demonstrated the apparent presence of active compound(s) in the filtrates. 6PP was found in both filtrates. This compound has a boiling point of 286°C (Alam et al. 2016). The initial concentration of this metabolite (before autoclaving) was 35 and 30 mg/kg for 356 and 1535 respectively.

A



B



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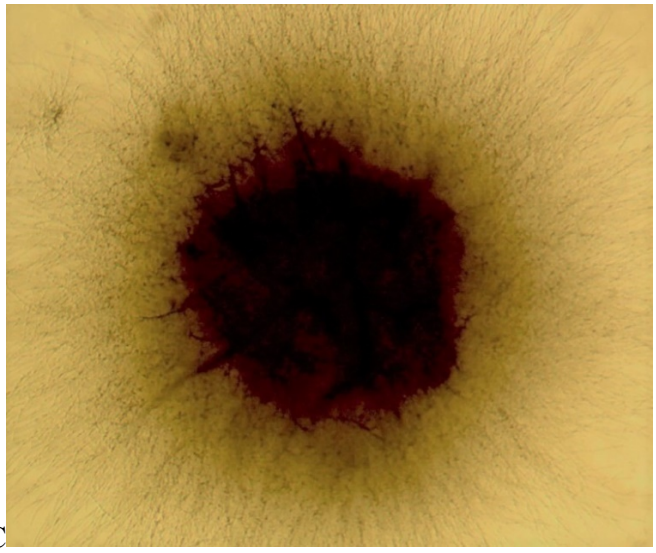

Fig. 4.15 Colonies of *M. panattonianun* cultured in A: Untreated PDA; and treated with filtrates from isolates B:1535 and C: 356, 10 days after inoculation X20

4.7 Vegetative compatibility/incompatibility

Under the experimental conditions, several "couplets" of incompatibilities were identified. Strong, medium, and weak barrages were observed (Table 4.7). Barrage formation from couplets producing the strongest reaction were least variable across three replications, e.g. isolates MACA (*T. longibrachaitum*) and CAROL (unidentified) formed strong barrages in each challenge showing they are strongly incompatible. Each block shown in Table 4.7 corresponds with 81 individual assessments, (27 from each of 3 replicates) between the same couplets located in the external ring (Fig. 3.7). The mean of challenges between isolates in the central position and individual radial neighbours, corresponded to 18 readings. Hundred and thirty-six couplets out of 378 (36%) possible combinations were identified as barrage producers. Barrage formation was not always reciprocal, e.g. isolates MACA and 71558 formed barrages when MACA was in the central position, but when 71558 was in the central position the barrage did not form

4.8 Trichoderma secondary metabolites (TSM)

Seventy-six previously reported and described chemical compounds were detected in this experiment (Appendix 20). Many more molecules were present in the liquid cultures but, to date, are described only by their chemical formulae. Some of these undescribed compounds share the same chemical formula but they have different retention times suggesting different chemical structures requiring chemical characterisation. Only named compounds were considered in this project results and discussion.

.

	355	71558	33681	PHY	NS	LUP	MACA	NUT	PMF	71556	356	CAROL	45899	VIR	66079	1535	1536	GAU	ANT	LM	PMR	59837	99	TC1	ANAND	1567	BIANCA
355			X	X							X				X	X	X	X	X								
71558			X				X				X	X										X	X	X			X
33681	X	X		X				X			X					X		X	X				X	X			
PHY	X		X		X		X			X	X		X			X	X	X	X	X	X		X			X	
NS				X					X	X			X	X		X	X	X		X	X						
LUP										X		X							X								
MACA		X		X				X	X			X	X				X	X	X		X	X		X	X		X
NUT			X				X		X	X		X				X	X	X		X	X		X				
PMF					X		X	X		X						X		X		X							
71556				X	X	X	X	X	X		X						X	X	X		X				X			
356		X	X	X						X			X					X	X		X						
CAROL		X				X	X	X					X		X						X		X				X
45899		X		X	X		X				X	X		X	X	X		X	X	X	X		X	X	X	X	
VIR					X								X			X	X						X	X			X
66079	X											X	X	X								X					X
1535	X		X	X	X		X	X	X				X				X	X	X	X	X			X	X		X
1536	X			X	X		X	X		X				X				X	X								
GAU	X		X	X	X		X	X	X	X	X		X			X	X		X	X	X					X	X
ANT	X		X	X		X	X			X	X		X			X	X	X		X	X			X		X	
LM					X			X	X				X			X		X	X		X	X					
PMR				X	X		X	X		X	X	X	X			X		X	X	X							
59837		X					X								X					X			X		X		
99		X	X		X			X				X	X	X								X		X			X
TC1		X	X				X						X	X		X			X				X				X
ANAND							X			X			X			X						X					X
1567				X									X					X	X								X
BIANCA		X					X					X		X	X	X		X					X	X	X	X	

Table 4.7 Trichoderma isolates showing incompatibility from barrage formation. Red blocks denote strongest incompatibility; X denotes medium incompatibility and empty blocks denote compatibility.

Liquid cultures of individual isolates produced fewer metabolites than challenged couplets. In some cases, e.g. liquid cultures from isolates 99, 1536, 71558 and VIR, contained no known metabolites. However, after these isolates were challenged, they produced up to 6 known metabolites. In general, only 33% of metabolites present in the liquid cultures were named. Liquid cultures from couplets with the strongest incompatibility in the vegetative incompatibility tests produced on average 3.7 known compounds out of 16.25 in total (20%). Liquid cultures from the single isolates forming those couplets produced on average 1.94 known compounds out of 11.6 in total (16.7%). Couplets produced 90% more known metabolites and 40% more total compounds than non-challenged isolates.

Known metabolites (Table 4.8) found in this experiment were:

Metabolite	**Biological activity**	**Literature Reference**	**Liquid cultures of:**
Zinnolide	Phytotoxic for lettuce seedlings	Ichihara et al. (1985)	356
Fusalanipyrone	Phytotoxic for lettuce seedlings	(Shimizu et al. (2005)	See appendix 20.
Mitorubrin	Phytotoxic	Büchi et al. (1965)	VIR, MACA
Asperfuran	Inhibits *Colletotrichum acutatum*	Batista et al. (2017)	355/356, 45899/TC1
PR toxin	Mutagenic	Dubei et al. (2018)	45899/66079
Citrinin	Nephrotoxic and Mutagenic	(Flajs and Peraica 2009) Pascual-Ahuir et al. (2014)	356
Pyrenochaetic acid	Phytotoxic against onion and lettuce seedlings	Ichikara et al. (1984)	356
Cytosporone S	Antifungal, antibacterial	Ishi et al. (2012)	66079, 66079/45899,

			CAROL/66079.
Fusarochromanone	Antiangiogenic, Anticancer	Mahdavian et al. (2014)	ANT, PHY, GAU, 355/ANT, MACA/GAU, PHY/ANT, PHY/GAU, PHY/NS
Mollicelin	Cytotoxic, antifungal.	Ibrahim et al. (2018)	1535/ANAND, 33681/ANT
Harzianic acid	Antifungal, plant growth promoter, phytosiderophore.	Vinale et al. (2013)	33681/TC1, 45899/VIR, MACA/TC1, VIR/NS, VIR/1535.
Oidiolactone E	Phytotoxic (herbicide)	Herath et al (2009).	45899/99, LM/PMR
Zearalanone	Mycotoxin, hepato, immuno and genotoxic	Maaroufi et al. (1996), Lioi et al. (2004), Berek et al. (2001).	45899/ANT, 45899/GAU, 45899/VIR, 45899/CAROL, BIANCA/CAROL
Xestodecalactone A, B and C	Antifungal	Bringmann et al. (2004)	MACA, MACA/71558
(+)-Harzialactone A	Antitumor, cytotoxic, antiaging	(Kothar et al. 2007), Yamashita (2000).	MACA/71558, MACA/45899, MACA/PMF
Gossipol	Toxic to animals, germicide, viricide	Coutinho (2002), Polski et al. (2002).	MACA/71558, MACA/45899, MACA/59837, MACA/CAROL.
Vertinolide	Mycotoxin	Li et al. (2019)	MACA/45899, MACA/BIANCA, ANT/LM, PMF/LM, NUT/GAU, LUP/71556

Phomalactone	Toxic to fruit fly and apple maggot. Herbicide and bactericide.	Krasnoff and Gupta (1994), Prathumpai and Kocharin (2014).	MACA/59837
Lucilactaene	Anticancer properties	Kayeya et al. (2001)	MACA/59837
Aspereline	Toxic to *S. sclerotiorum*	Brito et al. (2014)	MACA/NUT
Penicillide	Antifungal	Ferraz et al. (2008)	MACA/PMR
Ferricrocin	Phytosiderophore	Mukhergee et al. (2018)	CAROL/NUT
Harzianopyridone	Phytotoxic. Inhibitor effect against *R. solani* and *B. cinerea.* Antiviral against Herpes virus.	Dickinson et al. (1989) Ioca et al. (2016)	PHY/LM
Koningin B	Fungicide, Phytotoxic	(Singh et al. (2012) (Cutler et al. (1989)	BIANCA, BIANCA/1567

Table 4.8 Secondary metabolites with reported biological activity produced in the liquid cultures

Harzianolide, dehydroharzianolide and T39 butenolide, three well documented metabolites, are related chemically and synthesised only by couplets 1535/TC1, 45899/TC1 and NUT/99; and partially (one or two of them) in 99/BIANCA, 45899/99 and 59837/99. These metabolites were not produced in single liquid cultures by any of these isolates.

Fusalanipyrone was the second most produced TSM under experimental conditions in this project. Thirteen out of 27 (48%) individual isolates synthesised fusalanipyrone. When cultured under challenged conditions, 84 out of 137 (61%) of cultures contained this compound. Only strain 99 or any of its challenged couplets did not synthesise this chemical.

6PP, a volatile TSM, was the most identified compound in the liquid cultures (75%). Liquid

cultures produced by isolates 355 and 356, 71556, GAU, LM, LUP, NS and PMF and all their combinations contained 6PP. However, the highest concentrations (>100 mg/l) were found in cultures produced by PMR, PMF, LM LUP and 71556. Isolate 1535 and all its couplets except for 1535/TC1 also produced this metabolite (Fig 4.16). Many sets of PDA plates were cultured to assess several variables *in vitro*. "Coconut" smell associated with the presence of 6PP was present in many isolates.

Consistent results were achieved from organoleptic assessments performed three times with isolates cultured on PDA and incubated at 25°C. These results were consistent with those produced by the chromatography method (Table 4.9).

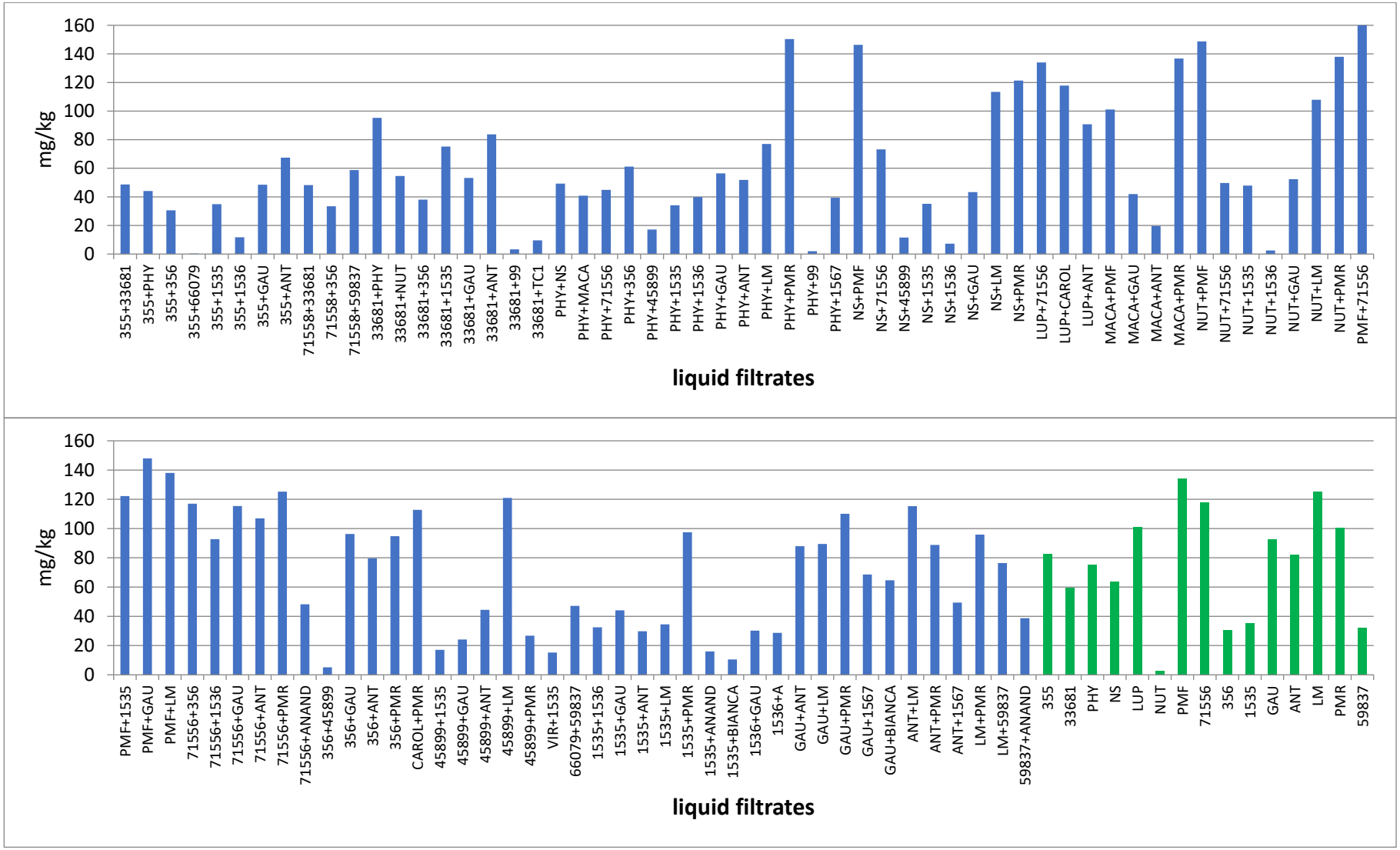

Fig. 4.16 Concentration of 6PP in experimental liquid cultures. Challenged cultures in blue columns, individual isolate cultures in green columns

N	Isolate	Species	Coconut aroma (0-5) (Smell)	6PP presence	6PP concentration (mg/l)
1	1535	*T. viride*	**2**	**Yes**	**35.01**
2	355	*T. neokoningii*	**2**	**Yes**	**82.63**
3	1536	*T.* sp	**1**	**No**	**0.0**
4	71558	*T.* sp	**0**	**No**	**0.0**
5	71556	*T. paratroviride*	**4**	**Yes**	**117.96**
6	33681	*T. composticola*	**0**	**Yes**	**59.6**
7	VIR	*T. harzianum*	**0**	**No**	**0.0**
8	66079	*T. aureoviride*	**0**	**No**	**0.0**
9	356	*T. composticola*	**3**	**Yes**	**30.40**
10	NUT	*T. paratroviride*	**1**	**Yes**	**2.74**
11	99	*T. harzianum*	**0**	**No**	**0.0**
12	LUP	*T. atroviride*	**3**	**Yes**	**100.75**
13	NS	*T. asperelloides*	**1**	**Yes**	**63.76**
14	PMR	*T atroviride*	**3**	**Yes**	**100.59**
15	PMF	*T. atroviride*	**3**	**Yes**	**134.11**
16	GAU	*T.* sp	**0**	**Yes**	**92.7**
17	ANT	*T.* sp	**0**	**Yes**	**81.99**
18	MACA	*T. longibrachiatum*	**0**	**No**	**0.0**
19	PHY	*T. sp*	**0**	**Yes**	**75.2**
20	TC1	*T.* sp	**0**	**No**	**0.0**
21	LM	*T atroviride*	**5**	**Yes**	**125.32**
22	59837	*T. viride*	**0**	**Yes**	**31.92**
23	45899	*T. polysporum*	**0**	**No**	**0.0**
24	CAROL	*T.* sp	**0**	**No**	**0.0**
25	BIANCA	*T. polysporum*	**0**	**No**	**0.0**
26	ANAND	*T. spirale*	**0**	**No**	**0.0**
27	1567	*T. viride*	**0**	**No**	**0.0**

Table 4.9 Coconut smell test. Average smell values come from a triplicated test. 6PP presence detected by chromatography analysis and concentration detected

Only in one challenged liquid culture, where isolates identified as not being 6PP producers, produced this metabolite; 1536/ANAND (28.65 mg/l) showed that the challenge stimulated TSM production. On the other hand, inhibition was detected when one 6PP producing individual isolate was inhibited by a non-producing isolate, as in: 66079 by 45899; 59837 by 99; 66079 by BIANCA; 59837 by MACA; NUT by MACA; NUT by 99; NUT by CAROL and 66079 by VIR. In general, production of 6PP was improved when isolates were challenged. Five individual isolate liquid cultures produced concentrations of 100-135mg/l (Fig. 4.17 in green columns): PMF, PMR, LM, LUP and 71556. Also, 16 challenged filtrates produced more than 100 mg/l and showed promising future practical use (blue columns).

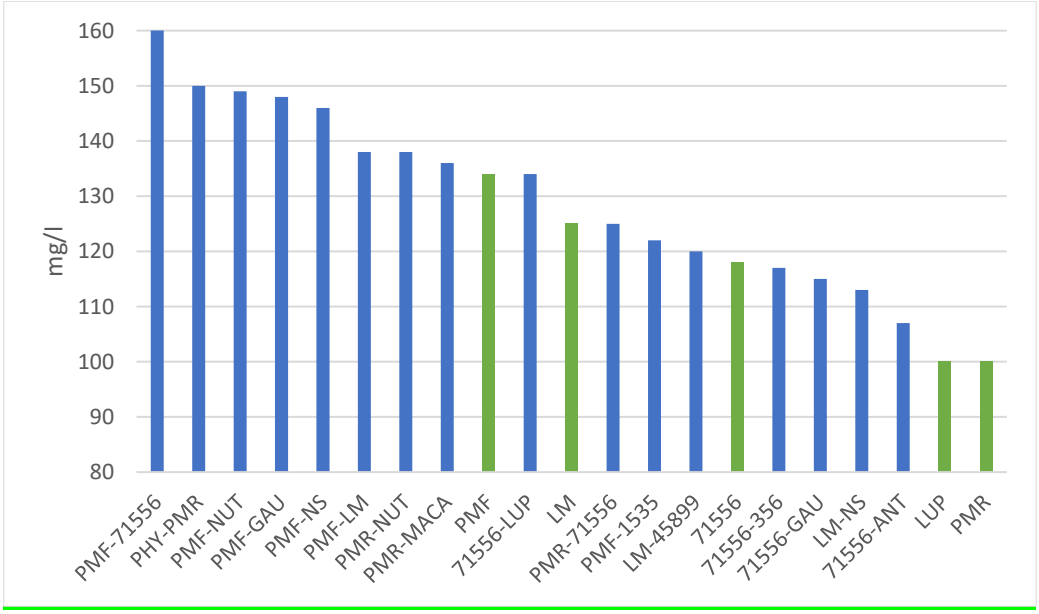

Fig. 4.17 Liquid filtrates from challenged and single cultures with ≥ 100mg/l 6PP

4.9 Pot, tray, and field trials

Before establishing the first field experiment, a tray trial was set up to assess efficacy of the cold tolerant isolate, 356, using two application techniques: pre inoculation in compost and conidial spray.

4.9.1 Trial with Trichoderma conidia and compost

In the tray trial, lettuce leaves showed no visible symptoms of MP after 3 months growth. Weather conditions were cold and wet enough to ensure pathogen virulence. Additional watering and splashing were used unsuccessfully to promote MP infection. However, plants were affected by other winter diseases such as *Sclerotinia minor* and *Botrytis sp.* which are also quite common and devastating diseases in lettuce crops. These pathogens caused the loss of 43.8% of plants.

Under trial conditions there were significant differences in the number of harvestable plants between treatments. Compost produced significantly more harvestable plants ($p < 0.05$). (Fig. 4.18) (Appendix 21). Anthracnose did not develop during this trial, however, Trichoderma conidial suspension, added previously to a humic acid suspension did not show control on the *Botrytis/Sclerotinia* complex but more dead plants. This level of infestation was comparable only with untreated plants.

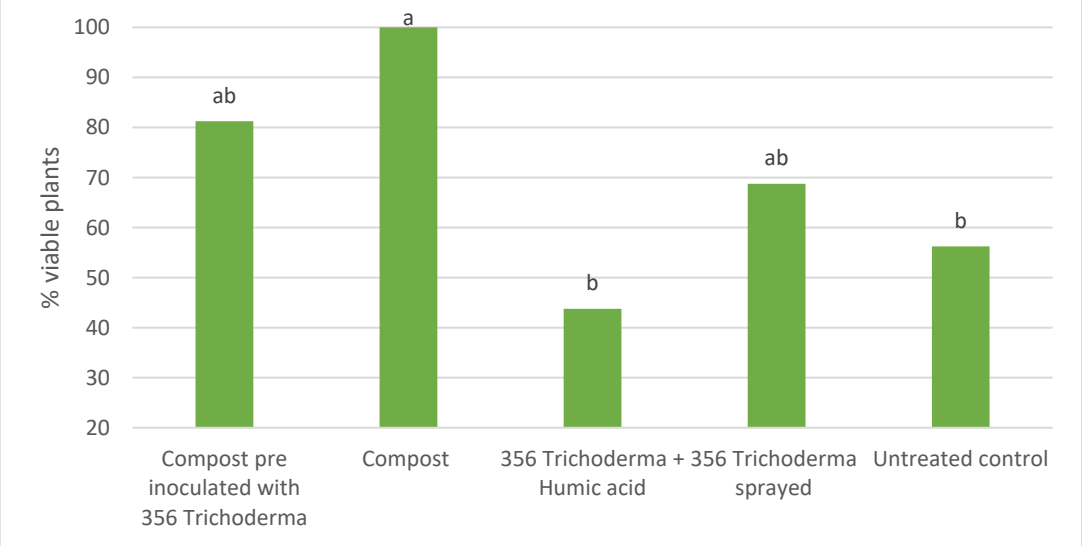

Fig. 4.18 Effect of *Trichoderma sp.* strain 356 on percentage of viable (marketable) plants of lettuce in trays trial (Appendix 21)

4.9.2 Pot trial in refrigerated incubator

Lettuce seedlings with obvious MP symptoms distributed evenly over the leaves were selected from a commercial nursery. Despite the application of the treatments (3.9.2.2), providing cold and wet conditions to encourage disease expression on plants in the refrigerated incubator, new leaves were not infected.

4.9.3 Second pot trial in refrigerated incubator

The second pot trial was set up to determine the minimum pathogen conidial concentration required to produce disease expression at 10°C in the refrigerated incubation. There were inconsistent results in two replicates. In the first trial, plants were incubated at 10°C and checked regularly for disease symptoms but no anthracnose spots were detected. In the second experiment the incubation temperature was raised immediately after pathogen inoculation from 10°C to 14°C for two more weeks to promote virulence. Disease was evident in this trial.

First MP spots were detected on midribs at the base of some leaves 5 days after treatment application with conidial concentrations between 10^4 - 10^5 cfu/ml. Table 4.10 shows results of disease incidence 28 days after application.

Treatment (cfu/ml)	N° of infected plants	Total number of plants	Percentage of incidence
Untreated (0)	0	21	0
1 x 10	0	20	0
1x 10^2	2	18	11
1x 10^3	2	22	9
1x 10^4	5	23	21
1x 10^5	9	17	53

Table 4.10 Incidence of MP: Number of plants with MP spots and percentage

4.9.4 First field trial

Under field experimental conditions there was no significant effect of 8 cold tolerant Trichoderma isolates or application technique on the reduction of incidence and severity of MP and the percentage of harvestable lettuce plants.

Samples of soil from the experimental plot were analysed two weeks before harvest. The distribution of MP propagules was uneven. Higher populations of MP propagules in the soil was correlated with high incidence of the disease on leaves (R= 0.9009) (Fig 4.19, Table 4.11).

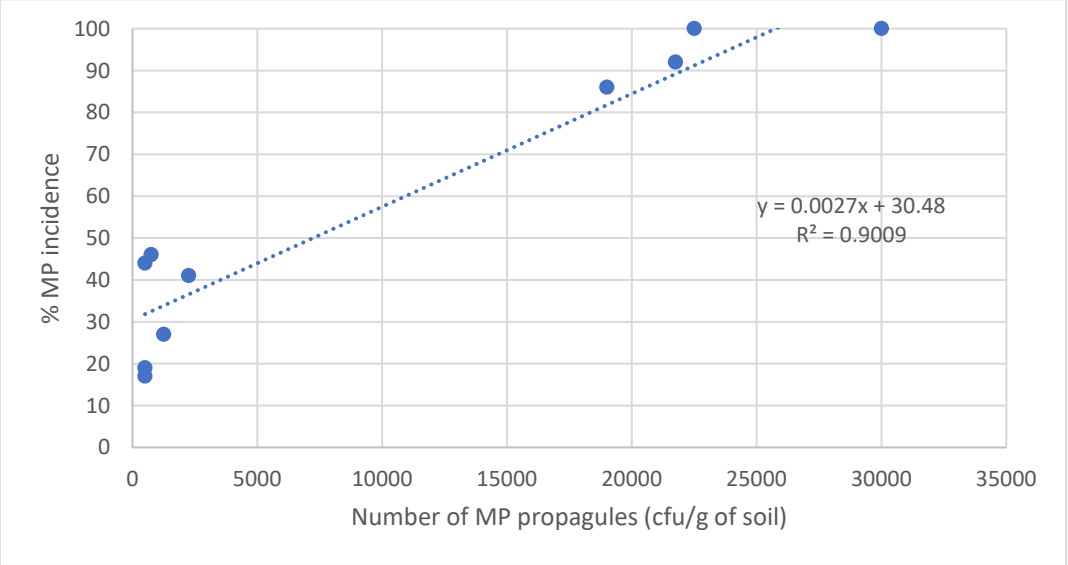

Fig. 4.19 Correlation between the number of MP propagules in soil and incidence of the disease

Sample	1	2	3	4	5	6	7	8	9	10
Treatment	T3	T3	T1	T14	T18	T3	T14	T11	T18	T11
Propagules (cfu/gr soil)	21750	1250	500	22500	19000	2250	500	30000	5000	750
Incidence (%)	92	27	19	100	86	41	17	100	44	46

Table 4.11 Population of MP in soil and incidence of the disease in ten spots (treatments)

4.9.5 Second field trial

In the second field experiment, lettuce varietal tolerance was found to be the most significant factor in reducing incidence and severity of lettuce anthracnose in field ($p<0.05$) (Appendix 22). Varieties, Lotus and Quintus, showed significantly less plants infected by the pathogen (incidence) ($P<0.05$) and the largest number of harvestable lettuce heads per hectare (64 and 85% respectively).

Varieties, Zazu and Gradara, produced significantly ($P<0.05$) more infested plants per experimental plot (28 and 81% respectively) and a larger number of non-harvestable viable heads per hectare at harvest (74 and 85% respectively) (Appendix 23). Fig 4.20 and 4.21 show lettuce varieties Lotus and Zazu were more tolerant to anthracnose.

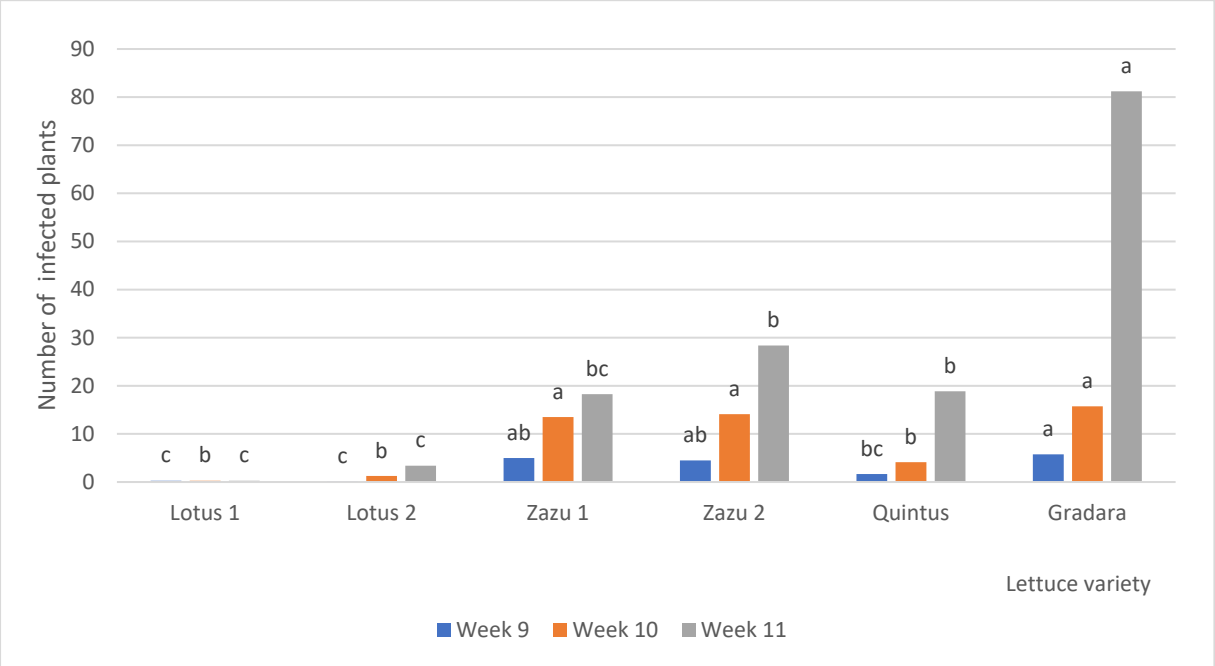

Fig. 4.20 Incidence: Number of lettuce plants out of 140 total plants, with at least one spot of anthracnose in one leaf (by variety) (Appendix 22)

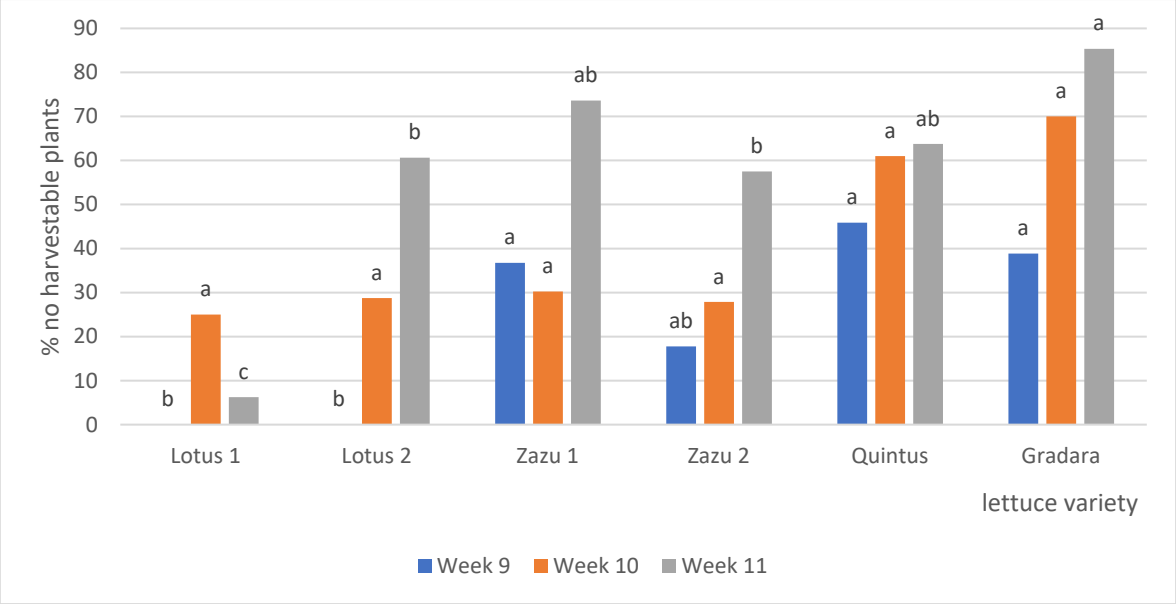

Fig. 4.21 Severity by lettuce variety: Percentage of infected plants with not commercial value (App. 23)

At week 9, the effect of applied treatments in the first assessment (in blue columns) showed no significant differences in disease incidence. At week 10, disease incidence increased, but was still not significant (orange column). At week 11 the incidence of anthracnose (grey columns) showed an increase throughout all experimental plots (Fig 4.22).

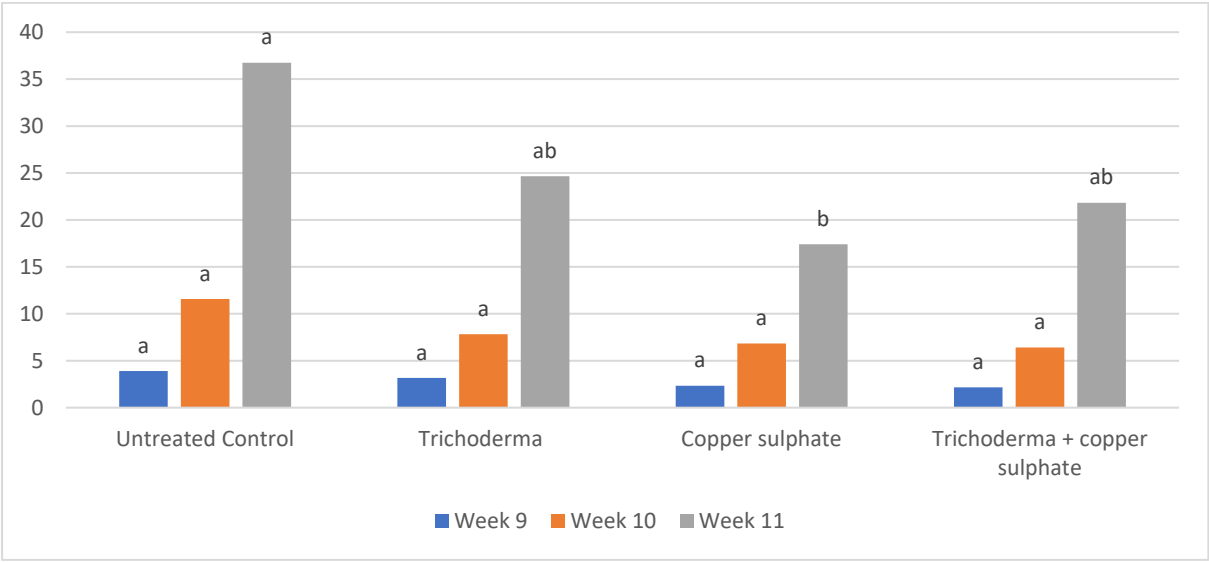

Fig. 4.22 Incidence: Number of plants (out of 140 per plot) with at least one anthracnose spot per leaf. (by treatments)

By harvest time (week 11), the percentage of plants with at least one spot of MP per replicate was significantly lower (P<0.05) in the plants treated with isolate 356, 356 + copper sulphate and copper sulphate (Table 4.12) (Appendix 22).

Treatment	Incidence (%)
Untreated control	26
Isolate 356	17
Copper sulphate	13
Isolate 356 + Copper sulphate	15

Table 4.12 Incidence of MP by treatment at harvest

Anthracnose severity level was low in the first assessment (week 9), but at week 10, severity was reduced significantly by all treatments (P<0.05) (Appendix 24). At harvest time, conditions favoured the disease, and in all treatments, the number of lost lettuce heads was dramatically higher. Copper sulphate showed least yield loss during the last week before harvest. Isolate 356 losses were 60% of lettuce heads while the untreated control resulted in 75% loss (Fig 4.23).

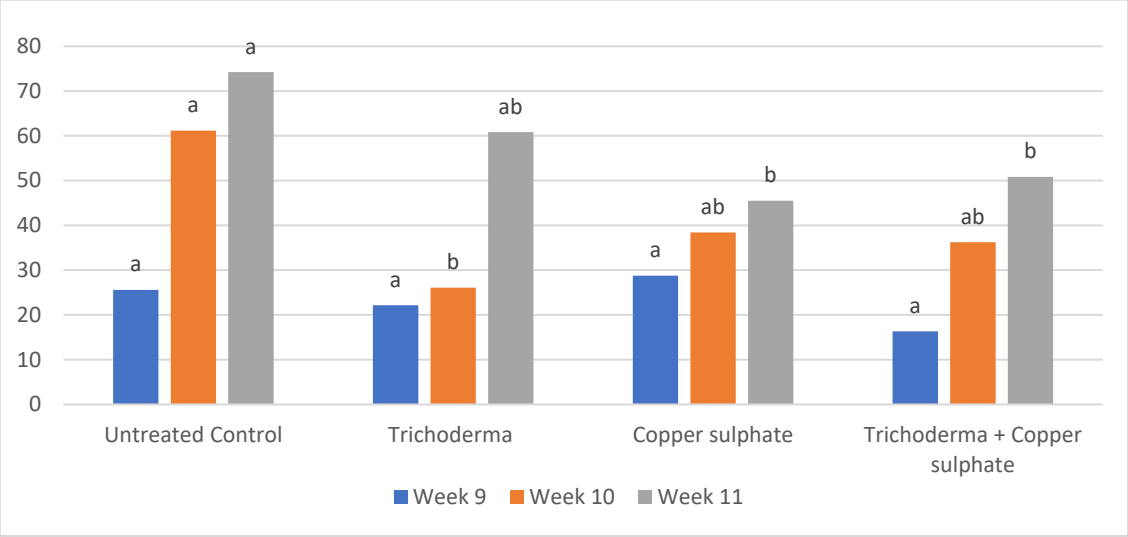

Fig. 4.23 Severity: Percentage of MP infected plants with no commercial value by treatment (App 24)

In both annual field experiments, symptoms of MP disease were noticeable at eight weeks after planting, three weeks before harvest. Therefore, the critical period for this disease was in the last three weeks before harvest under these conditions. The second soil test (3.9.4) from 16 soil samples showed the distribution of propagules of MP was uniform with an average of 1 colony per plate that corresponded to 500 cfu/g of soil.

Chapter 5

DISCUSSION AND CONCLUSIONS

5.1 Introduction

The first objective of this thesis was to isolate, characterise and identify cold tolerant Trichoderma isolates with the capacity to control lettuce anthracnose in soil. This objective was fulfilled by identifying 8 cold tolerant *Trichoderma* species which were selected, characterised, and identified using morphological and molecular techniques. It was found that temperature, pH, and media had a significant influence on the morphological characteristics of Trichoderma isolates. Cold tolerant isolates identified were classified as *T. viride*, *T. atroviride*, *T. polysporum* and *T. composticola* by molecular techniques. Four out of 8 cold tolerant isolates were identified as *T. atroviride*. All strains used in this project belonging to this species were cold tolerant. McBeath 2002) had success controlling *M. nivale* (a close relative of MP) by a strain of *T. atroviride* in its cold pathosystem. These findings suggest *T. atroviride*, formerly *T. lignorum,* is potentially useful in cold environments, but prior screening is always essential.

The second objective was to identify Trichoderma secondary metabolites for lettuce anthracnose control by foliar application. Fusalanipyrone, 6PP, cytosporone and cladoacetal A reduced growth of MP *in vitro*. Additional TSMs were produced by challenging isolates in PDB liquid cultures. Conidial suspension of the cold tolerant isolate, 356 (*T. composticola*), containing TSMs were applied to lettuce in the field significantly increasing the number of commercial lettuce heads ($p<0.05$) (Appendix 23).

Outcomes from this research have significant advantages for the control of MP in winter lettuce

crops. They provide a biological alternative for disease protection in cropping systems where the only control method now is chemical. Song Zhu, agronomist in Coolibah Herbs (pers. comm. 31st august 2020), identified a reduction in efficacy using Octave (prochloraz) to control MP in field. This suggested MP has developed resistance to this chemical leaving the industry without the only chemical with relative efficacy in controlling this pathogen. Prochloraz was sprayed 7 times on 10 weeks lettuce crops without success.

This integrated methodology has international application in disease protection for many soil borne and leaf borne pathogens. It gives farmers more options for sustainable food production. Some advantages include more economical production, more sustainable production, protection, and preservation of beneficial microbiology in agricultural soil, a safer and healthier working environment for farm workers, and finally, a healthier product for consumers.

Therefore, this thesis fulfilled its aims and showed it is possible to control lettuce anthracnose, a winter disease, using cold tolerant isolates of the biological control agent, Trichoderma. Control of lettuce anthracnose was achieved when a *Trichoderma* sp was applied to soil prior to planting to promote soil colonisation combined with post-planting foliar sprays of liquid filtrates at regular intervals during the winter crop season.

Not all the isolates of Trichoderma, wild type or from commercial formulations, that were tested could grow and express all required activity in the environmental conditions where MP shows maximum virulence. Therefore, morphological characterisation and molecular identification and *in vitro* tests in cold incubation to find effective isolates was carried out before field trials were begun.

The commercial production of effective TSMs able to restrict MP growth is feasible but not necessarily achieved using cold tolerant isolates that were essential for soil colonisation. As the liquid cultures were produced under controlled conditions where isolates reached optimal mycelia and TSM production, the ideal temperature for culturing conditions depended on specific isolate requirements (18-25ºC), adequate pH (5.0-6.0) and nutrient balance. Additionally, the process of challenging isolates in co-cultures used to promote the activation of their defence mechanisms achieved more diverse and abundant production of TSMs.

5.2 Identification and characterisation of suitable Trichoderma isolates to control MP in soil.

Assembling a Trichoderma culture collection was the first and essential step in this research project. Each culture was created from a single spore guaranteeing the genetic purity of each isolate. The validity of the morphological, molecular, biochemical, and pot and field experiment results relied on this premise. Because efficacy relies on particular characteristics of species, it is essential that isolates used to produce useful TSMs are of single spore isolate origin. Special care was taken to collect wild type isolates from different environmental conditions within Australia to ensure the broadest thermal, hydric, and nutritional spectrum. Isolates were collected from wet and dry areas, soil, mulch, roots, and plant buds, to achieve morphological and genetic diversity and a more diverse ability to produce TSMs. Cold tolerant isolates with consistent ability to control MP *in vitro* were isolated from temperate environments that correspond to the pathosystem in study corroborating the concept coined by Fravel (2005): the biocontrol must be successful in the environment where the pathogen is virulent.

In this research project the morphological characteristics, historically considered as fundamental for fungal identification, required molecular profiling to further separate the species. However, at standard *in vitro* conditions (25°C, PDA, and pH 5.0) characteristics such as colony growth speed, mycelia texture i.e felty, hairy or ricotta like, colony shape i.e. symmetrical or "fried egg" shape, or conidiation pattern, i.e. from centre or from edge of culturing plate; were necessary for grouping clades and predicting their performance as biocontrol agents.

Carbon and nitrogen food sources had a profound effect on the biological activity of Trichoderma isolates. Trichoderma colonies grew in different pattern when cultured on PDA and CZA media showing that some components in PDA favour Trichoderma growth and indirectly TSM production. It was evident from morphological studies that some species preferred monosaccharides and specific aminoacids (Atanasova 2014).

PDA is an undefined medium, amino acids, sugars, starch, and many other biologically active molecules are present in variable concentrations depending on the potato cultivars and on the growing and post-harvest management conditions (Leonel et al. 2017). For example, Griffith et al. (2017) found that low levels of copper in the tubers used to produce PDA, reduced the production of key enzymes associated with conidial pigmentation. In a similar way the production of 6PP was apparent only when Trichoderma 6PP producers were cultured on PDA under all experimental incubation temperatures and pH values used in this project. This suggested that this characteristic was not directly affected by changes in these variables. These changes can induce or reduce the hyphal growth confirming the result from Kubicek-Pranz et al (1998) that some monosaccharides and monosaccharides-related compounds support growing conditions for some species over others.

Czapek dox agar is a defined medium with all its components as salts at standard concentrations. Therefore, more reproducible results were possible than when organisms were cultured in PDA. However, PDA contains sources of nitrogen other than nitrates as in CZA. This may explain why Trichoderma isolates cultured on PDA produced abundant growth, while growth on CZA produced thin, meagre, and hard to distinguish colonies with few phialides and conidia (Fig 4.4 right). This confirmed results from Danielson and Davey (1973) and Rajput (2014) that ammonium and not nitrate was the preferred nitrogen source for Trichoderma growth. Therefore, nitrate applications in soil as a source of nitrogen for crop nutrition may impact negatively on the antagonistic performance of Trichoderma. High concentration of nitrates in the rhizosphere can cause a rise in pH which is not preferred by Trichoderma. N-NO_3^- is up taken by the plant and an OH^- is released to balance the charge (Hinsinger et al. 2003). This could be the reason why the microbial activity in the rhizosphere improves in presence of ammonium rather than in nitrate nitrogen.

Further research to confirm the effects of ammonium and nitrate ions on Trichoderma performance *in vitro* could be carried out substituting ammonium nitrate and ammonium sulphate, as they are popular agricultural fertilisers, in the CZA recipe instead of the standard sodium nitrate in its formulation.

Neither coconut aroma in the Trichoderma 6PP producers, nor yellow pigmentation in the agar by pigmenting isolates were noticed when Trichoderma isolates were cultured on CZA under any conditions of pH and temperature. These results also support the hypothesis that nitrate reduced the biological activity and antagonistic properties of Trichoderma. Czapek dox agar, however, induced Trichoderma isolates to produce less mycelium which tended to be more concentrated in small cottony balls that were easy to dislodge from the agar surface allowing

good quality slide mounts for clearer microscopic observations to be prepared.

Modifying the culturing conditions induced fungal morphological changes and biological control attributes, i.e. production of 6pp. In practical terms, the fertilisation (organic or inorganic), liming, gypsum application, ploughing, irrigation, and the seasonal conditions may also modify the behaviour and distribution of Trichoderma in the soil. These parameters must be considered before a selection and practical application of this BCA in field conditions.

During this research work, Trichoderma isolates showed preference for pH media between 5.0 and 6.0. Colony growth decreased with decreasing acidity, reducing their capacity to supress pathogens as found by Simon and Sivasithamparam in 1988. They determined that there was better control of the 'take all' pathogen by Trichoderma in moderately acid soil. These findings also correlated with the maximum inhibition of *Fusarium* sp colony growth obtained by a higher volatile TSM production reached at pH 6.0 (Abeyratne and Desahappriya 2018). This may be a result of more abundant Trichoderma biomass production.

The most sensitive isolates to changes in substrate acidity were 355 (*T. neokoningii)* and BIANCA (possibly *T. polysporum*) expressed by the difference in the rate of growth on PDA at pH 5.0 and 6.5 (Fig. 4.3). When pH increases, hyphae became stressed manifesting as distorted hyphae. Daryaei et al 2016 reported that Trichoderma, as other fungi, have the capacity to adapt themselves to external pH modifying it by activation of a pH-sensing system that is genetically activated in alkaline conditions by the zinc finger transcription factor PacC described by (Tilburn et al 1995). This system is inactive in acidic conditions. and varies between species and strains (Moreno-Mateos et al 2007). This may explain why 355 and BIANCA were affected by slight changes in acidity. More investigation is required to

determine the activity of factor PacC in all the isolates to predict their ability to modify their environmental pH and select those that perform better in alkaline soils.

As expressed above, Trichoderma is more often reported in the international literature as a warm temperature organism which contrasts with the MP thermal conditions. Temperature is a regulator of mycelial growth and is considered an indirect factor in metabolite production. The most effective Trichoderma isolates that control MP *in vitro* and in soil corresponded with locations characterised by cold temperatures. The species are related to *T. viride*, *T. atroviride*, *T. polysporum* and *T. composticola*. However, *T. polysporum* (BIANCA) and *T. composticola* (33981) are exceptions. This suggests that the ability to survive and develop in cold temperatures is not a species attribute but rather an environmental adaptation mechanism as stated by Ghildiyal and Pandey (2008). For this reason, it would be expected that the best isolates to control MP by soil inoculation should originate from cold regions. At Trichoderma suboptimal conditions like in winter when MP shows maximum virulence, the application of one or more fast growing biocontrol agents is critical to colonise more soil volume in the shortest time and destroy MP propagules before they become active.

Conidial formulations are used commercially as a benchmark around the world based on their easy dispersal and convenient storage. Conidia can be freeze dried for long shelf life and when applied to the soil they will germinate unless conditions are not ideal. Results from *in vitro* experiments suggested that when the Trichoderma cold tolerant isolates were inoculated on PDA as mycelia and incubated at low temperatures they formed colonies faster than when they were inoculated as conidial suspensions confirming that mycelia are less affected by fungistasis than are conidia (Papavizas, 1985). Isolate 33681 (*T. composticola*) conidia were unable to germinate in PDA at 10°C and for that reason is not considered a cold tolerant isolate. However,

when mycelia of this isolate were inoculated onto PDA, they produced healthy colonies under cold incubation. Based on this observation, mycelia from cold tolerant isolates were inoculated in an organic substrate, incubated, and then applied into soil in cold conditions in the first field experiment. Similarly, Wong et al (2002) applied Trichoderma conidia on straw infested with *Fusarium pseudograminearum*, covered them with soil and incubated *in vitro* with enough moisture at 15°C obtaining 90% of reduction of pathogen survival structures. This suggests that for better results, conidia or mycelia should be applied in a carrier that provide shelter, moisture, and nutrients for the BCA confirming the observations of Van Elsas and Postma (2006). A product based on hyphal fragments would cause farmers problems by blocking sprinklers, spray nozzles and filters as well as drippers in irrigation systems. Compost was selected as the carrier for Trichoderma cold tolerant isolates because it is relatively easy to find and manipulate. Kok et al (1996) obtained successful control of *R. solani* inoculating, incubating, and applying swine manure pellets with a *T. harzinaum* conidial suspension. This material could be high in ammonia which can result in toxicity for the conidia and mycelia as found by Montgomery et al (1980). For this reason, chicken manure pellets also high in ammonia, popular in Victorian agriculture, were not selected as the carrier.

The tray experiment set up to evaluate the efficacy of the inoculation technique in soil using a conidial suspension and mycelia pre-incubated in compost to control MP produced an unexpected result. MP was not evident despite the soil used to fill the trays was sampled from a heavily infested MP lettuce crop the winter before. Cold tolerant isolate 356 controlled *B. cinerea* and *Sclerotinia minor* and resulted in a significantly greater number of harvestable lettuce heads because of the improved control ($p<0.05$). Diverse microflora in the compost may have an important role in controlling these pathogens confirming the conclusions of Blaya et al (2016) demonstrating that the application of compost on farms is a valuable resource and

needs more research work. Commercial Humic acid (Humax) is highly alkaline (pH 9.5). This strongly alkaline input could negatively affect the Trichoderma capacity to control these two diseases confirming that alkaline pH may not allow Trichoderma to express their control mechanisms as reported by (Trushina et al 2013). In relative terms, thermal aerobic composts, have received less attention from researchers than, for example, a synthetic single nitrogen fertiliser, urea. This observation shows that there are good reasons for serious research into the potential disease control by these natural products.

The 8 cold tolerant isolates were tested in a commercial lettuce field under winter conditions using conidia and mycelia pre cultured in compost. Large visible, but not statistical, differences in the number of apparently harvestable plants were noticed when the soil was inoculated with Trichoderma cold tolerant isolates using both application techniques. This variation was due to the apparently uneven distribution of MP in the field which correlated with the observations of Paterson and Grogan (1991). The pathogen distribution analysis in soil showed that areas with more disease incidence and severity corresponded to soil with the highest infestation (R=90%). What cannot be explained from these results, despite not having significant differences in incidence and severity of MP from the application of cold tolerant isolates and inoculation techniques in the soil, was the higher number of extra viable lettuce heads in the crop treated with isolate 356. There were 40% more commercial heads per hectare than the untreated control.

The experimental design for the second field experiment considered 12 replicates rather than 4 as in the first field experiment to reduce the experimental error from the erratic distribution of the pathogen in the field. Unfortunately, the first plot was not available in the following winter because it was engaged with other crops. This was a limitation for this experiment because

ideally the same trial should have been replicated in the same plot to assess the pathogen dynamics one year after the treatments were applied.

Among the cold tolerant isolates, LM and PMF are remarkably close to *T. atroviride* in the phylogenetic tree and they are sold commercially using this name although no conclusive identification was obtained from the phylogenetic analysis. LUP, isolated from lupin roots near Robinvale, Victoria, was also located in this clade but in a separate branch next to PMR, which are sold by the same commercial company as LM and PMF (Fig. 4.6). The remaining cold tolerant isolates were identified as 45897 (*T. polysporum*) which formed hyaline conidia on PDA; 1535 and 59837 both (*T. viride*) and 356 (*T.composticola*).

Isolate 1536 (*Trichoderma* sp.) was not successfully identified to species and is not cold tolerant although it was isolated from the same substrate as isolate 1535. This fact confirmed that cold tolerant and warm tolerant Trichoderma isolates can share the same substrate and possibly alternate dominance through warm and cold seasons as explained by (Lo et al. 1997).

Isolates 356 from pistachio buds from South Australia and 33681 from the NSW herbarium belong to the same species (*T. composticola*) but, 356 is cold tolerant while 33681 is inactive at 10°C. Morphologically, their phialides produced by both isolates are similar; very long and pointy, but their conidiation pattern is different on PDA at 5.0 pH and 25°C (Fig 5.1). The cold tolerant 356 began conidiation with the radial development of mycelium at 25°C suggesting that it was not the preferred incubation temperature. Isolate 33861 did not formed conidia until mycelium covered the available nutrient suggesting that 25°C was a suitable growth temperature and sporulation was initiated only by depletion of nutrients. These morphological dissimilarities confirm that there is still more variability intraspecies than interspecies

(Druzhinina et al 2012).

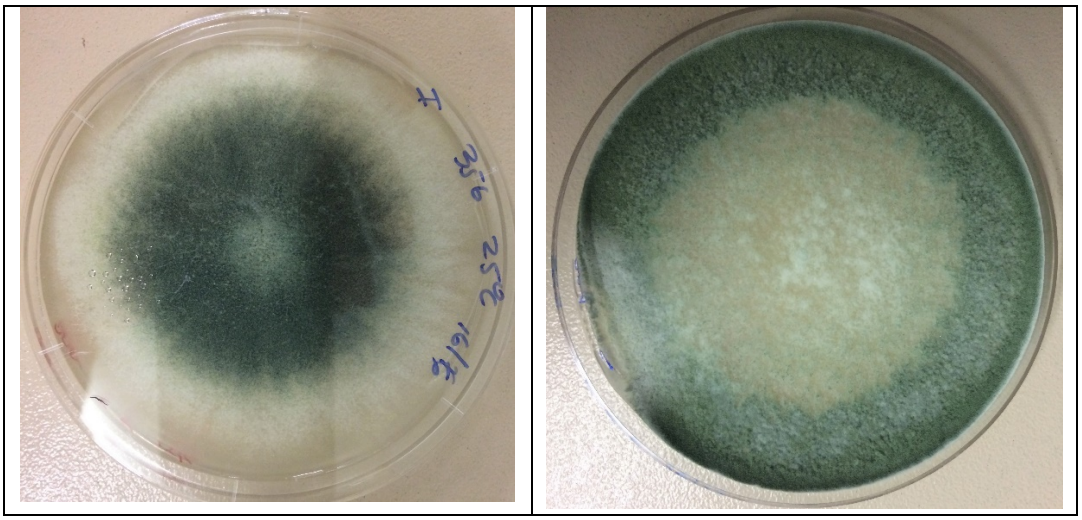

Fig. 5.1 *T. composticola*, isolate 356 (left) and 33861 (right) showing differences in their conidiation pattern at 25°C on PDA.

5.3 Identification of TSM efficacy to control MP

Results from the well experiment (see 3.6.1) were unexpected. The expected result was no growth of the pathogen at the well edges because of the toxic effect of the liquid filtrates containing TSM applied to the wells. The MP hyphal growth at the edges of wells from treated plates was no different from the hyphal growth at the well edges of the untreated control (sterile water). Banana-shaped, bi-celled conidia distinctive of MP were recovered from the bottom of the wells. Therefore, no control was achieved at the incubation temperature (10°C). Similar results were obtained by Konakovsky (2012) when agar plugs impregnated with peptaibols (a group of TSMs) were introduced onto PDA plates where *Fusarium graminearum* and *Penicillium citreonigrum* were in culture. He attributed the lack of efficacy to a metabolic concentration lower than that required to inhibit pathogen growth. Unfortunately, liquid chromatography tests for liquid filtrates were not performed at the same time to confirm TSM concentrations.

However, the wells experiment produced two by-products: the Trichoderma liquid filtrates that were stored at cold temperature, and the MP discs that were introduced as challengers in every culture. The washed agar discs containing MP were processed (3.5.5), inoculated on PDA and incubated at 10 °C expecting to have MP colonies characterised by the orange colour. MP colonies were formed only on the untreated control plates (pathogen cultured in sterile water) confirming that the pathogen survived the liquid culturing conditions. This was important because the fluctuating submerged conditions caused variation in the access to oxygen which is necessary for good fungal growth. The washed agar discs corresponding to isolates 66079 (*T. aureoviride*) and 33681 (*T. composticola*) when cultured on PDA showed neither growth of the pathogen nor of the Trichoderma isolates themselves. This suggests that at least one compound in each liquid culture killed the pathogen. At the same time, these two Trichoderma isolates remained inactive because they were incubated below their optimal temperatures. After 20 days of cold incubation, PDA plates containing these two Trichoderma isolates were transferred to a warm incubator at 25°C where they produced colonies confirming that these organisms were inactive but not dead (fungistasis).

The metabolite test results (Table 4.8) for 66079 (*T. aureoviride*) liquid filtrate showed the presence of cytosporone S, a compound with broad antibacterial and antifungal activity (Ishi et al. 2013). Cytosporone S was found in liquid filtrates produced only by isolate 66079. Liquid filtrates from isolate 33681 contained 6PP. Both cytosporone S and 6PP are potential candidates for MP control but further research is required to determine the effects of different conditions on the production of these two TSMs.

Results from wells and from MP agar disc experiments were contradictory. No effective control of MP was apparent on the well edges experiment while on the agar disc experiment evidence

of MP control was observed. Another experiment was required to confirm or dismiss the efficacy of the TSM to control MP. Having liquid filtrates from the wells experiment, a new trial was designed with PDA using filtrates instead of demineralised water. Two liquid filtrates from native cold tolerant isolates 1535 (*T. viride*) and 356 (*T. composticola*), were selected. Both liquid filtrates contained 35 mg/l and 30.4 mg/l (Fig 4.16) of 6PP in filtrates of 1535 and 356 respectively before autoclaving (3.9). According to The US National Centre for Biotechnology Information (2020), this compound has a boiling point of 287°C making it heat stable at autoclaving temperature. Before autoclaving the final volume of PDA was 60 ml. enough for 3 Petri dishes (replicates) but after autoclaving, the media volume was reduced to 40 ml. This decrease in volume may have resulted in a 33% increase in TSM concentration, but the final concentration was not determined. This was a limitation of this trial. MP was inoculated and incubated on this agar to determine efficacy of the TSMs from the filtrates on the MP colonies. The growth patterns on the amended agar were abnormal suggesting that at least one compound in each filtrate was affecting normal pathogen growth. Furthermore, this reaction proved that at least one effective compound was not heat labile. Additional investigation is needed to find minimum effective concentration of 6PP against MP.

After these trials there was a need to quantify the TSM production and determine if challenging between isolates induced them to produce more or different TSMs. Vegetative compatibility/incompatibility was a useful technique. Barrages formed by these couplets showed incompatibility and possibly/probably the activation of genes for TSM production as stated by Schirmböck et al. (1994) and Vinale et al. (2017). However, the position of the isolates on the carrousel layout (Fig. 3.7) may have influenced the barrage formation; some very fast-growing isolates surrounded and masked slow growers not allowing them to express antagonistic activity.

One of the most intense barrage-forming couplets was MACA-CAROL. This couplet produced cytosporin C, chaetoquadrim E and gossypol, this last compound is characterised by the intense yellow pigmentation with toxic activity against *P. ultimum*, *P. irregulare* and *F. oxysporum* (Mellon et al 2014). From these three metabolites, only chaetoquadrim E was produced by CAROL (*Trichoderma* sp.). This couplet could be used in further investigation to control these three plant pathogens.

MACA (*T. longibrachiatum*) was obtained from a mulching material employed extensively on a macadamia farm at Alstonville in northern NSW subtropical conditions. This contrasts with the origin of the isolate CAROL from McKinnon, Melbourne (cool temperature) which originated from composted kitchen waste. Isolates MACA and CAROL produced the most consistent incompatibility characterised by a thick barrage. One hundred percent of the interactions between these two isolates produced barrages and a wide and clear gap with no physical contact between colonies. Andrade et al. (2015) reported fungicidal compounds like trichodermolide and sorbiquinol from filtrates of *T. longibrachiatum,* but none of these TSMs were not found in MACA liquid filtrates during this research, confirming that TSM production can vary between strains within species (Hatvani et al 2014).

Isolates VIR and 45899 formed strong and consistent barrages. Isolate VIR (possibly *T. harzianum*), was isolated from Virginia, South Australia, from a piece of farm fence wood. It grew rapidly at 25°C but was inactive in cold temperatures and produced a slight yellow pigmentation on PDA. 45899 (*T. polysporum*), had a broader temperature growth range. The single isolate VIR liquid culture contained only unreported TSMs. Isolate 45899 produced trichodermatides A and D and mitorubrin, however, when VIR and 45899 were challenged an

additional TSM, harzianic acid, was produced by the couplet. This may be an additional clue identifying VIR as *T. harzianum* because harzianic acid is reportedly produced by *T. harzianum* (Vinale et al 2009). In this experiment harzianic acid was produced only through the challenging process demonstrating that challenging promotes more TSM production.

Isolate BIANCA (possibly *T. polysporum*) originated on plates from which lettuce anthracnose was first isolated (Pearcedale, Victoria). This isolate is aggressive under warm conditions but in cold temperatures it is almost inactive. Isolate ANAND (possibly *T. spirale*) was extracted from avocado tree samples infected by *Phytophthora cinnamomi* from subtropical Belthorpe, Queensland, 100 km north west of Brisbane. ANAND and BIANCA isolates developed strong and consistent barrages. ANAND, unchallenged, produced candidusin C, while BIANCA, unchallenged produced dermadin, trichodermatide A and D and koningin B. However, their challenged filtrate only produced dermadin. This result shows that less and not more TSMs are produced and that possibly they inhibited one another as reported by Serrano-Carreón et al (2002).

Couplets of isolates BIANCA, MACA, VIR, CAROL, 45899, 99 and 1536 developed strong barrages between each other but moderate reactions with other isolates. Strongly cold tolerant isolate, 1535 (*Trichoderma viride*), was incompatible with warm tolerant isolate 1536 (*Trichoderma* sp.). Both were isolated from the same mulch sample from a forest in Tween Peaks, Western Australia. This shows that Trichoderma isolates from the same environmental niche can exhibit different growth parameters and possibly alternate seasonally. Isolate 1535 produced fusalanipyrone, a fungicide and phytotoxic compound, and 6PP. Isolate 1536 did not produce identified TSMs. Their couplet liquid culture contained, additionally, the peptaibol Trichorzin PAU which, according to Leclerc et al. (1997), is almost exclusively synthesised by

T. harzianum strains, another clue to 1536 identification.

In general, TSM production is culture condition dependent and directly correlated with active mycelial growth. The progressive depletion of nutrients during culturing determined the diversity and concentration of the resulting TSMs, and efficacy as pathogen control agents (Ghisalberti and Sivasithamparan, 1990). Similarly, it is apparent that the same two parameters can be modified when strains are challenged in a liquid culture. Therefore, all these factors should be studied in further investigation to improve the effectiveness of liquid cultures containing TSMs to control not only MP but also other plant pathogens.

Challenged and unchallenged liquid filtrates of isolate 1535 were tested on lettuce seedlings. Unchallenged filtrates from isolate 1535, which stimulated atypical MP hyphal growth *in vitro*, were selected as foliar sprays on lettuce plants. Plants suspected of having MP and incubated under cold conditions (10°C) did not express the disease on new leaves despite their initial MP symptoms. The prior history of the seedlings (chemical treatments) was unknown.

Development of leaf disease depended on spore concentration in the environment, soil, or air. Lettuce seedlings were sprayed twice with 10^1-10^5cfu/ml MP conidial suspensions and maintained in the humidified refrigerated incubator. However, there were no observable signs of the disease. Galea et al. (1986) experimented with the effects of temperature, pH and water stress on conidial germination and germ tube growth *in vitro* and found the optimal temperature for conidial germination was 10-26°C after 24 hours of incubation. Field conditions are different from environmental chamber conditions; leaf temperature can be 4-5°C higher than air temperatures on sunny days (Taiz and Zeiger 2010). The experiment was set up again at 14-15°C. After 7 days, spots on the midribs of leaves inoculated with a conidia concentration of

$1x10^4$cfu/ml were observed. Typical MP lesions were identifiable after 20 days. Perhaps the incubation temperature for the curative test in refrigerated conditions also had to be raised. Further research is needed.

Finally, a second field experiment was set up to evaluate the efficacy of the cold tolerant isolate 356. The treatments included conidia and TSMs from isolate 356 sprayed on lettuce leaves alone and in combination with a copper-based fungicide. Also, different lettuce varieties were tested as a condition of using the commercial field.

The first and second field trial sites have similar climatic and soil conditions and presence of MP. Less lettuce anthracnose was observed in the second field experiment. Distribution of MP propagules was more uneven in the soil in field experiment 1 than in the soil of field experiment 2. This may be the reason why, despite large differences in the number of viable lettuce plants (yield), the effect of the application of cold tolerant Trichoderma and application technique was not significant in reducing the crop loss because of the disease.

Soil in field trial 2 had lower and more evenly distributed pathogen propagules than soil in field trial 1 which may explain why the incidence and severity were lower and significance was clearly shown ($p<0.05$). At the time of the second field experiment (winter 2018), TSMs had not been identified in the *in vitro* liquid cultures, so conidial/TSMs suspensions for spraying were not produced in the similar way as liquid cultures. However, the strong coconut smell in the solid cultures used to produce conidial suspension suggested the presence of 6PP. The TSM identification was available post 2018 winter season. This was one of the limitations of this experiment.

5.4 Integrated practices to control MP

As a result of this research project some practices to control MP in field have been identified:

1. Rotate lettuce crops in winter. If a lettuce crop was infested with MP in winter, the next two winter crops in that infested patch must not be lettuce. Therefore, operationally, the winter lettuce crop rotation in that infested patch may be triennially.
2. Identify and plant, selectively, the best MP tolerant variety lettuce in winter.
3. The chemical control based in Prochloraz is developing resistance. Farmers should not overuse this chemical. It is common that prochloraz is sprayed almost every week for up to 7 times in a winter lettuce crop.
4. Select and apply Trichoderma cold tolerant isolates in compost or in direct conidial spray as a soil application as a feasible and practical strategy. Selection of cold tolerant isolates can be commissioned from specialised microbiology laboratories following similar approaches trialled in this research project. Cold tolerant isolates can be purchased as lettuce mate (LM), plant mate root (PMR) or plant mate foliar (PMF) from Agrimm, New Zealand. Intellectual property rights reside with the owners of all registered isolates.
5. Produce and apply Trichoderma liquid cultures with validated efficacy against MP In field. Contractual arrangements are necessary for anyone to use the above isolates for TSM production. Isolates 1535, CAROL and LUP which were isolated from Australian environments must be identified fully before intellectual property registration. Finally, liquid, or solid products could be available as commercial products.

Additionally, control of lettuce anthracnose is an integrated strategy that involves every activity on the farm. For example, practices that avoid spreading propagules from field to field must consider vehicles (utes, tractors), implements and workers boots. Footbaths and vehicle washing pad should be a part of general farm practices.

5.5 Recommendations for further research

It is important to understand that each isolate of Trichoderma has defined growing parameters which include soil temperature, moisture, pH, aeration, light intensity, nutritional factors, and surrounding microbiota. These parameters affect their efficacy particularly in the MP pathosystem that is not the optimal for Trichoderma. As these factors fluctuate throughout the seasons, selection, production and use of isolates should consider these conditions for maximum efficacy. Further research is required to determine if the application of other isolates (warm tolerant) in warmer seasons on other crops as part of the rotation plan can contribute to reducing the MP inoculum. Minimum dose of effective TSMs such as 6PP and optimal parameters for its maximum production using appropriate Trichoderma isolates also need more research. General farming practices such as liming or N-nitrate applications have a strong negative impact on the establishment of Trichoderma in soil as a BCA. This approach can be implemented to control other soil borne diseases under unfavourable conditions such as salty or alkaline soils and dryland agriculture, etc. In areas where compost is available, the inoculation and incubation of selected isolates of *Trichoderma* spp. to compost before field application may add value and enhance the benefits of the selected Trichoderma isolates.

Technology transfer to farmers and agricultural scientists is critical to maximise cost effective investment and avoid loss of credibility in biological control systems.

APPENDICES

Appendix 1

Differences in colony size by Trichoderma 356 at different temperatures. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	4570.3	2285.13	256.67	0.000
Error	51	454.1	8.90		
Total	53	5024.3			

Appendix 2

Differences in colony size by Trichoderma 1535 at different temperatures. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	540.48	270.241	445.39	0.000
Error	51	30.94	0.607		
Total	53	571.43			

Appendix 3

Differences in colony size by Trichoderma 71556 at different temperatures. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	2232.1	1116.07	478.99	0.000
Error	51	118.8	2.33		
Total	53	2351.0			

Appendix 4

Differences in colony size by Trichoderma CAROL at different temperatures. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	3820.3	1910.17	662.71	0.000
Error	51	147.0	2.88		
Total	53	3967.3			

Appendix 5

Differences in colony size by Trichoderma 59837 at different temperatures. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	896.93	448.463	244.91	0.000
Error	51	93.39	1.831		
Total	53	990.31			

Appendix 6

Differences in colony size by Trichoderma LM at different temperatures. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	485.81	242.907	222.77	0.000
Error	51	55.61	1.090		
Total	53	541.43			

Appendix 7

Differences in colony size by Trichoderma LUP at different temperatures. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	5292.3	2646.17	546.37	0.000
Error	51	247.0	4.84		
Total	53	5539.3			

Appendix 8

Effect of temperature on Trichoderma isolates phialide length. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	269.2	134.576	16.04	0.000
Error	897	7525.5	8.390		
Total	899	7794.7			

Appendix 9

Effect of temperature on Trichoderma isolates phialide width. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	1.062	0.531	3.20	0.041
Error	897	148.77	0.166		
Total	899	149.83			

Appendix 10

Effect of temperature on Trichoderma conidia width. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Temperature	2	24.57	12.28	50.66	0.000
Error	897	217.48	0.2425		
Total	899	242.05			

Appendix 11

Effect of pH on Trichoderma isolates phialide width. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
pH	2	20.98	10.4913	73.03	0.000
Error	897	128.86	0.1437		
Total	899	149.84			

Appendix 12

Effect of pH on Trichoderma isolates conidia length. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Culture media	2	8.172	4.0859	7.97	0.000
Error	897	459.565	0.5123		
Total	899	467.737			

Appendix 13

Effect of pH on Trichoderma isolates conidia width. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Culture media	2	3.007	1.5037	5.64	0.004
Error	897	239.038	0.2665		
Total	899	242.046			

Appendix 14

Effect of culture media on Trichoderma isolates phialide length. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Culture media	2	823.8	823.786	106.12	0.000
Error	897	6970.9	7.763		
Total	899	7794.7			

Appendix 15

Effect of culture media on Trichoderma isolates phialide width. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Culture media	2	0.763	0.7633	4.60	0.032
Error	897	149.076	0.1660		
Total	899	149.839			

Appendix 16

Effect of culture media on Trichoderma isolates conidia length. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Culture media	2	30.84	30.8395	63.39	0.000
Error	897	436.90	0.4865		
Total	899	467.74			

Appendix 17

Colony size in 7 cold tolerant Trichoderma isolates incubated at 10°C. **10** days after inoculation. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Isolate	6	560.667	93.444	49.01	0.000
pH	2	9.270	4.635	2.43	0.098
Error	54	102.952	1.907		
Total	62	672.889			

Appendix 18

Colony size in 7 cold tolerant Trichoderma isolates incubated at 10°C. **14** days after inoculation. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Isolate	6	5245.7	874.286	109.17	0.000
pH	2	321.5	160.762	20.07	0.000
Error	54	432.5	8.009		
Total	62	5999.7			

Appendix 19

Effect of pH on colony radius in 7 cold tolerant Trichoderma isolates incubated at 10°C. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Isolate	6	5245.7	874.286	109.17	0.000
pH	2	321.5	160.762	20.07	0.000
Error	54	432.5	8.009		
Total	62	5999.7			

Appendix 20

List of TSM in liquid filtrates

LIQUID FILTRATE	99	99 vs BIANCA	BIANCA	355	355 vs 356	356	356 OLD	355 vs 1535	1535	355 vs 1536	1536	1535 vs 33681	33681	355 vs 66079	66079
METABOLITE															
Dehydro harzianolide		X													
Harzianolide		X													
T39 Butenolide		X													
Mitorubrin				X									X	X	X
4-deoxovermiculic acid				X											
Phomolide A				X											
Fusalanipyrone				X	X			X	X			X	X		
6-n-pentylα pyrone				X	X	X	X	X	X	X		X	X	X	X
Mycosinol					X		X								
(3S,4R)-6-Acetyl-3,4-dihydroxy-2,2-dimetylchromane					X		X								
Asperfuran					X		X								
Pyrenocine B						X	X								
Pyrenochaetic acid A						X	X								
Citrinin						X									
Cladoacetal A						X							X		
Dermadin			X												
Trichodermatide A			X												
Trichodermatide B															
Trichodermatide D			X												
Koninginin B			X												
Zinnolide							X								
Corymbiferan lactone B												X			
460 A															
Chaetoquadrin E															
Cytosporone S															
Fusarochromanone															
6-Desmethylmonacolin J															
Ganoderic acid K															
Deacetyl HT 2 toxin															
Tricorzin PAU 4															
Mollicellin F															
WF 14861															
(3alpha, 4beta)-4,15-bis(acetyloxy)-12,13-epoxy-3-hydroxy trichotec-9-en-8-one (3AB)															
Cytosporin C															
Mollicellin C															
Corymbiferan lactone D															

LIQUID FILTRATE	355 vs ANT	ANT	355 vs GAU	GAU	355 vs PHY	PHY	356 vs 45899	356 vs ANT	356 vs GAU	356 vs PMR	1535 vs 1536	1535 vs ANAND	1535 vs ANT	1535 vs BIANCA	1535 vs GAU
METABOLITE															
Dehydro harzianolide															
Harzianolide															
T39 Butenolide															
Mitorubrin		X		X								X			X
4-deoxovermiculic acid															
Phomolide A															
Fusalanipyrone	X	X	X	X	X	X		X	X	X	X		X		X
6-n-pentylα pyrone	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Mycosinol															
(3S,4R)															
Asperfuran															
Pyrenocine B															
Pyrenochaetic acid A															
Citrinin															
Cladoacetal A															
Dermadin															
Trichodermatide A															
Trichodermatide B							X							X	
Trichodermatide D							X							X	
Koninginin B							X								
Zinnolide															
Corymbiferan lactone B															
460 A															
Chaetoquadrin E															
Cytosporone S															
Fusarochromanone	X														
6-Desmethylmonacolin J							X								
Ganoderic acid K							X								
Deacetyl HT 2 toxin								X							
Tricorzin PAU 4											X	X	X	X	X
Mollicellin F												X			
WF 14861												X		X	
(3AB)												X			
Cytosporin C															
Mollicellin C															
Corymbiferan lactone D									X						

LIQUID FILTRATE	1535 vs LM	1535 vs PMR	1535 vs TC1	1536 vs ANAND	153 6 vs GAU	1567	1567 vs BIANCA	33681 vs 99	33681 vs 356	33681 vs ANT	33681 vs GAU	33681 vs NS	33681 vs PHY	33681 vs TC1
METABOLITE														
Dehydro harzianolide			X											
Harzianolide			X											
T39 Butenolide			X											
Mitorubrin	X					X	X							
4-deoxovermiculic acid														
Phomolide A														
Fusalanipyrone	X	X		X	X				X	X	X	X	X	
6-n-pentylα pyrone	X	X		X	X			X	X	X	X	X	X	X
Mycosinol														
(3S,4R)-6-Acetyl-3,4-dihydroxy-2,2-dimetylchromane														
Asperfuran														
Pyrenocine B									X			X		
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A														
Trichodermatide B							X							
Trichodermatide D														
Koninginin B							X							
Zinnolide														
Corymbiferan lactone B														
460 A														
Chaetoquadrin E				X	X			X						X
Cytosporone S														
Fusarochromanone														
6-Desmethylmonacolin J														
Ganoderic acid K														
Deacetyl HT 2 toxin														
Tricorzin PAU 4														
Mollicellin F														
WF 14861	X			X										
(3alpha, 4beta)-4,15-bis(acetyloxy)-12,13-epoxy-3-hydroxy trichotec-9-en-8-one								X						
Cytosporin C						X		X						X
Mollicellin C								X						
Corymbiferan lactone D								X	X	X	X	X		X
Harzianic acid														X

LIQUID FILTRATE	45899	45899 vs 99	45899 vs 1535	45899 vs 1567	45899 vs 66079	45899 vs ANAND	45899 vs ANT	45899 vs GAU	45899 vs LM	45899 vs PMR	45899 vs TC1	45899 vs VIR	59837	59837 vs 99
METABOLITE														
Dehydro harzianolide		X									X			X
Harzianolide											X			
T39 Butenolide		X									X			
Mitorubrin	X							X					X	X
4-deoxovermiculic acid							X			X				
Phomolide A							X							
Fusalanipyrone							X	X	X	X			X	
6-n-pentylα pyrone			X				X	X	X	X			X	
Mycosinol														
(3S,4R)-6-Acetyl-3,4-dihydroxy-2,2-dimetylchromane														
Asperfuran											X			
Pyrenocine B														
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A	X							X						
Trichodermatide B							X	X		X				
Trichodermatide D	X				X		X	X		X		X		
Koninginin B														
Zinnolide														
Corymbiferan lactone B														
460 A												X		
Chaetoquadrin E		X	X			X	X	X		X		X		
Cytosporone S					X									
Fusarochromanone														
6-Desmethylmonacolin J														
Ganoderic acid K														
Deacetyl HT 2 toxin														
Tricorzin PAU 4														
Mollicellin F														
WF 14861								X						
(3alpha, 4beta)-4,15-bis(acetyloxy)-12,13-epoxy-3-hydroxytrichotec-9-en-8-one														X
Cytosporin C														
Mollicellin C														
Corymbiferan lactone D														
Harzianic acid												X		
Didiolactone E		X												
PR toxin					X									
Mortivinacin B							X		X		X			
Zealaranone						X	X				X			
Versiol						X	X							

LIQUID FILTRATE	59837 Vs ANAND	59837 vs 66079	66079 vs BIANCA	71556	71556 vs 356	71556 vs 1536	71556 vs ANAND	71556 vs ANT	71556 vs GAU	71556 vs PMR	71558	71558 OLD	71558 vs 99	71558 Vs 356
METABOLITE														
Dehydro harzianolide														
Harzianolide														
T39 Butenolide														
Mitorubrin														
4-deoxovermiculic acid					X									
Phomolide A					X	X								
Fusalanipyrone	X	X		X	X	X	X	X	X	X				X
6-n-pentylα pyrone	X	X		X	X	X	X	X	X	X				X
Mycosinol														
(3S,4R)-6-Acetyl-3,4-dihydroxy-2,2-dimetylchromane														
Asperfuran														
Pyrenocine B														
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A														
Trichodermatide B														
Trichodermatide D														
Koninginin B														
Zinnolide														
Corymbiferan lactone B														
460 A														
Chaetoquadrin E						X	X	X		X				
Cytosporone S														
Fusarochromanone														
6-Desmethylmonacolin J														
Ganoderic acid K														
Deacetyl HT 2 toxin						X							X	
Tricorzin PAU 4														
Mollicellin F														
WF 14861														
Cytosporin C													X	
Mollicellin C														
Corymbiferan actone D														X
Mortivinacin B														
Zealaranone														
Versiol														
3,5-Dimetyl-8-hydroxy-7-methoxy-3,4-dihydroisocoumarin				X										
Mollicellin B											X			
Terrestrol H													X	
Gibberol A 10														
[4Beta(Z)]-12,13-epoxythricothec-9-en-4-ol-2-butenoate														
Gibberelin A 10													X	
Kestodecalactone A														
Chaetoquadrin H														
(+)-Harzialactone A														
Sohirnone B														
Gossipol														
Candidusin														
Dermadin														

LIQUID FILTRATE	71558 vs 33681	71558 vs 59837	71558 vs BIANCA	71558 vs CAROL	71558 vs MACA	71558 vs TC1	ANAND	ANAND vs BIANCA	ANT	ANT vs 1567	ANT vs LM	ANT vs PMR	BIANCA	CAROL
METABOLITE														
Dehydro harzianolide														
Harzianolide														
T39 Butenolide														
Mitorubrin	X								X		X	X		
4-deoxovermiculic acid											X			
Phomolide A										X	X			
Fusalanipyrone	X	X							X	X	X	X		
6-n-pentylα pyrone	X	X							X	X	X	X		
Mycosinol														
(3S,4R)-6-Acetyl-3,4-dihydroxy-2,2-dimetylchromane														
Asperfuran														
Pyrenocine B														
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A													X	
Trichodermatide B			X											
Trichodermatide D			X										X	
Koninginin B													X	
Zinnolide														
Corymbiferan lactone B														
460 A						X								
Chaetoquadrin E														X
Cytosporone S														
Fusarochromanone														
6-Desmethylmonacolin J														
Ganoderic acid K														
Deacetyl HT 2 toxin														
Tricorzin PAU 4														
Mollicellin F														
WF 14861														
Cytosporin C			X	X	X	X								
Mollicellin C														
Corymbiferan lactone D														
Mortivinacin B														
Zealaranone														
Versiol														
3,5-Dimetyl-8-hydroxy-7-methoxy-3,4-dihydroisocoumarin														
Mollicellin B														
Terrestrol H														
Gibberol A 10														
(4Beta(Z))-12,13-epoxythricothec-9-en-4-ol-2-butenoate		X												
Gibberelin A 10	X	X												
Kestodecalactone A				X										
Chaetoquadrin H				X										
(+)-Harzialactone A				X										
Sohirnone B				X										
Gossipol				X										
Candidusin						X								
Dermadin														
Vertinolide							X			X				

LIQUID FILTRATE	ANT vs TC1	CAROL vs 99	CAROL vs 45899	CAROL vs 66079	CAROL vs BIANCA	CAROL vs PMR	GAU vs 1567	GAU Vs ANT	GAU vs BIANCA	GAU vs LM	GAU vs PMR	LM	LM vs 59837	LM vs PMR
METABOLITE														
Dehydro harzianolide														
Harzianolide														
T39 Butenolide														
Mitorubrin							X	X				X		X
4-deoxovermiculic acid									X	X				
Phomolide A									X		X			X
Fusalanipyrone						X	X	X	X	X	X	X	X	X
6-n-pentylα pyrone						X	X	X	X	X	X	X	X	X
Mycosinol														
(3S,4R)-6-Acetyl-3,4-dihydroxy-2,2-dimetylchromane														
Asperfuran														
Pyrenocine B														
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A														
Trichodermatide B			X		X									
Trichodermatide D			X		X									
Koninginin B														
Zinnolide														
Corymbiferan lactone B														
460 A	X			X	X	X								
Chaetoquadrin E		X			X					X			X	X
Cytosporone S				X										
Fusarochromanone														
6-Desmethylmonacolin J														
Ganoderic acid K														
Deacetyl HT 2 toxin			X											
Tricorzin PAU 4														
Mollicellin F														
WF 14861								X	X					
Cytosporin C														
Mollicellin C														
Corymbiferan lactone D														
Mortivinacin B														
Zealaranone		X		X										
Versiol		X		X										
3,5-Dimetyl-8-hydroxy-7-methoxy-3,4-dihydroisocoumarin														
Mollicellin B														
Terrestrol H														
Gibberol A 10														
[4Beta(Z)]-12,13-epoxythricothec-9-en-4-ol-2-butenoate														
Gibberelin A 10		X		X	X									
Kestodecalactone A														
Chaetoquadrin H														
(+)-Harzialactone A														
Sohirnone B														
Gossipol														
Candidusin														
Dermadin	X													
Vertinolide														
Myrothenone B														
Uridine								X					X	
Didiolactone E														X

LIQUID FILTRATE	LUP	LUP vs 71556	LUP vs ANT	LUP vs CAROL	MACA	MACA vs 1536	MACA vs 45899	MACA vs 59837	MACA vs ANAND	MACA vs ANT	MACA vs BIANCA	MACA vs CAROL	MACA vs GAU	MACA Vs NUT
METABOLITE														
Dehydro harzianolide														
Harzianolide														
T39 Butenolide														
Mitorubrin	X				X									
4-deoxovermiculic acid														
Phomolide A														
Fusalanipyrone	X		X	X						X			X	
6-n-pentylα pyrone	X		X	X		X				X			X	
Mycosinol														
Bisvertinol						X				X				
Asperfuran														
Pyrenocine B														
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A														
Trichodermatide B														
Trichodermatide D														
Koninginin B														
Zinnolide														
Corymbiferan lactone B														
460 A														
Chaetoquadrin E			X			X	X	X	X	X		X		
Cytosporone S														
Fusarochromanone													X	
Okaramine M						X				X				
Ganoderic acid K								X						
Deacetyl HT 2 toxin														X
Tricorzin PAU 4														
Mollicellin F														
WF 14861										X			X	
Tropolactone B						X				X				
Cytosporin C							X				X	X		
Lucilactaene							X							
Chaetoquadrin I								X						
Mortivinacin B														
(+)-Ganoderic acid K														
Terricoline														
Phomalactone							X							
Mollicellin B														
Terrestrol H														
Gibberol A 10														
AK toxin II							X							
Gibberelin A 10			X											
Kestodecalactone A				X										
Chaetoquadrin H				X										
(+)-Harzialactone A						X								X
Sohirnone B				X										
Gossipol						X	X				X			
Candidusin										X				
Dermadin														
Vertinolide	X					X				X				
Roquefortine D										X			X	
Aspereline A													X	
Penicillide														

LIQUID FILTRATE	MACA vs PMF	MACA vs PMR	MACA vs TC1	NS	NS vs 1535	NS vs 1536	NS vs 45899	NS vs 71556	NS vs GAU	NS vs LM	NS vs PMF	NS vs PMR	NS vs VIR	NUT
METABOLITE														
Dehydro harzianolide														
Harzianolide														
T39 Butenolide														
Mitorubrin				X										X
4-deoxovermiculic acid														
Phomolide A				X										
Fusalanipyrone	X	X		X	X			X	X	X	X	X		
6-n-pentylα pyrone	X	X		X	X	X	X	X	X	X	X	X	X	X
Mycosinol														
Bisvertinol														
Asperfuran														
Pyrenocine B														
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A														
Trichodermatide B							X							
Trichodermatide D							X							
Koninginin B														
Zinnolide														
Corymbiferan lactone B														
460 A			X											
Chaetoquadrin E	X	X	X	X									X	
Cytosporone S														
Fusarochromanone														
Okaramine M														
Ganoderic acid K														
Deacetyl HT 2 toxin									X					
Tricorzin PAU 4														
Mollicellin F														
WF 14861														
Tropolactone B														
Cytosporin C					X	X	X						X	
Lucilactaene														
Chaetoquadrin I														
Mortivinacin B														
(+)-Ganoderic acid K														
Terricoline														
Phomalactone														
Mollicellin B														
Terrestrol H														
Gibberol A 10														
AK toxin II														
Gibberelin A 10														
Kestodecalactone A														
Chaetoquadrin H														
(+)-Harzialactone A														
Harzianic acid			X										X	
Sohirnone B														
Gossipol														
Candidusin														
Dermadin														
Vertinolide														
Roquefortine D														
Aspereline A														
Penicillide	X													
3-α-hydroxy-α-ergokriptine													X	

LIQUID FILTRATE	NUT vs 99	NUT vs 1535	NUT vs 1536	NUT vs CAROL	NUT vs GAU	NUT vs LM	NUT vs PMF	NUT vs PMR	NUT vs 71556	PHY vs LM	PHY vs 356	PHY vs 1535	PHY vs 1536	PHY vs 1567
METABOLITE														
Dehydro harzianolide	X													
Harzianolide	X													
T39 Butenolide	X													
Mitorubrin														
4-deoxovermiculic acid														
Phomolide A					X									
Fusalanipyrone		X			X	X	X	X	X	X	X	X	X	
6-n-pentylα pyrone		X	X		X	X	X	X	X	X	X	X	X	X
Mycosinol														
Bisvertinol														
Asperfuran														
Pyrenocine B														
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A														
Trichodermatide B														
Trichodermatide D														
Koninginin B														
Zinnolide														
Corymbiferan lactone B									X					
460 A														
Chaetoquadrin E	X	X	X			X						X		
Cytosporone S														
Fusarochromanone														
Okaramine M														
Ganoderic acid K														
Deacetyl HT 2 toxin	X													
Tricorzin PAU 4														
Mollicellin F														
WF 14861	X											X		
Tropolactone B														
Cytosporin C												X		
Lucilactaene														
Chaetoquadrin I														
Mortivinacin B														X
(+)-Ganoderic acid K														
Terricoline														
Phomalactone														
Mollicellin B														
Terrestrol H														
Gibberol A 10														
AK toxin II														
Gibberelin A 10														
Kestodecalactone A														
Chaetoquadrin H														
(+)-Harzialactone A														
Harzianic acid														
Sohirnone B														
Gossipol														
Candidusin														
Dermadin														
Vertinolide				X										
Roquefortine D														
Aspereline A														
Penicillide														
Terricrocin									X					
8-α-hydroxy-α-ergokriptine														

LIQUID FILTRATE	PHY vs 45899	PHY vs 71556	PHY vs ANT	PHY vs GAU	PHY vs LM	PHY vs MACA	PHY vs NS	PHY vs PMR	PHY	PMF vs 1535	PMF vs 71556	PMF vs GAU	PMF vs LM	PMF
METABOLITE														
Dehydro harzianolide														
Harzianolide														
T39 Butenolide														
Mitorubrin														
4-deoxovermiculic acid														
Phomolide A										X	X		X	X
Fusalanipyrone		X	X	X	X	X	X	X	X	X	X	X	X	X
6-n-pentylα pyrone	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Mycosinol														
Bisvertinol														
Asperfuran														
Pyrenocine B														
Pyrenochaetic acid A														
Citrinin														
Cladoacetal A														
Dermadin														
Trichodermatide A														
Trichodermatide B	X													
Trichodermatide D	X													
Koninginin B														
Zinnolide														
Corymbiferan lactone B														
460 A														
Chaetoquadrin E	X											X	X	
Cytosporone S														
Fusarochromanone			X	X			X	X						
Okaramine M														
Ganoderic acid K														
Deacetyl HT 2 toxin														
Tricorzin PAU 4														
Mollicellin F														
WF 14861	X		X	X		X								
Tropolactone B														
Cytosporin C	X							X						
Lucilactaene														
Chaetoquadrin I														
Mortivinacin B														
(+)-Ganoderic acid K														
Terricoline														
Phomalactone														
Mollicellin B														
Terrestrol H														
Gibberol A 10														
AK toxin II														
Gibberelin A 10														
Kestodecalactone A														
Chaetoquadrin H														
(+)-Harzialactone A														
Harzianic acid														
Sohirnone B														
Gossipol														
Candidusin														
Dermadin														
Vertinolide												X		
Roquefortine D					X									
Aspereline A														
Penicillide														
8-α-hydroxy-α-ergokriptine														
Harzianopyridone									X					

LIQUID FILTRATE	TC1	PMR	TC1 vs BIANCA	VIR	VIR vs 99	VIR vs 1535	VIR vs 1536	VIR vs 66079	VIR vs BIANCA	VIR vs TC1	CONTROL
METABOLITE											
Dehydro harzianolide											
Harzianolide											
T39 Butenolide											
Mitorubrin	X	X	X		X			X		X	
4-deoxovermiculic acid											
Phomolide A											
Fusalanipyrone		X									
6-n-pentylα pyrone		X				X					
Mycosinol											
Bisvertinol											
Asperfuran											
Pyrenocine B											
Pyrenochaetic acid A											
Citrinin											
Cladoacetal A											
Dermadin											
Trichodermatide A											
Trichodermatide B											
Trichodermatide D											
Koninginin B											
Zinnolide											
Corymbiferan lactone B											
460 A			X								
Chaetoquadrin E					X		X		X		
Cytosporone S											
Fusarochromanone											
Okaramine M											
Ganoderic acid K											
Deacetyl HT 2 toxin							X				
Tricorzin PAU 4											
Mollicellin F											
WF 14861											
Tropolactone B											
Cytosporin C											
Lucilactaene											
Chaetoquadrin I											
Mortivinacin B											
(+)-Ganoderic acid K											
Terricoline											
Phomalactone											
Mollicellin B				X							
Terrestrol H											
Gibberol A 10											
AK toxin II											
Gibberelin A 10											
Kestodecalactone A											
Chaetoquadrin H											
(+)-Harzialactone A											
Harzianic acid						X					
Sohirnone B											
Gossipol											
Candidusin											
Dermadin											
Vertinolide											
Roquefortine D											
Aspereline A											
Penicillide											
9-α-hydroxy-α-ergokriptine											
1,8-Dihydroxynaphthalene	X										
R-(-)-Mellein	X										

Appendix 21

Effect of Trichoderma application on number of viable plants in pot trial. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Treatment	4	12.20	3.05	3.05	0.005
Error	15	15.00	1.00		
Total	19	27.20			

Appendix 22

Effect of lettuce variety and treatments to reduce the **incidence** of MP in second field trial at harvest. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Lettuce variety	5	35274	7054.8	25.67	0.000
Treatments	3	2467	822.8	2.99	0.042
Error	39	10717	274.8		
Total	47	48459			

Appendix 23

Effect of lettuce variety and treatments on **severity** of MP in the second field trial, at harvest. One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Lettuce variety	5	29693	5938.7	22.75	0.000
Treatments	3	5755	1918.5	7.35	0.001
Error	39	10181	261.1		
Total	47	45630			

Appendix 24

Effect of lettuce variety and treatments on **severity** of MP in field trial, at week 10 (one week before harvest). One-way ANOVA

Source	DF	Adj SS	Adj MS	F-value	P-value
Lettuce variety	5	15466	3093.2	3.20	0.016
Treatments	3	7888	2629.4	2.72	0.057
Error	39	37698	966.6	1.08	
Total	47	61052			

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