Conidium “fitness” in *Trichoderma*

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of

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A major constraint for use of biocontrol agents (BCAs) is inconsistent performance under changeable environmental conditions. This study aimed to develop new knowledge of effects of growth conditions, to increase persistence and efficiency of the key agent *Trichoderma atroviride* LU132, and to understand the factors which may influence conidium "fitness" for biocontrol formulations of conidia, that are robust (long-surviving) and active against target plant pathogens. Effects of culture conditions, (incubation period, temperature, nutrients, water activity and pH) productivity, germinability and bioactivity of *T. atroviride* LU132 conidia were assessed, in assays against the soil-borne plant pathogen, *Rhizoctonia solani*. Conidium fitness was assessed after storage and in glasshouse pot experiments. Biochemical and ultrastructural characteristics of conidia produced in different culture conditions were also examined, in relation to conidium fitness.

The influence of incubation temperatures (20, 25, or 30°C) on the production of conidia was assessed under constant light over a 25 d period. Two measures of quality of the resulting conidia were also determined; - germination and subsequent bioactivity against *Rhizoctonia solani*. Maximum conidium production occurred at 25°C after 20 d but was less at 25 d. Conidia produced at 30°C germinated more rapidly and gave the greatest bioactivity against *R. solani* in comparison with incubation at 20 or 25°C. An incubation period of 25 d gave the greatest bioactivity compared with shorter incubation periods. To examine the effects of extending incubation time on conidium production, germination and bioactivity, the experiment was extended at 25°C for up to 50 d, which resulted in a second peak of conidium production at 45-50 d. These conidia had optimum germination after 20 and 25 d incubation, and optimum bioactivity was achieved with conidia harvested after 15 d. Therefore, temperatures near 25°C and incubation period of 15 d were shown to be optimum for production of *T. atroviride* LU132. Formulations of *T. atroviride* based on optimised production of conidia may not result in optimal bioactivity. This is the first report indicating that the temperature at which conidia of *T. atroviride* are produced affects germination and bioactivity. Conidium production of this biocontrol strain was shown to be a continuous process, and a scheduled dark/light regime increased conidium production. Furthermore, conidium production is likely to be on 20 d base cycle, which is probably

dependent on colony age rather than abiotic factors. This is also the first report of bimodal conidium production in a *Trichoderma* biological control agent.

Identification of the production and storage factors that affect conidium fitness can assist the success of biological control agents. Conidia from the culturing regimes which resulted in greatest and least bioactivity against *R. solani* in dual culture were selected to assess effects of storage conditions on conidium fitness. Conidia were examined after storage at 30˚C and at 0 or 50% relative humidity (RH) over six months. Fitness declined over time, and the decline was greater for 50% RH than 0% RH. The greatest number of conidia and greatest germination resulted from C to N ratios of 5:1 or 160:1, amended with sucrose at 25°C, but greatest bioactivity resulted from conidia produced at 30˚C. However, fewer conidia were produced at 30˚C, and the least germination and bioactivity resulted from conidia produced at 20°C, both amended with dextrose. The conidia adapted to high temperature of 30°C (amended with dextrose) or nourished at C to N ratio of 5:1 (amended with sucrose) showed the greatest conidium fitness.

Further experiments assessed effects of temperature and hydrocarbon type. Interactions of temperatures (20 or 30°C) *vs* sugars (dextrose, 4.2 g/L or sucrose, 4.2 g/L in constant C:N ratios of 5:1) were examined for bioactivity and colonisation potential in pot experiments with ryegrass in the presence of *R. solani*. Conidia produced at 20°C with dextrose (4.2 g/L in constant C:N ratio of 5:1) gave the greatest bioactivity, where rhizosphere and bulk soil assessments were carried out. The bimodal population cycle in *T. atroviride* LU132 recurred in pot experiments (recorded as colony forming unit (CFU)) in a manner similar to that observed in agar plates, but in an approx. 15 d cycle, indicating that simulated natural conditions shortened the *Trichoderma* life cycle.

Biochemical and ultrastructural studies were carried out to determine relationships between quality variations and cellular characteristics for conidia produced in different culturing conditions. The effect of culture conditions on trehalose accumulation was most marked, while differences in arabitol and mannitol were much less. The least trehalose accumulation was detected in conidia produced at 20°C (13 mg/g dry conidia). This could justify the least conidium survival and bioactivity during storage. Fatty acids detected in conidia by gas chromathography were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 *c*9) and linoleic acid (18:2 *c*9, 12). Linoleic acid was the most abundant (overall mean of 26%), and stearic acid was the least abundant (8%). The conidium production treatment at 20°C gave the greatest amount of fatty acids (66 µg/g of dry conidia), giving conidia deep dormancy or other deterioration effects, while the C:N 5:1 treatment which gave high bioactivity after storage gave the least conidium fatty acid content (12 µg/g dry conidia).

Ultrastructural differences of conidia were linked to differences in conidium survival and successful biocontrol establishment. Low electron density of conidium contents and accumulation of lipid droplets were associated with less integrity and viability. Conidia produced at 20°C showed significant disorganisation of cellular structures.

This research has provided new insights which can form the basis of efficient production of Trichoderma-based biocontrol agents. Additional insights into the basis of conidium fitness in *T. atroviride* LU132 have also been provided.

Keywords: *Trichoderma atroviride* LU132, biocontrol agent, optimum production, bioactivity, germination, culture condition, bimodal conidium production, *Trichoderma* life cycle, *Rhizoctonia solani,* C:N ratio, dextrose, sucrose, lipid droplet.

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Abbreviations

The international system of abbreviations was used for chemicals, formulae, and elements. Other abbreviations used in the text are listed below.

× g	Relative centrifugal force
ANOVA	Analysis of variance
a_w	Water activity
AUC	Area Under the Curve
BCA(s)	Biocontrol Agent(s)
bp	Base Pair
BLAST	Basic Local Alignment Search Tool
BLR	Blue Light Regulator
cAMP	Cyclic Adenosine Monophosphate
CFU	Colony Forming Unit
DDT	Dichlorodiphenyl trichloroethane
DNA	Deoxyribonucleic Acid
EPA	Eicosapentaenoic Acid
Exp.	Experiment
FA	Fatty Acid
GB	Glycine-betaine
HPLC	High Performance Liquid Chromatography
ITS	Internal Transcribed Spacer
LF	Liquid Fermentation
Log	Logarithm
LSD	Least Significance Difference
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Centre for Biotechnology Information
P	Probability
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
qPCR	Quantitative Polymerase Chain Reaction
RH	Relative Humidity
RNA	Ribonucleic Acid

rpm	Revolutions per minute
SDW	Sterile Distilled Water
SmF	Submerged Fermentation
sp.	Single species
spp.	Multiple species
SSF	Solid-State Fermentation
TEF-1	Transcription enhancer factor-1
TEM	Transmission Electron Microscopy
Tre	Trehalose
TSM	*Trichoderma* Selective Medium
var.	Variety
vs	*Versus*
vvm	Volume per volume per minute

Preface

This thesis is presented in six chapters. Each chapter is written as a separate paper, including relevant literature citations and appendices.

Chapter 1 describes microbial biological control in crop production, with presentation of appropriate definitions. Key biocontrol agents used for management of plant diseases, and their typical modes of action, are outlined, with particular emphasis on the fungal genus *Trichoderma*. Some illustrative examples of biocontrol products are highlighted, describing difficulties faced for their registration, production, variability and constraints in application and efficacy for management of plant diseases. Information on *Trichoderma* as an important biological control agent is summarised, with aspects of taxonomy, nutrition and ecology discussed. Applicability of this fungus for biological control is then considered, with consideration of variability, constraints on performance and methods of production to increase quantity and quality. This chapter concludes by outlining the aims of the present study.

Chapter 2 describes a series of experiments designed to assess effects of different culture conditions (temperature, nutrient, pH and water activity) on conidium production, germination and bioactivity for *T. atroviride* LU132 against *Rhizoctonia solani* colony growth. This was to provide key knowledge as a basis for optimising commercial production of this fungus as a biological control agent.

Chapter 3 describes an experiment to define the intrinsic stability of *T. atroviride* LU132 conidia produced under different incubation and nutritional conditions, and to identify the influence of storage conditions on conidium viability and bioactivity.

Chapter 4 describes experiments that explored effects of *T. atroviride* LU132 conidium incubation conditions on ryegrass host plant growth parameters, rhizosphere and endophytic colonization in presence or absence of *R. solani*.

Chapter 5 describes experiments that examined biochemical and ultrastructural changes in conidia of *T. atroviride* LU132, to determine key cellular characteristics of conidia that vary in relation to bioactivity as indicated in previous sections of this study.

Chapter 6 presents a summary of the outcomes from the study, a general discussion, some general conclusions and suggestions for further research.

Chapter 1

Introduction and literature review

1.1 Importance of biological control

Current farming practices rely heavily on chemical inputs including fertilizers and pesticides. This has delivered very substantial improvements in crop production (yield and quality) in the last 50 years. However, the actual and potential negative ecological impacts, as well as air and water pollution, brought about by the increased use of agrochemicals has caused widespread concerns. This has given impetus to substantial investment in research that identifies more environmentally-friendly alternatives to these high input agrarian systems (Pal & McSpadden Gardener, 2006). Biological control using microbial antagonists has been identified as a promising and environmentally-friendly option for management of pests, pathogens and weeds. Furthermore, the number of manufactured biological control agents (BCAs) is increasing, although these still only account for approx. 1% of the agricultural pesticide market (Gerbore *et al*., 2013). However, widespread use of BCAs is still limited due to their lack of consistency of efficacy in commercial crop environments (Alabouvette *et al*., 2006; Gerbore *et al*., 2013).

1.2 What is biological control?

Biological control is a concept largely applicable to plant pathology (using microbial antagonists to suppress diseases), entomology (using predatory insects, entomopathogenic nematodes, and microbial pathogens to suppress insect pests), and weed science (to manage weed populations by host-specific pathogens) (Pal & McSpadden Gardener, 2006). Biological control of plant diseases is a relatively recent area of research activity in comparison with biological control of insects (Fravel, 2005).

Baker & Cook (1974) described biological control of plant diseases as “the reduction of inoculum density or disease-producing activities of a pathogen or a parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host or an antagonist, or by mass introduction of one or more antagonists.” A later definition presented by the same authors is “the reduction in the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man” (Cook & Baker, 1983). A more recent definition refers to “the purposeful utilization of introduced or resident living

organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens" (Mukherjee *et al*., 2008; Pal & McSpadden Gardener, 2006).

The present study focuses on biological control of plant diseases, particularly the use of fungi for the suppression and management of plant pathogens to reduce the harmful effects of plant diseases on crop yields and quality of produce.

1.3 Key biological control organisms

Organisms involved in biological control of plant diseases are categorised as competitive saprophytes, facultative plant symbionts and facultative hyperparasites. Key organisms reported as BCAs include the bacterial genera *Bacillus*, *Burkholderia*, *Lysobacter*, *Pantoea*, *Pseudomonas*, and *Streptomyces*, and the fungal genera *Ampelomyces*, *Coniothyrium*, *Dactylella*, *Gliocladium*, *Paecilomyces*, and *Trichoderma* (Pal & McSpadden Gardener, 2006). Among these genera, *Bacillus* and *Trichoderma* are the most commonly reported, and these are the genera that have been developed for the greatest number of commercial biological control products (Stewart, 2001).

1.4 Mechanisms of biological control

Biological control can be achieved through a single mechanism of action, but is more likely to be achieved *via* multiple mechanisms (Alabouvette *et al*., 2006). There are three main types of interspecies antagonism leading to biological control of plant pathogens (Mukherjee *et al*., 2008; Pal & McSpadden Gardener, 2006). These are outlined below.

1.4.1 Direct antagonism

Direct antagonism involves hyperparasitism of the pathogen by the microbial antagonist. In this situation, the BCA kills the pathogen or its propagules (Pal & McSpadden Gardener, 2006). Fungal parasites of plant pathogens are more often grouped into two types based on their mechanisms – necrotrophic parasites which kill their host and then feed off the dead host tissues (*e.g. Trichoderma, Pythium oligandrum* and *Coniothyrium minitas*), and biotrophic mycoparasites which obtain their nutrients from living host tissues before eventually killing them (Jeffries & Young, 1994). This second group are often obligate parasites as they have very intimate relationships with their host fungi. Examples are *Sporidesmium sclerotivorum* – an obligate biotrophic mycoparasite of sclerotia of *Sclerotinia* spp. (Fravel, 1998), *Verticillium biguttatum* on *R. solani* (van den Boogert & Deacon, 1994) and *Ampelomyces mycoparasites* on powdery mildew pathogens (Kiss *et al*., 2004).

1.4.2 Antibiosis

Antibiosis involves the action of antibiotics, containing lytic enzymes which suppress the growth of plant pathogens. Antibiotics, such as 2,4-diacetylphloroglucinol and phenazines produced by *Pseudomonas fluorescens* strains, can control the wheat diseases damping-off, caused by *Pythium* spp., and take-all, caused by *Gaeumanomyces graminis* var. *tritici* (Shanahan *et al*., 1992; Thomashow *et al*., 1990). Lytic enzymes produced by BCAs can hydrolyze a broad range of polymeric compounds such as chitinases, glucanases, and proteases that directly affect cell walls of microbial plant pathogens containing, for example, chitin. *Serratia marcescens* is an example, which suppresses *Sclerotium rolfsii* by producing chitinase (Ordentlich *et al*., 1988). Waste products (organic amendments) can also inhibit plant pathogens (Bailey & Lazarovits, 2003). For example, hydrogen cyanide produced by *Pseudomonas fluorescens* suppressed black rot of tobacco caused by *Thielaviopsis basicola* (Voisard *et al*., 1989). Similarly, volatile compounds such as ammonia produced by *Enterobacter cloacae* have been reported to suppress damping-off of cotton caused by *Pythium ultimum* (Howell *et al*., 1988). Goss *et al*., (2013) described other examples of antibiotic producing potential BCAs.

1.4.3 Indirect antagonism

Indirect antagonism can involve competition (*e.g.* for nutrients, iron, or physical niche occupation) (Pal & McSpadden Gardener, 2006), or induction of host plant resistance mechanisms. Induction of host resistance can occur *via* systemic acquired resistance (Voisard *et al*., 1989), which is mediated by salicylic acid, or induced systemic resistance, which is mediated by ethylene and/or jasmonic acid (Mukherjee *et al*., 2008).

1.5 Examples of biological control products

Table 1.1 presents examples of commercial products marketed as BCAs against plant diseases. Some of these products may not be available as they have been registered for application probably in a specific period of time (see also Woo *et al*., 2014). These products are the most recently registered BCAs, and are also representative of bacterial and fungal BCAs, mostly *Trichoderma*-based agents. There can be challenges associated with the registration of BCAs. For example, up to 2009 only four strains of BCAs (*Ampelomyces quisqualis*, *Coniothyrium minitans*, *Paecilomyces fumosroseus*, and *Pseudomonas chlororaphis*) were listed by the European Union as registered BCA products. This was because of strict regulations imposed by Council Directive 91/414/EEC on BCA products, to characterise their biology, potential effects on human health and non-target organisms (Montesinos, 2003; Alabouvette *et al*., 2006). Due to the limitations on BCA registration, however, there are many

non-registered products or preparations available through governmental agencies to control plant pathogens or pests (Harman *et al*., 2010). Due to growing public concern about healthy foods, studies on BCAs have increased dramatically, so that there are 14 genera of BCAs that have recently been listed as registered or pending registration under European regulation no. 1107/2009 (Woo *et al*., 2014).

1.6 Variability in biological control performance

In contrast to chemical pesticides, many BCAs show inconsistent performance in disease control after application in field environments (Berger *et al*., 1996; Stewart, 2001; Alabouvette *et al*., 2009 Gerbore *et al*., 2013). Since they are living organisms, they are more sensitive to many different biotic abiotic (environmental) conditions than synthetic chemicals (Alabouvette *et al*., 2006). Furthermore, an environment that is favourable for the target pathogen may not be optimum for the BCA to elicit its best biological control activity (Mew *et al*., 2004). Factors that can constrain BCA performance include unfavourable climatic conditions, large pathogen diversity, and lack of diversity in the mode of action on the target pathogens (Alabouvette *et al*., 2006). Unsuitable soil temperature, moisture, pH or organic substances can also limit the BCA's growth and development (Stewart, 2001). Other factors that can restrict the activity of BCAs include insufficient BCA in the product, and incorrect time and place of application (Alabouvette *et al*., 2006; Weller, 1988; Weller *et al*., 1995). A further issue may be inconsistency of biological control activity because of the narrow specificity of effect to a particular pathotype of a pathogen, rather than to all pathotypes (Schisler *et al*., 2000). Researchers have attempted to address these problems by improving the quality and vigour of inocula through the development of optimised culturing conditions to increase their ability to withstand environmental variations (Desai *et al.,* 2002; Verma, 2007). However, there is little available literature adequately describing optimization of BCAs for practical application (Fravel *et al*., 1999).

1.7 *Trichoderma*, a beneficial fungus

Since Bisby (1939) studied the taxonomy of *Trichoderma viride*, using morphological characteristics, there has been a significant body of knowledge obtained about *Trichoderma* spp. as BCAs against plant pathogens (Harman, 2006), and as industrial sources of enzymes (Mukherjee *et al*., 2008). *Trichoderma reesei* has been efficient in production of broad spectrum cellulase enzymes for second generation biofuels (*e.g.* bioethanol) from cellulosic waste (Kumar *et al*. 2008), paper, pulp (Buchert *et al*., 1994) and textile industries (Galante *et al*., 1998), protein secretion (El-Gogary *et al*., 1998) and also degradation of agricultural and industrial byproducts such as cellulose, xylan, plant polymers, and lactose to cellulase and hemicellulose (Schuster & Schmoll 2010).

Table 1.1 Details of the active organisms, trade names, manufacturers, target pathogens and crops for representative commercial biological control agents (BCAs).

BCA/s	Trade name/s	Manufacturer/s, Country	Target pathogen/s	Crops	Reference
Agrobacterium radiobacter	Dygall	AgBio Research, New Zealand	*Agrobacterium tumefaciens*	Fruit and ornamental trees	Stewart (2001)
Bacillus subtilis	Kodiak (HB, AT)	Gustafson Inc, USA	*Rhizoctonia*, *Fusarium*, *Alternaria*	Cotton and legumes	Stewart (2001)
Conithyrium minitans	Contans	Prophyta Ltd, Germany	*Sclerotinia sclerotiorum* and *S. minor*	Vegetables and field crops	De Vrije *et al*. (2001)
Fusarium oxysporum (Non-pathogenic)	Fusaclean L and G [Fo47]	NPP, France	*F. oxysporum*	Carnation and tomato	Desai *et al*. (2002)
Pseudomonas spp.	Proradix	Sourcon Padena, Germany	*Rhizoctonia solani*	Tomato	Berg, (2009)
T. harzianum & *T. viride*	Trichopel, Trichojet, Trichodowels, Trichoseal	Agrimm Technologies, New Zealand	*Amillaria*, *Botrysphaeria* and other fungal diseases	Various	Verma *et al*. (2007a)
T. harzianum & *T. polysporum*	Binab	Bio-Innovation, U.K., Sweden	Tree-bound pathogens	Various	Verma *et al*. (2007a)
Trichoderma sp.	Biofungus	De Ceuster, Belgium	A wide range of pathogens	Flowers, strawberry, tree, vegetables	Stewart (2001)
Trichoderma spp.	Monitor SD	M/s Agriland Biotech Pvt. Ltd, India	Soil-borne plant pathogens	Various	Desai *et al*. (2002)
T. viride	Trieco	Ecosense Labs, India	*Rhizoctonia* spp., *Pythium* spp., *Fusarium* spp., and *Botrytis cinerea*	Various	Desai *et al*. (2002)
T. atroviride	Esquive WP	Agrauxine, ZA de Troyalac'h, Europe Union	*Eutypa, Botryosphaeria spp., Phaeomoniella, Phaeoacremonium*	Vineyards, nursery, Grapes- root, dieback	Klaic *et al*. (2013)
T. asperellum	T34 Biocontrol	Biocontrol Technologies S.L., Fargro Ltd, European Union	*F. oxysporum*	Various	Fernández *et al*. (2014)

Many *Trichoderma* strains are resistant to a wide range of toxic compounds, including herbicides, fungicides and pesticides, as well as plant defence compounds such as phytoalexins, flavonoids, terpenoids, phenolic derivatives and aglycones (Benitez *et al*., 2004). In addition, *Trichoderma* can tolerate toxic pollutants from wastewater or sludges, such as metal complexes (by chelating metal ions), and can degrade organic compounds such as pentachlorophenol, endosulfan, and dichlorodiphenyl trichloroethane (DDT) *via* the production of hydrolases, peroxidases, laccases, and other lytic enzymes (Gadgil *et al*., 1995; Katayama & Matsumura, 1991; Srinivasan *et al*., 1993; Molla *et al*., 2004). For example, *Trichoderma* strains can catabolise cyanide by producing degradative enzymes (i.e. cyanide hydratase and rhodanese) (Ezzi & Lynch, 2002). However, some *Trichoderma* spp. are important crop pathogens, such as *T. pleuroticola*, which causes mushroom green mould resulting in significant crop losses of *Agaricus bisporus* and *Pleurotus ostreatus* (Hatvani *et al*., 2007). Recently, pathogenicity of several *Trichoderma* species on four strains of *Agaricus bisporus* was tested in mushroom farms in Poland. Except *T. atroviride*, the rest of species examined, including *T. aggressivum*, *T. europaeum, T. hamatum, T. harzianum, T. inhamatum, T. koningii*, and *T. longibrachiatum* caused significant crop loss (Górski *et al*., 2014). Moreover, some *Trichoderma* spp. have been reported to have deleterious impacts on human (*e.g.* respiratory disorders) and animal health (Nichols *et al*., 1999; Nunez-Otano *et al*., 2013; Kredics *et al*., 2003a; Thornton & Wills, 2013). For example, *T. longibrachiatum* can grow at human body temperature, which may put users at risk of potentially lethal aplastic anaemia and prolonged neutropenia specifically in immunocompromised patients (Kuhls *et al*., 1997; Munoz *et al*., 1997; Richter *et al*., 1999; Samuels, 2006). Furthermore, some *Trichoderma* spp. affect non-target microorganisms such as mycorrhizae by reducing plant root colonisation, or *rhizobacteria* by disturbance of root nodulation (Brimner & Boland, 2003). However, the inhibitory effects by *Trichoderma* on beneficial soil inhabitants is apparently temporary until *Trichoderma* establishment in rhizospheres. For example, the non-target impacts of *Trichoderma atroviride* on mycorrhizae, rhizobacteria and also on plant growth parameters have been shown to not adversely affect physical changes in plant health, and no disturbance was observed on beneficial effects of mycorrhizae or rhizobacteria (McLean *et al*., 2013).

1.7.1 Characterisation of Trichoderma

1.7.1.1 Taxonomy

In 1794, Persoon described *Trichoderma* as a fungal genus with four species, of which only *T. viride* remains taxonomically valid. The relationship between the anamorph genus *Trichoderma* and its teleomorph genus *Hypocrea* was established in 1865 by the Tulasne brothers (Druzhinina *et al*., 2006; Hanson & Usda, 2000; Samuels, 2006). Since then, more than 200 *Hypocrea* spp. have been described,

of which at least 100 have *Trichoderma* anomorphs (Samuels, 2006). It is very likely that more new *Trichoderma* spp. will be discovered as new geographical areas and new niches are explored (Kubicek *et al*., 2003). For many years, *Trichoderma* spp. (teleomorph *Hypocrea*, *Hypocraceae*, *Hypocreales*, *Ascomycota*) (Kubicek *et al*., 2008) were classified using characteristics of phenotype, cytology, physiology, and ultrastructure (Samuels, 2006). These classical methods have limitations, however, and better resolution at the species level can be obtained using DNA sequence analysis (Druzhinina *et al*., 2006). The molecular phylogenetic approach has revealed 83 species, forms, and varieties of *Trichoderma* (Samuels, 2006). Multiple gene sequencing can be used to study phylogenies at all levels of taxa (Taylor *et al*., 2000), and using DNA barcoding technology allowed study of diversity in *Trichoderma* and *Hypocrea* in more detail (Kubicek *et al*., 2008). More recently, high-throughput procedures have provided new tools using gene knockouts in *Trichoderma* spp. to assess their biological activity (Schuster *et al*., 2012).

1.7.1.2 Nutrition

Trichoderma spp. grow by means of vegetative mycelia, and reproduce through production of asexual conidia. Some species also produce thick-walled intercalary chlamydospores which may assist long term survival (Amsellem *et al*., 1999). Many species have been reported to produce a wide range of antagonistic metabolites during vegetative growth (Verma *et al*., 2007a). *Trichoderma* spp. are saprophytic fungi that can utilise a large variety of biochemical compounds for their nutritional requirements (Danielson & Davey, 1973c; Papavizas, 1985). This genus utilises a wide range of carbon (monosaccharides, disaccharides, and polysaccharides) and nitrogen (nitrates, ammonium, and amino acids) sources. Danielson & Davey, (1973d) reported that *Trichoderma* growth on ammonium nitrogen sources was superior to growth on nitrate nitrogen sources. *Trichoderma* growth and conidiation is mostly affected by C:N ratio rather than carbon concentration. Gao *et al*. (2007) reported that optimal conidiation for *Trichoderma* and several other fungi occurred with sucrose as the carbon source at concentrations of 8 - 12 g/L, and soy peptone as the nitrogen source at C:N ratios between 10:1 and 20:1. Hydrogen ion concentration (pH) can affect conidiation, conidium germination and pigmentation. In general, conidium yield and volumetric productivity is greater at pH 7.0 than 4.0 (Steyaert *et al*., 2010a). In contrast, faster vegetative growth has been recorded under acidic than alkaline conditions (Agosin & Acuilera, 1998). *Trichoderma* strains can change the external pH of the surrounding environment to provide optimal conditions for the production of metabolites. This is achieved *via* a pH-sensing response system, which is modulated by the PacC protein. This system activates the transcription of alkaline-responsive genes and suppresses acidic-responsive genes under alkaline conditions, and reverses this process under alkaline conditions (Arst & Penalva, 2003).

Trichoderma harzianum senses external pH and regulates growth, conidiation, colony morphology, and enzyme activity for mycoparasitism and protein secretion (Moreno-Mateos *et al*., 2007).

1.7.1.3 Biology and ecology

Trichoderma spp. are imperfect fungi which commonly reproduce through asexual fruiting bodies and have no sexual stages, particularly those that have been considered for biological control. If there is a sexual stage, it is within the Ascomycetes and the teleomorph is in the *Hypocrea* genus. These fungi form typical hyphae (5-10 µm in diameter) from green, single-celled conidia (3-5 µm in diameter). The conidia germinate and produce hyphae and chonidiophores which terminate in phialides, which produce conidia (Rifai, 1969).

Trichoderma spp. are ubiquitous free-living saprophytes that commonly decompose organic substances (Druzhinina *et al*., 2006; Mukherjee *et al*., 2008). They are also capable of colonizing plant root surfaces and may be avirulent plant symbionts. Furthermore, they can act as parasites of other fungi (Mukherjee *et al*., 2008). Some species are limited in their geographic distribution, while others, such as *T. viride* and *T. harzianum,* occur very widely (Samuels, 2006). *Trichoderma* spp. are predominant components of soil fungal communities in agricultural land, pastures, meadows, forests and deserts. They are found in a broad range of climatic areas because of their ecological adaptability (Klein & Eveleigh, 1998; Kubicek *et al*., 2008), although there is some species specificity. For example, *T. hamatum* and *T. pseudokoningii* are prevalent in conditions of high soil moisture. *Trichoderma viride* and *T. polysporum* are restricted to cool temperature areas, and *T. harzianum* is well adapted to warm climatic regions (Danielson & Davey, 1973a, b). *Trichoderma* adaptation to cool temperature protects plant seed germination, when cool damp conditions are not favourable for germination and subsequent seedling growth. The window of opportunity available for damping-off pathogens to attack is longer when conditions are cool and moist (Kredics *et al*., 2003b). Antal *et al*. (2000) isolated 360 *Trichoderma* strains and evaluated them for cold-tolerance, demonstrating that the *T. viride* species group had the greatest incidence of cold tolerant isolates, and that the production and activities of extracellular enzymes was affected by low temperatures. A study by Antal *et al*. (2001) revealed that many enzymes produced by cold tolerant strains at 10°C were probably involved in mycoparasitic processes. These included; β-1,4-N-acetyl-glucosaminidase (NAGase), β-glucosidase and trypsin- and chymotrypsin-like proteases, which remained highly active even at 5°C. The low osmotolerance of *Trichoderma* strains is an important limitation to the use of this fungus as a biofungicide (Kredics *et al*., 2003b). Kredics *et al*. (2000) studied the influence of water potential on the growth, secretion and activities of *T. harzianum* enzymes such as β-glucosidase, cellobiohydrolase, β-xylosidase, NAGase and chymotrypsin-like protease at different temperatures. They showed greater

growth rates at the higher temperature and higher water potentials that at lower levels of these factors. *Trichoderma* spp. are able to grow across a wide range of pH conditions, but are predominantly acid-tolerating fungi. As such, they are less commonly used to control pathogens that grow and infect hosts under alkaline rather than acid conditions (Kredics *et al*., 2003b).

1.7.2 *Trichoderma* as a biological control agent

Trichoderma has been reported to be antagonistic towards a range of soil-borne phytopathogenic organisms, including fungi, bacteria, and invertebrates (Verma *et al*., 2007a). *Trichoderma* spp. can suppress pathogens by different modes of action, including competition for space and nutrients (Verma *et al*., 2007a), parasitism, and antibiosis (Harman, 2006; Mukherjee *et al*., 2008). Different modes of biocontrol action are listed below.

Antibiosis: *Trichoderma* spp. produce several enzymes and antibiotics during interactions with pathogen targets to inhibit and disrupt pathogen growth and development. The inhibition is achieved by the production of proteases, chitinases, cellulases and β-1, 3-glucanases (Samuels *et al*., 2002). Some *Trichoderma* species employ a combination of hydrolytic enzymes and antibiotics to elevate antagonistic activity (Monte, 2001). For example, *T. harzianum* showed synergistic effects of hydrolytic enzymes and peptaibols on inhibition of conidium germination in *Botrytis cinerea* (Howell, 2003).

Mycoparasitism: *Trichoderma* species parasitise a wide range of plant pathogens and remotely sense pathogens by gene expression of pathogenesis-related proteins such as proteases, glucanases and chitinases (Harman *et al*., 2004a). Parasitism will generally involve several steps including detecting and growing towards pathogens, making contact, coiling around the pathogen body, forming appresoria, secreting hydrolytic and degrading enzymes, then dissolving the pathogen cell wall (Chet *et al*., 1981). For example, *T. harzianum* and *T. asperellum* parasitised several plant pathogenic fungi by efficient production of secondary metabolites and hydrolytic enzymes (Qualhato *et al*., 2013). Successful mycoparasitic *Trichoderma* strains usually coil around the target pathogens followed by extensive hydrolytic enzyme production. However, dos Reis Almeida *et al*, (2012) tested 15 *T. harzianum* isolates for antagonistic activity against *R. solani* and showed the strains did not differ in coiling frequency and performance, and there was no correlation between hydrolytic enzyme production and coiling frequency.

Competition: *Trichoderma* strains usually compete with pathogens in rhizospheres through rhizosphere competence for availability of space and nutrients (Kumar, 2013), which is a strain-specific characteristic (Hoyos-Carvajal *et al*., 2009). Greater degrees of biological control activity may be achieved by higher colonization proportion, as has been indicated by application of *T. harzianum* at different populations on rice straw in soil (Cumagun *et al*., 2009). *Trichoderma* rhizosphere

competency involves growth and development in close associations with host root systems, although these are not necessarily indices of bioactivity (Howell, 2003). For instance, *Trichoderma koningii* has shown ability to colonise roots of cotton seedlings while providing little or no biological control activity against *R. solani* (Howell, 2007). Similarly, *Trichoderma* species may be able to supress and replace endogenous fungi on root surfaces (Kumar, 2013).

Induced resistance: *Trichoderma* spp. are also known to induce systemic or localised resistance in plants (Harman, 2006). For example, *T. virens* reduced Verticillium wilt of cotton by inducing terpenoid synthesis in host plants (Hanson & Usda, 2000). In another study, bean leaves were protected against *Colletotrichum lindemuthianum* and *Botrytis cinerea* 10 d after root inoculation with a strain of *T. harzianum* (De Meyer *et al*., 1998). *Trichoderma* spp. produce a wide range of enzymes, including cellulases, hemicellulases, proteases, and β-1,3-glucanase (Mukherjee *et al*., 2008; Verma *et al*., 2007a). Identification of the genes encoding these enzymes has been exploited in plant breeding to produce transgenic plants with resistance to certain diseases (Howell, 2003; Harman, 2006). For example, expression of the chitinase Chit42 from *T. harzianum* resulted in a wide spectrum of host resistance to both foliar and soil-borne plant pathogens in tobacco and potato plants (Lorito *et al*., 1998). In another study, enhanced resistance to apple scab has been demonstrated in transgenic apple plants through expression of endo- and exo-chitinase genes from *T. atroviride* (Bolar *et al*., 2001). Induction of resistance in plants through expression of inducer genes in *T. hamatum* has been related to biotic and abiotic stresses in addition to metabolism of RNA, DNA and proteins (Alfano *et al*., 2007). Under favourable growth conditions, defence mechanisms mediated by jasmonic acid and salicylic acid will be suppressed following defence activation caused by threats (environmental stress or pathogen attack), while host growth will be suppressed. Kazan and Manners (2012) proposed a model for the contribution of *Trichoderma* to balance between plant growth promotion and induced resistance. This balance comes with some costs through up-regulation or down-regulation of related genes because the host plant's sources of energy are limited. Therefore, when plants are under environmental stress or attack by pathogens, allocation of limited energy sources will be compensated *via* suppression of plant growth (Heil, 2002). For example, *Trichoderma* production of indole-3-acetic acid stimulates up-regulation of defence-related genes to increase the level of jasmonic acid/ethylene and/or salicylic acid. In turn, suppression of plant growth will occur and the host will compensate the cost with increased lateral root production (Kazan & Manners, 2012).

Endophytic colonisation: Endophyte microorganisms ubiquitously colonize almost all vascular plant species, and have also been reported in ferns, mosses and marine algae (Tan & Zou, 2001). They are bacteria or fungi which show a range of relationships with host plants, from latent phytopathogenesis to mutualistic symbiosis (Sieber, 2007). They live inside host plants intra- and/or inter-cellularly, and

spend all or part of their life cycles entirely within plant tissues without causing symptoms of disease (Vega Fernando *et al*., 2010). Some endophytes produce alkaloids which are toxic to livestock, but the major positive effects of these compounds are to increase resistance to insect herbivores, by inducing naturally adapted defence systems (Clay, 1988). Endophytes can also improve photosynthetic efficiency, nutrient uptake, water usage and tolerance to abiotic stresses (Singh *et al*., 2011). Uptake of nutrients by plants requires complex processes to enable them to be accessed by roots. Some soil elements such as inorganic nutrients are not in the form that can be absorbed by plants or are in very low concentrations. Endophytes can assist in the capture of nitrogen resulting in a reduction in the need for chemical fertilizers (Lyons *et al*., 1990; Lewis *et al*., 1996; Hurek *et al*., 2002). Endophytes such as *Trichoderma* spp. improve nutrient uptake and make these elements available for plants but inaccessible to plant pathogens (Monte, 2001). Endophytes can alleviate water shortage and also capture nitrogen in order to reduce the need for chemical fertilizers. Therefore, plants inoculated with beneficial endophytes also adapted to extreme environmental conditions can increase crop tolerance to unfavourable conditions such as heat and drought (Hurek *et al*., 2002). Furthermore, using endophytes to enhance plant growth is often more efficient and less costly than plant genetic modification to withstand abiotic stresses (Malinowski & Belesky, 2006).

Growth promotion: *Trichoderma* strains colonise root systems to reduce disease severity and also improve the growth of the plants (Harman & Bjorkman, 1998; Bae *et al*., 2009). The ability of these fungi to colonise plant roots is related to their ability to tolerate plant secretions and other toxic compounds, and also their ability to utilise plant root exudates. Some strains can establish long-lasting root colonization and penetration into root epidermis cells (Benitez *et al*., 2004; Harman *et al*., 2004). They are able to produce metabolites that enhance plant growth through rhizosphere competency and endophytic colonisation. However, the production of metabolites is a strain-specific characteristic in *Trichoderma* (Hoyos-Carvajal *et al*., 2009). The promotion of plant growth or induction of resistance is not always in correlation with biological control activity. For example, *T. stromaticum* as a mycoparasite of the cacao witches' broom pathogen *Moniliophthora perniciosa*, has shown endophytic colonisation in shoots and roots, resulting biological control capability against the pathogen (De Souza *et al*., 2008).

Most *Trichoderma* spp. use a combination of mechanisms to control plant pathogens (Cotxarrera *et al*., 2002). For example, tomato wilt caused by *Fusarium oxysporum* was suppressed by *T. asperellum* through a combination of mechanisms including antibiosis, mycoparasitism, and competition for nutrients (Cotxarrera *et al*., 2002). Furthermore, the biological control activity may be elevated in presence of particular pathogens. For example, Cooney & Lauren (1998) showed that the presence of pathogenic fungi induced metabolite production and bioactivity of BCAs, and reported 300 - 700%

increases in antagonistic metabolite production by *Trichoderma* sp. in the presence of plant pathogenic fungi.

1.7.3 Variability and constraints on performance

Like all BCAs, *Trichoderma* spp. can provide variable biological control efficacy in the field. Some *Trichoderma* spp. do not persist in rhizospheres because of soil type and season (Kumar, 2013). This variability can be brought about by intrinsic deficiencies in the ecological fitness and/or biological control activity of the particular *Trichoderma* strain, but also by fluctuating environmental conditions – some not conducive to *Trichoderma* activity. The deficiencies may be related to high pathogen pressure (large amounts of inoculum), or indirect factors such as sub-optimal production, formulation and/or application methods for the BCA (Agbenin, 2011). For example, *T. atroviride* strain C52 could only decrease onion white rot incidence (caused by *Sclerotium cepivorum*) where disease levels were low (McLean *et al*., 2012). Where pellet and solid formulations plus a soil drench of the BCA in combination with fungicides were employed, significant decrease in disease incidence was observed compared with any of these treatments alone or untreated experimental controls. Also, *Trichoderma* supressed the disease at low and high disease pressure as fungicide did, regardless of *Trichoderma* formulations. Furthermore, disease progress over time showed that the BCA was apparently affected by the timing of disease onset (McLean *et al*., 2012). However, it is not realistic to expect a BCA such as *Trichoderma* to be effective in fluctuating environmental conditions even for an effective isolate. The best formulation, application methods and environmental conditions may not be ideal for a particular BCA. Therefore, all of the factors listed above need to be optimum for BCAs to operate effectively.

1.7.3.1 Strain performance

Finding the best microbial strain for commercial development requires intensive screening and selection. In the past, *Trichoderma* strains were often selected from laboratory and glasshouse trials utilising artificial substrates, but this did not select strains best suited for field conditions. More recent screening systems have attempted to reproduce field conditions as closely as possible (Mathre *et al*., 1999). Improving the intrinsic properties of a strain can be achieved in a number of ways. For example, protoplast fusion technology can be used to enhance biological traits, such as increased cellulase production (Sumeet & Mukerji, 2000). Ogawa *et al*. (2000) developed an improved strain using protoplast fusion from heterokaryons of *T. harzianum*, which exhibited antagonism towards multiple pathogens, resistance to the fungicide benomyl, and enhanced growth. Engineering strains using recombinant DNA technologies is another technique that has been used for strain improvement (Mathre *et al*., 1999). Huang *et al*. (1997) used genetic modification to introduce phloroglucinol

production capability into a strain that produced phenazine-l-carboxylate. As a result, the new strain had antagonistic activity to the wheat diseases Rhizoctonia root rot and take-all. However, inconsistent field performance has often resulted from application of single strains into different agro-ecosystems (Raupach & Kloepper, 1998).

It has been suggested that multiple strains from the same or different species may provide better control and broader activity spectrums than single strains (Stewart, 2001). Furthermore, application of multiple strains probably mimics natural conditions, and reduces variability and increases biological control efficacy (Xu & Jeger, 2013). With multiple strains, the biological control activity is likely to be supported by several modes of action, and the strains may be able to adapt to variable environmental and rhizosphere conditions (Larkin & Fravel, 1998). *Trichoderma* spp. can be applied in combinations with other *Trichoderma* strains or species, or with bacterial BCAs (Afzal *et al*., 2013). For example, to exploit potential synergistic effects obtained from a combination of biological control agents, the three products Sentinel® (*T. atroviride* C52, Agrimm, New Zealand), Trianum^{G} (*T. harzianum* T22, Koppert, Netherlands) and Serenade® (*Bacillus subtilis*, Agraquest, USA) were used for control of *Botrytis cinerea* on strawberry leaves. The BCAs showed increased biological control efficacy in combination rather than as single applications (Xu *et al*., 2010). However, there have also been recent reports that synergistic effects of combined BCAs may not always result (Lutz *et al*., 2004; Xu *et al*., 2011), but antagonistic interactions among BCAs occurred. Xu & Jeger (2013) noted that combination treatments using two BCAs with different biological control mechanisms may result in less than expected efficacy for control of foliar pathogens under fluctuating conditions.

1.7.3.2 Product performance

In addition to obtaining consistent field performance there are a number of key factors that must be addressed in the development of a commercial biological control product. These include production yield, costs of production, product shelf life, practical biological control efficacy, ease of handling, and toxicology related to non-target organisms (Alabouvette *et al*., 2006). Expensive raw ingredients for production and/or formulation can limit commercial viability, prompting investigation of cheap alternatives such as growth substrates from agricultural, industrial and/or municipal wastes. For example, Verma *et al*. (2007b) used starch industry wastewater as a low cost substrate supplemented with 1 or 2% soluble starch to maximise production of *T. viride* conidia and achieved concentrations of $\geq 10^{10}$ colony forming units (CFUs)/mL. Type, quantity, and age of inoculum can also have significant impacts on the economic viability of the production system. Some fungal tissues, such as mycelia and chlamydospores, possess effective biological control activity due to their ability to produce various essential metabolites (*e.g.* antibiotics). However, they may not tolerate the final processing steps in

production such as drying (Amsellem *et al*., 1999; Hanson & Howell, 2002; Hutchinson, 1999), and this limits their potential for commercial use. BCAs should possess similar shelf life and require comparable storage requirements to those of conventional pesticides, and be similarly packaged. Where the BCA produces biologically active secondary metabolites, the same toxicological and safety requirements to those of pesticides must be met (Butt & Copping, 2000; Mathre *et al*., 1999). It is also important that BCAs can be applied using standard equipment, because farmers are reluctant to spend extra money on new equipment (Butt & Copping, 2000). Furthermore, to be effective the BCA must be applied at the optimum rate at the right time and in the right place (Warrior *et al*., 2002). For example, Kovach *et al*. (2000) delivered *T. harzianum* on strawberry flowers through spray application and bumblebees. Although the population of the fungus was half as much in the bee-delivered treatments, bee application gave better disease control than that achieved from sprays. Similarly, Shafir *et al. (*2006) used successive applications of *T. harzianum* by honey bees to control grey mould (caused by *Botrytis cinerea*) on strawberry. Sampled flowers up to 200 m from the hives showed effective inoculum levels of *T. harzianum* distributed by bees.

1.7.4 Production methods to enhance *Trichoderma* conidium production, stability, and survivability

Significant research activity is now aimed at developing optimised production and formulation methods to maximise biological control performance of BCA strains. Development of *Trichoderma*-based products requires satisfactory survivability and stability during storage as well as effective application to the soil and/or plant phylloplane. Ability to withstand environmental variations such as desiccation, excess moisture and extreme temperatures are important characteristics necessary for successful BCAs (Agosin & Aguilera, 1998).

Definition of conidium "fitness": For this study, conidium fitness in biocontrol fungi is defined as ability to germinate, retain viability for long periods (in storage), and capability for effective biological control of plant pathogens and/or growth promotion of host plants.

1.7.4.1 Enhancement of stress tolerance

Desiccation can be a limiting factor causing rapid decreases in viability of BCAs. Bonaterra *et al*. (2005) designed a system for osmoadaptation of *Pantoea agglomerans* as a BCA against *Penicillium expansum*, the causal agent of postharvest fruit diseases. Inoculum preparation in sodium chloride plus trehalose and glycine-betaine increased tolerance to desiccation. Similarly, harvesting *T. harzianum* conidia in the stationary conidiation phase rather than the exponential conidiation phase

increased the trehalose content almost 20 times, and probably enhanced tolerance to desiccation (Pedreschi *et al*., 1997).

1.7.4.2 Improvement of shelf life

One of the most important factors determining the success of a biological control agent in commercial markets is shelf life. Agosin & Aguilera (1998) studied the effect of culture conditions (pH, C:N ratio, carbon content) and harvesting time on conidium shelf life, viability, and ultrastructure of *T. harzianum*. They showed that carbon concentrations did not affect maximum conidium yields, and only had limited effects on conidium shelf life, while shelf life was more greatly affected by pH, harvesting time and C:N ratio. Conidium viability also increased under low relative humidity because in a nearly dehydrated state there would be a delay in metabolic or deterioration reactions. Pedreschi & Aguilera (1997) evaluated the viability of dry *T. harzianum* conidia with or without heat shock after fermentation. They showed that storage of dried conidia in sealed containers under refrigeration and at low moisture contents extended their shelf life. Similarly, Thangavelu *et al*. (2004) mass-produced *T. harzianum* using dried banana leaves. This substrate produced high propagule numbers (4.6×10^{32} CFU/g of leaf) with long shelf life at room temperature compared with talc powder. The mass production on banana leaves was cost effective, easy to formulate, and readily adopted by farmers for effective control of Fusarium wilt of banana.

1.7.4.3 Improvement of production

To be successful as a commercial BCA, an effective mass production protocol must be developed for the product (Verma *et al*., 2007a). Liquid fermentation (LF) and solid-state fermentation (SSF) are the two main methods used for production of *Trichoderma* spp. (Khurana *et al*., 1993). More recently, Verma *et al*. (2007b) reported an integrated method of SSF and LF, where 2 - 3-d-old broth culture from LF was used as inoculum for SSF containing bran, rice, and grain-husk.

Submerged fermentation (SmF) in liquids has been used for the production of microbial biomass (Nigam & Singh, 1994), although mostly for production of industrial enzymes from genetically transformed organisms (Holker *et al*., 2004). Said (2009) reported extensive conidiation of *T. harzianum* in SmF with an agitation speed of 500 rpm and aeration rate of 1.5 to 2.0 vvm. Conidium viability was not significantly influenced by agitation or aeration. Similarly, an optimal medium was developed by Al-Taweil *et al*. (2009) for high production of *T. viride* mycelial biomass in SmF, including 45 g/L carbon, 0.35 g/L nitrogen, temperature of 30°C, 175 rpm agitation speed and pH 6. In another study, high agitator speed of 224 rpm increased cell growth of *T. harzianum* in a stirred tank bioreactor, but this resulted in decreased chitinase production (Felse & Panda, 2000).

Three novel fungal cultivation systems have been developed, including liquid-surface immobilization, liquid-liquid interface bioreactor, and extractive liquid-surface immobilization (Oda *et al*., 2009). These methods are considered suitable alternatives to previous production methods because they result in increased BCA yields, and the fungal cells form physically strong mats from addition of some micro-materials. The extractive liquid-surface immobilization method was developed for the production of 6-pentyl-α-pyrone (a fungicidal secondary metabolite) from *T. atroviride* (Oda *et al*., 2009).

1.8 Research background

Research at Lincoln University has involved many studies on biological control agents using *Trichoderma* spp. Achievements in this research are summarised below.

- Control of *Sclerotium cepivorum*, the causal agent of onion white rot, using a pellet formulation of *T. atroviride* strain LU132 (previously, *T. atroviride* strain C52), through commercialization as Tenet® (previously, Trichopel™ Ali 52), and applied in-furrow planting (McLean *et al*., 2005).
- Control of Sclerotinia lettuce drop with the commercialised product Trichodry™ 6S (dry flake formulation) and Trichoflow™ 6S (wettable powder formulation), based upon *T. hamatum* strain 6SR4 (Rabeendran *et al*., 2006).
- Fungicide-tolerance in *T. atroviride* LU132 developed using protoplast technology, as part of an integrated disease management programme for use in high disease pressure environments (Kandula *et al*., 2008b).
- Selection of a cold tolerant form of *T. atroviride* LU132 using protoplast fusion technology (in progress).

Trichoderma atroviride strain LU132 has been commercialised in New Zealand as Tenet® (McLean *et al*., 2005) for control of onion white rot caused by *Sclerotium cepivorum*, and Sentinel® (Stewart & McLean, 2007) for control of grey mould of grapes and tomatoes caused by *Botrytis cinerea* (Figure 1.1). Further, *T. atroviride* strain LU132 has been shown to improve growth and establishment of ryegrass in the presence of *Rhizoctonia solani* (Kandula *et al*., unpublished). The effects of culturing conditions (including light, pH and nutrients in combination with and without mycelial injury) on *T. atroviride* LU132 conidium production have been studied in detail (Steyaert *et al*., 2010a, b). However, there is no information regarding the effects of culture conditions on viability and fitness of the conidia. Since conidium quality is an important factor for production of a biological control agent, the present study aimed to increase the understanding of factors which may influence conidium fitness for biological control formulations of *Trichoderma* conidia that are robust (long-surviving) and active against target plant pathogens. The soil-borne pathogen, *R. solani* was chosen as a model system to

examine effects of conidium production conditions on biological control activity of *T. atroviride* LU132, both in controlled laboratory assays and in soil environment. This pathogen has shown pathogenicity on ryegrass and susceptibility to *T. atroviride* LU132, and has amenability for use in dual plate bioassays, for rapid screening to enable differences in bioactivity to be identified.

Figure 1.1 Product labels for the bio-fungicides Tenet® (left) and Sentinel® (right), both developed from research at Lincoln University and Agrimm Technologies Ltd in New Zealand.

1.9 Research objectives

The major aim of the research presented in this thesis was to increase the shelf life efficiency of *T. atroviride* strain LU132 conidia and soil biological control activity, through increased knowledge of the factors that influence conidium survival and bioactivity of this important biological control strain.

The key objectives of the research were:

I. Determine the optimum culturing conditions for *T. atroviride* LU132 conidium production, germination, and bioactivity against *R. solani*.

II. Define the intrinsic stability of *T. atroviride* LU132 conidia, and identify key factors that affect conidium fitness (stability and viability) during storage, and biological control activity as measured by interaction with *R. solani* in dual culture assays.

III. Determine the below ground colonization potential of *T. atroviride* LU132 from conidia produced under different culturing conditions, and quantify biological activity either without, or in competition with, *R. solani*.

IV. Understand the cellular basis of conidium fitness of *T. atroviride* LU132 as affected by different growth and storage conditions, using assessment of metabolic activity, measurement of fatty acid/lipid profile and using ultrastructural analysis.

1.10 References

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Chapter 2

The effects of culture conditions on conidium production, germination and bioactivity of *Trichoderma atroviride* LU132

2.1 Introduction

Widespread concerns over agrichemical residues in food and the environment along with the development of pathogen strains resistant to pesticides has resulted in greatly increased interest in the development of environmentally-friendly alternatives for management of plant diseases. These include biological control, using antagonistic microbes (Thrall *et al*., 2011). The fungus *Trichoderma* is one of the most commonly reported biological control agents (BCAs), with numerous commercial products available that contain strains of these fungi (Stewart, 2001). *Trichoderma atroviride* strain LU132 is the active organism in the commercialised biological control formulations Tenet®, developed to control *Sclerotium cepivorum*, the causal agent of onion white rot, and Sentinel®, for control of *Botrytis cinerea*, the causal agent of grey mould of grapes (McLean *et al.,* 2005; Stewart & McLean, 2007). Recently, this *T. atroviride* strain has also been shown to improve growth and establishment of ryegrass in the presence of *Rhizoctonia solani* (Kandula *et al*., 2014, submitted).

Many BCAs show inconsistent disease control after applications in field environments (Stewart, 2001; Alabouvette *et al*., 2006; Gerbore *et al*., 2013). Since they are living organisms, BCAs are more sensitive to fluctuating environmental conditions than synthetic pesticides (Alabouvette *et al*., 2006). Researchers have attempted to address this by improving the quality and vigour of BCA inocula through development of optimised culture conditions to increase capabilities of the agents to withstand environmental variations (Verma, 2007). There is little available information, however, regarding optimization of BCAs for practical application.

The influence of different culture conditions, including nutrients, water activity, light, pH, and temperature, have been assessed in several studies for effects on conidium quality and quantity of biological control fungi (*e.g.* Xue *et al*., 2013). A study on *Colletotrichum truncatum* and *Paecilomyces fumosoroseus* demonstrated the effect of nutrients on the quality of conidia for use as biological control agents (Jackson, 1997). Similarly, the effect of nutrients on the quality of *T. harzianum* conidia confirmed that the efficacy of the fungus can be improved by activation of conidia in nutrient rich culture prior to application (Hjeljord *et al.,* 2001). Steyaert *et al*. (2010a; b) demonstrated that conidium production of *T. atroviride, T. hamatum*, and *T. pleuroticola* was affected by nutrients, light, and different pHs in combination with and without mycelial injury.

Trichoderma reproduction and growth are heavily influenced by temperature and culture age (*e.g.* Hallsworth & Magan, 1996). Temperature is probably the principal abiotic factor determining the quality and quantity of BCA colonies (Hallsworth & Magan, 1996). Existing literature reveals a wide range of temperatures at which *Trichoderma* species can grow, from 0°C in *T. polysporum* to 40°C in *T. koningii* (Tronsmo & Dennis, 1978). However, the optimum temperature for most *Trichoderma* spp. is in the 25 - 30°C range (Klein & Eveleigh, 1998). For example, Knudsen & Li (1990) demonstrated that conidium production of *T. harzianum* was different at 10, 15 or 28°C with optimum production at 28°C. They also showed that conidium production of this fungus increased with increasing culture age (i.e. incubation time) up to 14 d. Similarly, Gupta & Sharma (2013) determined that maximum colony growth and conidium production of *Trichoderma* occurred at 28°C.

Trichoderma atroviride LU132 was chosen for the present research as it has been commercialised**.** The potential significance of culture conditions on biological control activity of this strain was examined, with the aim of increasing the quality of conidium production for biological control products. Although the effects of culture conditions on conidium production of this strain have been previously studied (Steyaert *et al*., 2010a; b), there is no information regarding the effect of culture conditions on fitness of the conidia. Since conidium quality is an important factor for production of a BCA, the present study aimed to examine temperature and culture age as the principal factors affecting conidium fitness for *Trichoderma* biological control formulations. Elevated production temperature has been studied previously for entomopathogenic fungi, showing that an increase of 10°C occurred during solid-substrate culture (Hallsworth & Magan, 1996). In the present study, conidia obtained from cultures grown at different temperatures and for different periods were evaluated for germination and bioactivity against the soil-borne plant pathogen *Rhizoctonia solani*, in laboratory-based experiments. This pathogen has shown pathogenicity on ryegrass and was selected as a model system due to its known susceptibility to *T. atroviride* LU132, and its amenability for use in dual plate bioassays, for rapid identification of differences in bioactivity.

A series of experiments is described here, which assessed effects of different culture conditions (temperature, nutrient, pH and water activity after 15 to 25 d incubation) on *T. atroviride* LU132 conidium production, germination and bioactivity. In light of results obtained, a subsequent experiment was carried out to assess effects of extended incubation period (50 d) and different light/dark regimes. This was to provide key knowledge as a basis for optimising commercial production of this fungus as a BCA.

2.2 Temperature and incubation period affect *Trichoderma atroviride* LU132 conidium production, germination and bioactivity

2.2.1 Materials and methods

2.2.1.1 Fungal cultures

Cultures of *T. atroviride* (strain LU132) and *R. solani* (strain R73-13b) were obtained from the Biocontrol Microbial Culture Collection (Bio-Protection Research Centre, Lincoln University, New Zealand). These fungi were derived from New Zealand soil or plant material, and were previously identified on the basis of morphology and ITS/tef1 sequence analysis for *T. atroviride* LU132, or morphological characteristics and pathogenicity tests for *R. solani* R73-13b (Sneh *et al*., 2004). Original cultures of *T. atroviride* LU132 were maintained as conidium suspensions stored in 20% glycerol at −80°C, while those of *R. solani* were maintained as mycelium on sterile parboiled rice grains stored at −20°C. Working cultures of both isolates were maintained on potato dextrose agar (PDA, Difco™; Appendix A) at 25°C in constant light.

2.2.1.2 Conidium production

Trichoderma atroviride LU132 conidia were harvested in sterile distilled water (SDW) from a 10-d-old culture grown on PDA. Conidia were dislodged from colonies with a sterile glass rod, then filtered through a 10 mL pipette tip plugged with sterile dental cotton (Defend®, Mydent International). Conidium numbers in the suspension were determined using a microscope slide haemocytometer and adjusted to 2×10^5 conidia/mL. Twenty µL aliquots from this suspension were evenly distributed over the medium surfaces in 9 cm diam. Petri dishes each containing 40 mL of PDA. Medium was poured precisely using an automatic dispenser (Jencons Scientific Ltd, Perimatic GP II). Petri dishes were incubated unsealed under constant light (Cool white fluorescent) at 20, 25, or 30°C for up to 25 d. After 5, 10, 15, 20 or 25 d, conidia were harvested separately from three replicate plates for each temperature, by adding 10 mL SDW to each plate, dislodging conidia with a glass rod, and then counting them in the resulting suspension with a haemocytometer.

2.2.1.3 Conidium germination

Conidium concentration in suspensions obtained at each incubation time from the plates incubated at each temperature was adjusted to 2×10^6 conidia/mL, and each suspension was incubated for 24 h at 4°C. This was to place the conidia into common physiological status for later synchronous germination, as recommended by Hjeljord & Tronsmo (2003). For each experimental treatment, 30 µL of suspension was added to a 600 µL capacity microtube containing 270 µL of half-strength potato dextrose broth (PDB, Difco™; Appendix A), then placed in a hybridization oven/shaker (Stuart-SI30H)

at 6 rpm and 22°C. Conidium suspensions were then observed using a light microscope (×20 magnification). The conidia were assessed as germinated when the germ tube lengths exceeded the conidium diameters (Hjeljord & Tronsmo, 2003). Germinated conidia (out of 100 conidia) were counted after 12 h incubation, and then every 2 h for a further 10 h. The proportion of conidia germinated at 22 h was recorded.

2.2.1.4 Bioactivity

A dual culture method in agar plates was used to examine the bioactivity of *T. atroviride* LU132 against *R. solani*. Suspensions of conidia of *T. atroviride* LU132, harvested from the conidium production test (Section 2.2.1.2), were adjusted to a concentration of 2 × 10^7/mL. Dual inoculation studies were conducted in Petri plates (9 cm diam.) each containing 35 mL PDA. Each plate was inoculated by applying 3 µL aliquots of conidium suspension and a 5 mm diam. mycelial disc of the *R. solani* colony, taken from the edge of an actively growing fresh fungal culture (5-d-old), and placing these two inoculum sources 4 cm apart on the agar surface. Dual inoculations with *R. solani* and SDW were used as experimental controls. Three different medium concentrations of PDA (full, half, and quarter strength) were tested. The Petri plates were incubated at 22°C, in constant light. Assessment of inhibition activity was made when the *R. solani* colonies in control plates completely covered the agar surfaces. The percentage inhibition of the *R. solani* growth was determined using the formula:

$$I\% = \frac{C - R}{C} \times 100$$

where I% is inhibition percentage, C is the radius of the colony of the control (*R. solani* with SDW) and R is the radius of the *R. solani* colony in confrontation with *T. atroviride* LU132, growing from the test conidia. After 12 d, the percentage of the *Rhizoctonia* colony covered with mature *Trichoderma* conidia was determined visually assessing the area covered, by placing transparent paper printed with a grid (4 mm^2 divisions) over the colony. The overgrowth area of *Trichoderma* on each *Rhizoctonia* colony was compared to dual inoculation of *R. solani* with *R. solani* as experimental controls.

2.2.1.5 Extended incubation time

To examine the effects of extending incubation time on conidium production, germination and bioactivity of *T. atroviride* LU132, experiments were set up as previously described, except that the strain was grown for 50 d in deep Petri plates (9 cm diam, 2 cm depth), each containing 70 mL of PDA. This was to provide enough nutrients for prolonged incubation. The plates were incubated at 25°C, under constant light. Conidium germination and bioactivity assays were conducted as previously described (Sections 2.2.1.3 and 2.2.1.4), at 5 d intervals during the 50 d period. The bioactivity assays were carried out using half strength PDA plates.

2.2.1.6 Light/dark regimes

The effect of light/dark regimes on conidium production of *T. atroviride* LU132 was assessed in the extended incubation experiment. Conidia were prepared and inoculated onto PDA plates as previously described Section 2.2.1.2 in the extended (50 d) incubation experiment. Incubators with light sources were the same as for the previous experiments. To provide dark conditions, containers were wrapped in tinfoil. A preliminary experiment was performed using three different light/dark regimes applied over 15 d. These were: 15 d constant light; 5 d light then 10 d dark; or 15 d constant dark. Temperature was maintained at 25°C for all light regimes. The numbers of conidia harvested from culture plates were assessed before transferring from light to dark at day 5, and at day 15 for each treatment.

Two subsequent experiments were then carried out, both at 25°C, using deep Petri plate cultures for 50 d. Plates were exposed to light (L) for 20 d in the first experiment (Exp 1) or 25 d in the second (Exp 2). They were then transferred to the dark (D) for a series of different periods, and were then transferred to the light until 50 d (Figure 2.1). Plates in constant dark or light were included as experimental controls. The numbers of conidia harvested from the cultures were counted before the first transfer to the dark incubator at 20 d for Exp 1 or 25 d for Exp 2, before each transfer from dark to light (LD1 for Exp 1 and LD2 for Exp 2), and also at day 50 for each treatment (LDL1 for Exp 1 and LDL2 for Exp 2). The two different initial incubation periods (20 d in Exp 1 and 25 d in Exp 2) were chosen to assess the possible effect of aging on conidium production, irrespective of the light/dark regimes applied.

2.2.1.7 Averaging over time

For each variable that was assessed, the trapezoidal area under the curve (AUC) for each batch of test conidia in each block was calculated, then divided by the time, to obtain an average throughout the whole assessment time, rather than having a single value for each assessment time. The AUC for each period was measured by the following equation (Campbell & Madden, 1990):

$$\sum_{i=1}^{n-1} [(y_i + y_{i+1})/2 \times (t_{i+1} - t_i)]$$

where t_1, t_2, ... t_n are the assessment times, and y_1, y_2, ... y_n are the data values of the particular variable at these times. AUC averaging was carried out both for 22 h assessments of conidium germination at each time period, (5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 d), and for data across all the relevant assessment periods (25 d for the temperature experiment and 50 d for the extended incubation time experiment).

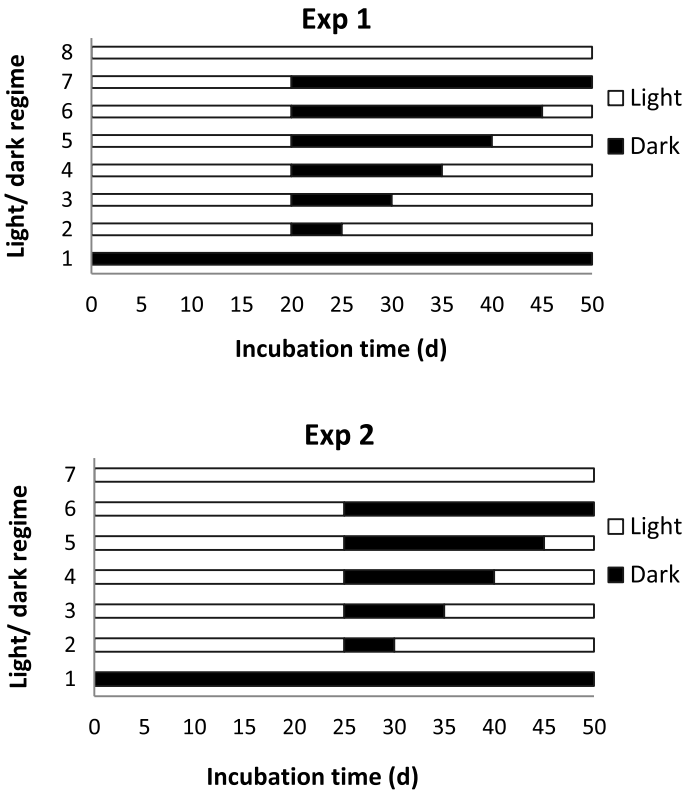

Figure 2.1 Graphic representations of light/dark regimes applied in two experiments (Exp 1 and Exp 2) to measure *Trichoderma* conidium production in culture. The plates incubated in complete dark or light were experimental controls (regimes 1 and 8 in Exp 1 and regimes 1 and 7 in Exp 2).

2.2.1.8 Experimental design and statistical analyses

The conidium production assessments were set up as completely randomised experiments. The germination, bioactivity and light/dark regime assays were set up as randomised complete block experiments, with three replicates for each treatment. Data from the experiments were subjected to analysis of variance (ANOVA), and treatment means were separated using unprotected Fisher's test of LSD at $P = 0.05$. All analyses were performed using GenStat® 16th edition (VSN International Ltd). To investigate how reproducible the results were, all of the experiments were performed twice, with very similar results. Results presented are from the second set of experiments.

2.2.2 Results

2.2.2.1 25 day incubation period

***Conidium production*:** The number of *T. atroviride* LU132 conidia/mL was significantly affected ($P < 0.001$) by different temperatures and incubation times (Table 2.1). There was a strong temperature

effect, with optimum conidium production at 25°C, and progressively fewer conidia were produced at 20°C or 30°C (Figure 2.2). The population of conidia after 5 d incubation was less than other incubation times at each temperature, and the greatest conidium production occurred after 20 d incubation. Furthermore, across all three temperatures, there was a clear trend of increasing numbers of conidia with increasing incubation time up to 20 d, and then a decline in numbers of conidia after 25 d.

Germination: In the germination assessments, after 12 h, conidia from each temperature and incubation time were swollen, and some had small germ tubes, but signs of obvious germination appeared after 14 h at 22°C. After 22 h, conidia from the temperature and incubation time treatments had large proportions of germination. The germination percentage of conidia assessed using AUC showed a significant effect of temperature, with an increasing trend in the germination percentage with increasing temperature. Mean germination of conidia produced at 20°C was 55%, and 72% for those produced at 30°C (Table 2.1). Incubation time affected ($P < 0.001$) conidium germination. The conidia harvested at 20 d across the different temperatures had the least germination (mean = 58%), while conidia harvested at 10 d had the greatest germination (68%).

Bioactivity: *Trichoderma atroviride* LU132 conidia produced at different temperatures and incubation times showed inhibition and overgrowth activity against *R. solani* on dual culture agar plates. Colonies which developed from conidia produced at 30°C gave small but statistically significant increases in inhibition of the *Rhizoctonia* colonies (mean = 78%), compared with colonies from conidia produced at 25°C (76%; Table 2.1). The greatest inhibition of *R. solani* growth was for *T. atroviride* LU132 from conidia harvested at 25 d (mean = 80%), which was greater than mycelial growth inhibition from conidia harvested at all other incubation times (74 - 76%). Macroscopic observations revealed that the growth of *R. solani* mycelia was inhibited soon after contact with *T. atroviride* LU132, in all of the three PDA medium concentrations tested (Figure 2.3). The three strengths of PDA were tested to determine which medium concentration gave the clearest confrontation reaction between *T. atroviride* LU132 and *R. solani*. The different medium strengths did not affect ($P > 0.05$) the percentage inhibition of *R. solani* colonies in the dual culture plates.

Table 2.1 Main effect means of numbers of conidia, germination and bioactivity for *Trichoderma atroviride* LU132, grown for five incubation times up to 25 d, at different temperatures and at three medium strengths (full, half or quarter strength PDA).

		Conidia no. (×10^6/mL)			Germination% (Average AUC)[1]				Bioactivity												
									Inhibition%							Overgrowth%						
		20°C	25°C	30°C	20°C	25°C	30°C	Main effect mean	20°C	25°C	30°C	Full	Half	Quarter	Main effect mean	20°C	25°C	30°C	Full	Half	Quarter	Main effect mean
Incubation time (d)	**5**	811	951	419	52	58	70	60	73	74	78	75	76	74	75	44	40	37	16	61	44	38
	10	1247	1333	627	60	66	79	68	74	75	77	75	76	75	76	40	46	38	25	57	42	40
	15	1493	1640	837	55	62	74	64	74	75	77	76	76	75	76	32	37	30	17	43	39	41
	20	1573	1877	888	51	56	67	58	74	73	76	74	74	75	74	40	48	47	24	69	40	33
	25	1437	1567	710	55	61	71	63	79	80	80	80	80	79	80	41	36	39	10	58	47	45
Main effect mean		1312	1474	696	55	61	72		75	76	78	76	76	76		39	41	38	18	58	43	
$LSD_{0.05}$		37.7	76.9	10.9	0.7			0.9	0.5			0.5			2.1	1.2			1.2			1.6
***Significance*[2]**		***	***	***	***			***	***			ns			ns	***			***			***

[1] Values averaged over time based on the trapezoidal area under the curve (AUC).

[2] *** : the effects of treatments are statistically significant at $P = 0.001$; ns= not significant ($P > 0.05$).

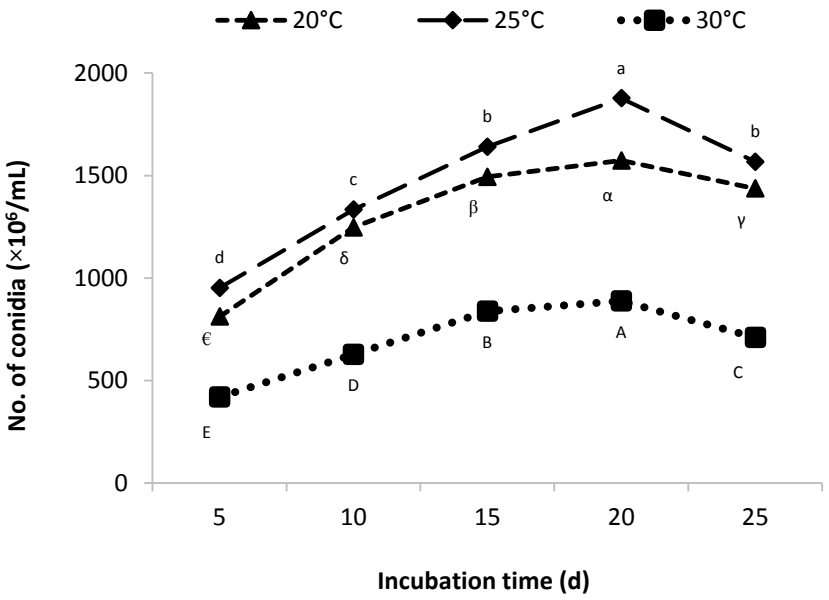

Figure 2.2 Mean numbers of *Trichoderma atroviride* LU132 conidia produced in cultures grown at different temperatures (20, 25, or 30°C) for five incubation times (5, 10, 15, 20, or 25 d). Values for each temperature accompanied by a common letter are not significantly different, according to unprotected Fisher's test of least significant difference ($LSD_{0.05}$ = 38 for 20°C, 77 for 20°C and 11 for 30°C).

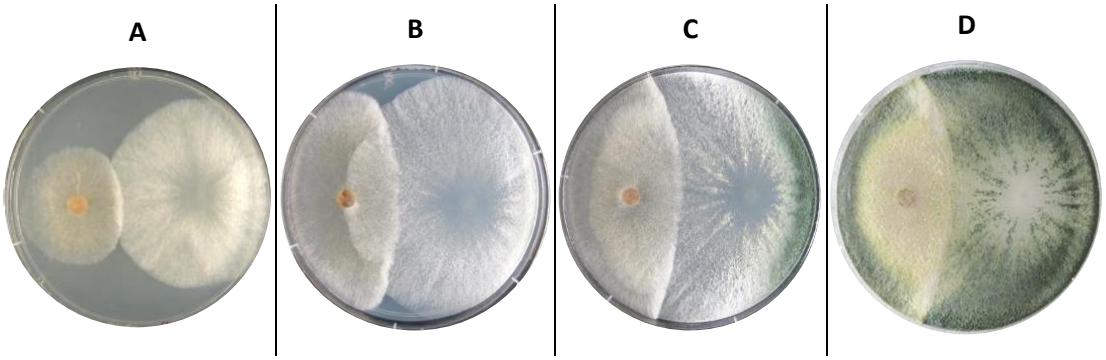

Figure 2.3 Examples of different degrees (A to D) of inhibition of *Rhizoctonia solani* colony growth (left colony in each Petri plate) after contact with *Trichoderma atroviride* LU132 (right colony), in dual inoculated agar plates.

The overgrowth of *R. solani* by the mycelial growth of *T. atroviride* LU132 was significantly affected ($P < 0.001$) by medium strength, incubation time, and temperature (Table 2.1). There was a small but significant temperature effect of *T. atroviride* LU132 mycelial growth over the *R. solani* colonies, whereby mycelium from conidia grown at 25°C overgrew the *R. solani* colony areas by a mean of 41%,

significantly more than the 38% overgrowth achieved from conidia produced at 30°C. There was a significant incubation time effect of mycelial growth over the *R. solani* colonies, with colonies originating from conidia harvested at 25 d overgrowing by a mean of 45%, which was greater than for conidia from all other incubation times (33 - 41%). Percentage overgrowth of the *R. solani* colonies by *T. atroviride* LU132 mycelia was significantly greater on half strength PDA (mean = 58%), compared with quarter (43%) or full strength (18%) PDA.

2.2.2.2 50 day incubation period

Conidium production: In the experiment where a total incubation period of 25 d was used, a decline in the number of conidia produced was detected between the 20 d and 25 d assessments. The series of experiments with extended incubation times was carried out to determine the effect of extended culture age on conidium production, conidium germination and bioactivity of *T. atroviride* LU132. Incubation time affected conidium production ($P < 0.001$) after extended culturing. Numbers of conidia increased from 5 d to 20 d, but decreased slightly from 25 to 40 d (Table 2.2; Figure 2.4), then increased again to 45 d and then remained similar to the end of the experiment. Two peaks of conidium production, one at 20 d and the other at 45 d, were therefore evident.

Table 2.2 Main effect means for conidium production, germination and bioactivity of *Trichoderma atroviride* LU132 grown at 25°C for different incubation times over a 50 d period time, on conidium production, germination and bioactivity of, at 5 d intervals for a period of 50 d.

Treatment		Conidia no. (×10^6/mL)	Germination% (average AUC)[1]	Bioactivity	
				Inhibition%	Overgrowth%
Incubation time (d)	**5**	865	29	65	57
	10	1417	40	68	64
	15	1583	41	74	73
	20	1963	51	65	57
	25	1797	50	67	63
	30	1867	38	68	53
	35	1617	33	61	50
	40	1830	30	66	46
	45	2123	35	67	39
	50	2117	28	64	41
$LSD_{0.05}$		64.6	1.3	2.3	3.2
Significance[2]		***	***	***	***

[1] Values averaged over time based on the trapezoidal area under the curve (AUC).
[2] ***: the effects of treatments are statistically significant at $P = 0.001$.

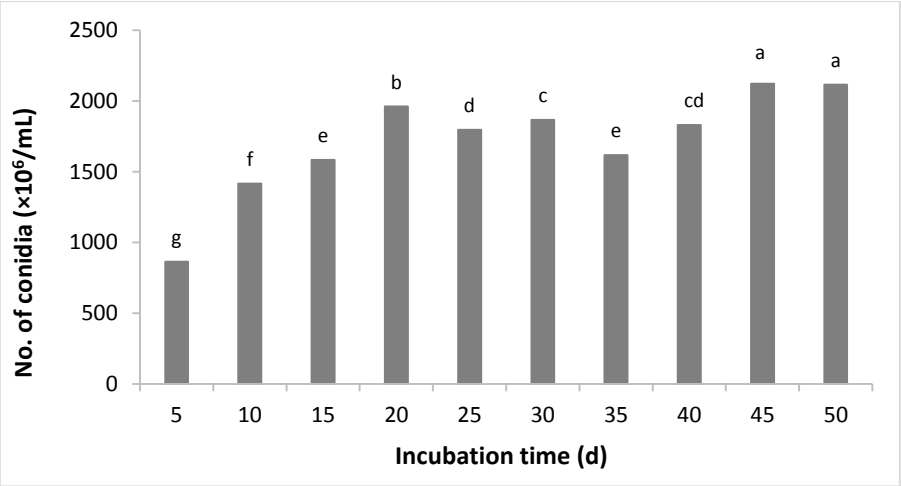

Figure 2.4 Mean numbers of *Trichoderma atroviride* LU132 conidia produced at 25°C after different incubation periods (5 - 50 d). Values with a letter in common are not significantly different according to unprotected Fisher's test of least significant difference ($LSD_{0.05}$ = 65).

Germination: There were significant differences ($P < 0.001$) in percentage germination for conidia produced at 25°C and harvested at ten incubation times (Table 2.2). The greatest mean germination percentage (50 - 51%) was recorded for conidia produced after 20 and 25 d incubation. The mean percentage germination of conidia harvested after incubation for 50 d (28%) and 5 d (29%) was significantly less than for all of the other incubation times (33 - 51%) apart from 40 d (30%). The germination trends across the 50 d incubation period showed increasing germination percentage for conidia harvested at 5 d to 20 d, then a subsequent general decline until 50 d, but with a small increase at 45 d.

Bioactivity: Incubation period had a significant effect on the percentage inhibition of *R. solani* colony growth by *T. atroviride* LU132 ($P < 0.001$). The greatest mean inhibition of 74% occurred with colonies from conidia harvested at 15 d (Table 2.2). There was an increase in mean colony inhibition with increasing incubation period from 5 to 15 d (from 65% to 74%), but after that inhibition fluctuated. The conidia harvested at 35 d provided the least *R. solani* colony inhibition (mean = 61%). The overgrowth of *Rhizoctonia* colonies by *T. atroviride* LU132 mycelia was affected by incubation period ($P < 0.001$). Conidia produced after 15 d gave the greatest overgrowth of 73%, greater than the mean values from mycelia from conidia produced after 45 - 50 d, which gave the least overgrowth of 39 - 41%. Colonies from conidia produced up to 15 d showed increased coverage of *R. solani* by *T. atroviride* LU132 (mean from 57 to 73%), then this generally declined to 41% at 50 d.

2.2.2.3 Different light/dark regimes

In the extended incubation time experiment, there was evidence of a second cycle of conidium production after a decline in conidium numbers between 25 and 35 d. Conidium production was originally monitored for cultures growing under constant light, so experiments were carried out using different light and dark regimes to determine if these affected conidium production in *T. atroviride* LU132.

Conidium production in the preliminary experiment was less in constant dark than where light treatments were applied, but was not completely suppressed by the constant dark treatment. For example, numbers of conidia were low in the dark after 15 d (mean = 1×10^6 conidia/mL) in comparison with the other incubation periods from 5 to 15 d in light/dark (Figure 2.5). Transferring the agar plates from light to dark did not result in large differences in the numbers of conidia produced between colonies incubated for 5 d (8.5×10^8 conidia/mL) in light and the colonies incubated for 5 d in light then 10 d in dark (8.7×10^8 conidia/mL). However, conidium production increased when the colonies were incubated for 15 d in continuous light (1.51×10^9 conidia/mL).

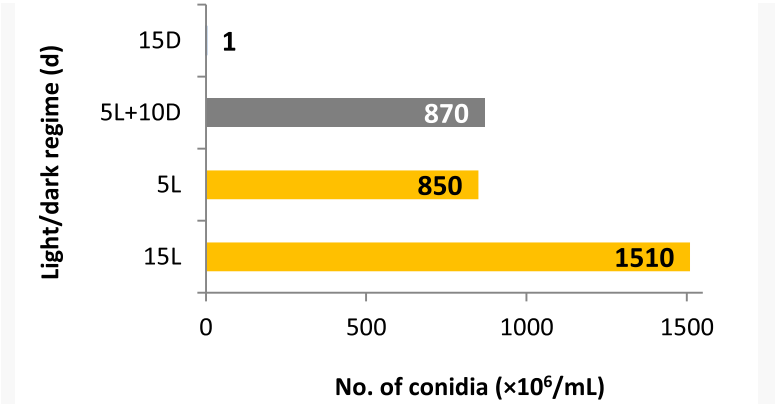

Figure 2.5 Mean number of *Trichoderma atroviride* LU132 conidia produced from different light (L)/dark (D) regimes, in a preliminary experiment.

Averaging of conidium production over time using AUC means is illustrated in Figure 2.6, for an equal period of 25 d incubation in different light/dark regimes including: LD1, LD2, LDL1 and LDL2 (Figure 2.1). There was reduced conidium production in the LD1 regime, where the agar plates were transferred from light to dark for a period of 5 d, then conidium production increased in a constant trend, except for 50 d in the dark. In the LD2 regime, conidium production was also reduced after transfer from light to dark, followed by an increase in conidium production then a slight reduction after 20 d in the dark (Figure 2.6, LD2). Comparison of the average conidium production over time (based on AUC) in the light/dark regime experiments (Exp 1 & Exp 2; Table 2.3), showed that there

was greater conidium production from LD1 than from LD2 (LD1 – LD2 = 4.82 × 10^8 conidia/mL). This was also evident where the colonies were transferred back to light (LDL1 – LDL2 = 4.28 × 10^8 conidia/mL). Figure 2.7 illustrates the amount of conidium production as polynomial curves with different intercepts, where colonies were incubated in constant light or transferred to dark after 20 d or 25 d in light. The least conidium production occurred in the LD2 regime, followed by greater production in constant light (L) and even greater production in the LD1 regime. The mean number of conidia after a period of darkness in LD1 was greater than LD2 and also for extended incubation time in constant light, in the order of regimes LD1 > L > LD2.

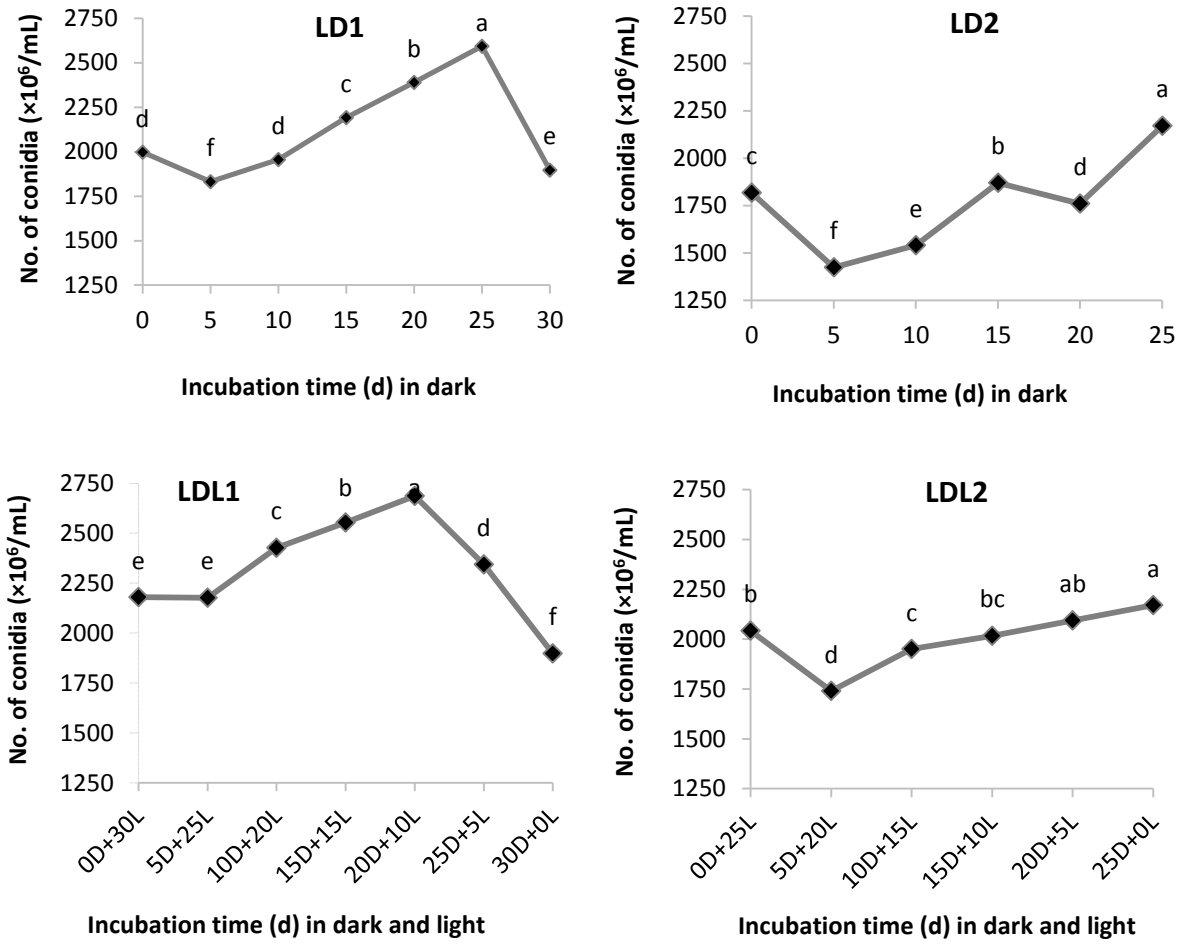

Figure 2.6 Mean numbers of *Trichoderma atroviride* LU132 conidia produced in different light (L) /dark (D) regimes. Graphs LD1 and LD2 show conidium production from colonies initially incubated for, respectively, 20 d or 25 d in light, after transfer to dark for different time periods. Graphs LDL1 and LDL2 show conidium production from colonies initially, respectively, for 20 d or 25 d in light, after transferring to dark and then light. Values with letters in common are not significantly different according to Fisher's unprotected test of least significant difference. $LSD_{0.05}$ for LD1 = 58, for LD2 = 50, for LDL1 = 49 and for LDL2 = 82.

Table 2.3 Average increase in numbers of conidia (mean number conidia/mL) of *Trichoderma atroviride* LU132 produced in different light (L)/dark (D) regimes, assessed using AUC means.

Average increase[1]	Difference
LD1: 2.2×10^9 LD2: 1.72×10^9	LD1 − LD2 = 4.82×10^8
LDL1: 2.41×10^9 LDL2: 1.98×10^9	LDL1 − LDL2 = 4.28×10^8

[1] LD1, colonies initially 20 d in light then transferred to dark.
LD2, colonies initially 25 d in light then transferred to dark.
LDL1, colonies from LD1 transferred back to light.
LDL2, colonies from LD2 transferred back to light.

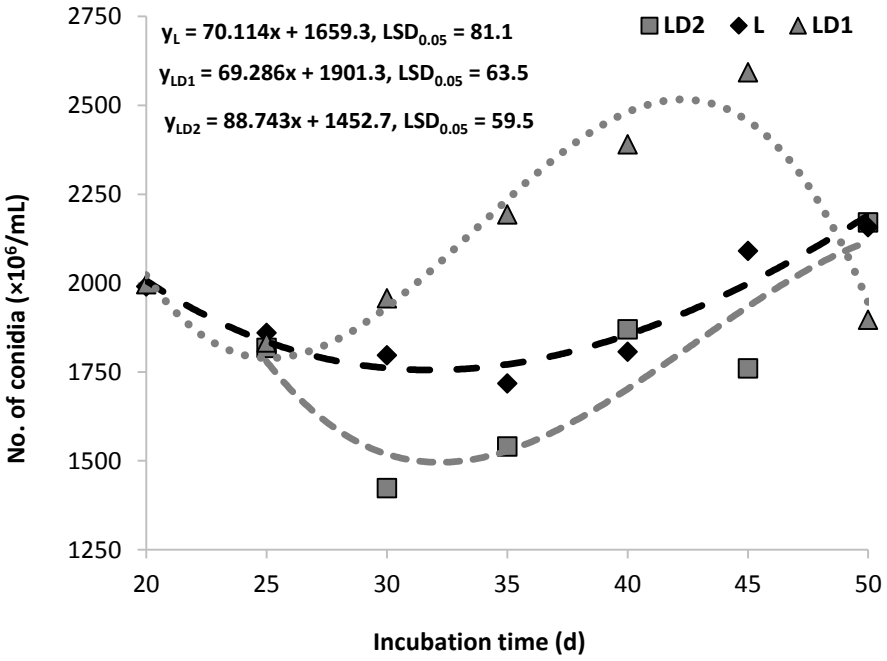

Figure 2.7 Polynomials of numbers of *Trichoderma atroviride* L132 conidia produced in different light regimes. These were: L, colonies continuously in light for 50 d ($LSD_{0.05}$ = 81); LD1, colonies initially incubated for 20 d in light ($LSD_{0.05}$ = 64); and LD2, colonies initially incubated for 25 d in light then transferred to dark ($LSD_{0.05}$ = 60).

2.2.3 Discussion

This study examined the effects of temperature and incubation time on conidium production, germination and bioactivity of *T. atroviride* LU132. Conidia developed over the tested temperature range of 20 - 30°C, and optimum conidium production occurred at 25°C. This optimum temperature was similar to that reported by Jakubikova *et al*. (2006) for large scale conidium production of *T. atroviride* during submerged cultivation. Furthermore, Longa *et al*. (2008) studying ecophysiological requirements of *T. atroviride* strain SC1, demonstrated that the strain grew best at 25°C. However, the temperature requirements for optimum conidium production in many fungi may not be the same as the optimum temperatures for conidium germination, fungal growth or bioactivity (*e.g.* Thomas & Jenkins, 1997). The differences in optima may exist between or within fungal species (Griffin, 1994). For example, Widden (1984) demonstrated that different *Trichoderma* species showed the greatest competition with other fungi, at 5°C for *T. polysporum*, 10°C for *T. viride*, and 20°C for *T. koningii*.

The low (20°C) and high (30°C) temperatures examined in the present study resulted in less conidium production than occurred at the optimum temperature (25°C). This is similar to conidium production for other biological control fungi, including *Zoophthora radicans* (Milner & Lutton, 1983), *T. harzianum* (Knudsen *et al*., 1991), *Metarhizium flavoviride* (Thomas & Jenkins, 1997), and *T. atroviride* (Jakubikova *et al*., 2006). Study of the bioherbicide pathogen *Rhynchosporium alismatis* has similarly shown that while conidium production and germination were decreased at low temperature, high temperature (35°C) inhibited growth and conidium production but not germination (Jahromi *et al*., 1998).

In the present study, fewer conidia were produced at 20°C in comparison with optimum production at 25°C. This could be related to the lower metabolic activity of *Trichoderma* at 20°C, resulting in fewer conidia being produced. The ability of *T. atroviride* LU132 to produce conidia decreased at higher temperature (30°C). Similar results were reported by Abbas *et al*. (1995) for the mycoherbicide *Alternaria helianthi*, whereby at high temperatures, degeneration and clumping of cell contents such as carbohydrates, lipids, and proteins reduced metabolic activity of the fungus followed by a decrease in conidium production.

Also in the present study, conidium production of *T. atroviride* LU132 increased similarly at different temperatures until 20 d of incubation, then reduced at 25 d. The extended incubation time experiment conducted at 25°C revealed a similar trend until 25 d. Then there was a decline in conidium production until 35 d, followed by an increase to a maximum between 45 and 50 d. The trend of conidium production in the extended culture experiment suggested bimodal conidium production. If this were the case, then the first cycle occurred at approx. 5 to 25 d and the second cycle at approx. 25 to 50 d

(Figure 2.8). Bimodal conidium production has been reported previously for pathogenic fungi including *Botryosphaeria dothidea*, *B. obtusa*, *B. rhodina* (Copes & Hendrix, 2004), and *Fusicladium carpophilum* (Scherm *et al*., 2008), in respect of the seasonal dynamics of conidium production.

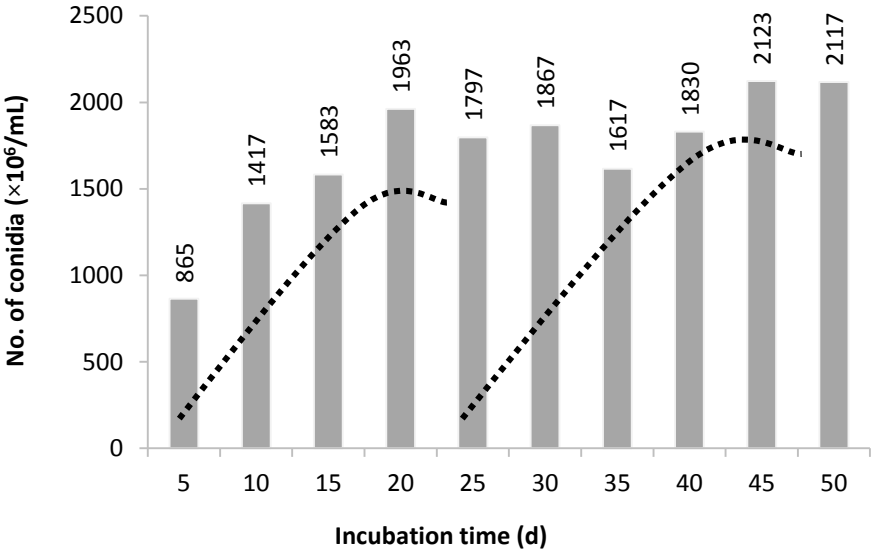

Figure 2.8 Bimodal conidium production in *Trichoderma atroviride* LU132 during 50 d incubation time at 25°C. Dotted lines show two hypothetical cycles of conidium production. Numbers above each bar represent the mean numbers of conidia (x10^6) determined in the 50 d experiment reported here.

Two peaks in conidium production were observed after 20 and 45 - 50 d culture periods. Previous studies have demonstrated that factors such as volatile metabolites stimulate growth, conidium production and germination of fungi (Bruce *et al*., 1996). For example, volatile metabolites in *T. atroviride* stimulated conidium production by 50 to 1,500% (Nemcovic *et al*., 2008). Stoppacher *et al*. (2010), examining dynamics of volatile metabolite profiles of *T. atroviride*, demonstrated fluctuations in production of these compounds, to maximums after 3 - 4 d. Optimum production of two volatile metabolites, 1-octen-3-ol and 3-octanone, occurred simultaneously with conidium production. In the present study, the first peak in conidium production at 20 d was probably related to mycelial maturation and differentiation into phialides to produce conidia. The effect of volatile metabolites was not studied, but could also have contributed to maturation and conidiation at this stage of conidium production.

As nutrients in the growth medium were used over time, the nutrient deprived conditions that resulted could have stimulated conidium production (Betina, 1995). For example, Steyaert *et al*. (2010b) demonstrated that nutrient-rich media containing carbon and nitrogen repressed carbon/nitrogen catabolite genes in *Trichoderma* spp., while under nitrogen or carbon starvation

conidiation was induced by carbon/nitrogen catabolite derepression genes of the fungus. In the present study, the conidium production peak at 20 d in the first incubation experiment (25 d) and also the second conidium production peak at 45 to 50 d under extended incubation (50 d) were probably related to starvation stimuli. However, Chovanec *et al*. (2001) postulated that aging-induced conidiation is initiated by gene regulation in microorganisms rather than by nutrient status.

Trichoderma atroviride LU132 conidia produced at different temperatures and incubation times were able to germinate, and the most rapid germination occurred with conidia produced at the high incubation temperature, 30^{o}C, followed by 25 and 20^{o}C. Fungal cells are dehydrated under high temperatures, and dehydration coincides with accumulation of intracellular polyols such as trehalose and glycerol. Other stress factors are also likely to occur following high temperature treatments, including oxidation, and pH alteration. The transfer of these conidia to fresh media allows them to show maximum germination and growth (*e.g.* Sterflinger, 1998). The polyol content of conidia has been correlated with changes in germination percentage (*e.g.* Hallsworth & Magan, 1996). Elevated germination percentage in *T. atroviride* LU132 in the present germination assays could be due to optimization of polyol concentration in conidia during conidium production at 30^{o}C. Pedreschi *et al*. (1997) noted that the concentration of trehalose (a typical polyol) in conidia did not normally increase under steady-state culture conditions, with heat shock and destabilizing enzymes being required for accumulation of trehalose in conidia. Therefore, high temperatures may not guarantee increased trehalose content in conidia (Hallsworth & Magan, 1996). However, the greater germination at 30°C detected in the present study did not agree with the results of Abbas *et al*. (1995), who demonstrated that high temperature caused reduced metabolic activity, followed by reduced conidium germination and bioactivity, for the bioherbicide fungus *Alternaria helianthi*. The decline in conidium germination and bioactivity coincided with decreased content of polysaccharides and proteins but increased lipid content.

Based on the observed bimodal profile of conidium production, one possible explanation for the trend for a decline in germination after 20 d incubation is that these conidia were probably a mix of older conidia from the first cycle of production and young conidia from the second cycle, which affected the overall conidium germination. Medium composition has been shown to greatly influence the physiology and vigour of fungi (Darby & Mandels, 1955). After 20 d, vegetative hyphae growing under nutrient exhausted conditions could have produced less vigorous or immature conidia, a response suggested by Hallsworth & Magan (1996). Based on their findings that the polyol content of conidia decreased with increasing culture age for the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus*, they suggested that late-produced conidia may contain less polyols than those produced earlier. Furthermore, they concluded that the decline in

viability of harvested conidia as cultures aged was associated with this decline in polyol content. In the present study, the average germination of conidia harvested at 5 and 50 d incubation were the least in the extended culture age experiment. These results are similar to those of Darby & Mandels (1955) with the bioherbicide agent, *Myrothecium verrucaria*, where very young conidia were not mature enough to germinate rapidly, while old conidia failed to germinate because of additional nutrient requirements for germination or a second dormancy. In addition, the respiratory activity of *M. verrucaria* increased with age to a maximum level, followed by a decline, and these two stages were identified as maturation and senescence, respectively.

Inhibition and overgrowth of *R. solani* cultures by *T. atroviride* LU132 were the only visible evidence of bioactivity observed by naked eyes in the present study. Die-back or lysis of *R. solani* hyphae caused by *T. atroviride* LU132 was not observed. The effect of temperature on bioactivity was small and statistically significant, but was probably biologically insubstantial. Optimum inhibition of *R. solani* occurred for conidia produced at 30°C, and the optimum overgrowth was from conidia produced at 25°C. These different temperature optima for inhibition and overgrowth activity are similar to those observed by Jahromi *et al*. (1998). They showed that the temperature at which conidia were formed did not influence the bioactivity of the colonies from these conidia, while the medium composition was the most important factor affecting conidium bioactivity. The greatest bioactivity (overgrowth) consistently occurred on half strength PDA, indicating that *R. solani* did not compete with *T. atroviride* LU132 in these nutrient conditions. Intermidiate bioactivity occurred on quarter strength PDA, suggesting that in these conditions both organisms were possibly nutrient stressed.

Maximum conidium production occurred at 25°C, but differences in bioactivity from *Trichoderma* colonies from conidia produced at different temperatures and incubation times were not biologically significant. Similarly, Jones *et al*. (2003) reported that for the sclerotial mycorparasite *Coniothyrium minitans*, fewer conidia were produced during solid state fermentation at the high temperatures (27 - 30°C) recorded at the top of a fermenter, compared with the constant 18°C found at the bottom of the fermenter, and although germination of the conidia produced under high temperatures was also less, sclerotial parasitism was similar from conidia produced at both temperature regimes.

In the light/dark regime experiments, periods of darkness increased conidium production by *Trichoderma* colonies, but reduced conidium production occurred in both the LD1 and LD2 regimes after 5 d in darkness. This is similar to the results of Flaherty & Dunkle (2005), who showed that continuous light hindered development of conidiophores in *Exserohilum turcicum,* the pathogen causing Northern leaf blight in maize, while mature conidia were formed by cultures grown under light/dark cycles or continuous dark. In addition, analysis of ergosterol content and genomic DNA showed that vegetative growth was much greater in cycles of light/dark than in constant light or

constant darkness. The expression of 12 putative regulatory genes during dark-induced conidiation showed that for the majority of these genes, maximum expression occurred 2 h after initiation of the dark period, which coincided with the period of darkness required to initiate conidiation. For most of the genes this was followed by a decline to initial levels after 4 - 24 h in darkness. However for two genes, the expression remained increased when in darkness for 24 h, but decreased back to initial levels when the cultures were placed back under light. These genes were indicated to be involved in asexual development. In the present study conidium production occurred in constant light, but was increased by changing the light/dark regime. It is therefore possible that further increases in conidium production could be achieved by further manipulation of the dark/light conditions.

The need for a dark period for conidium production in *T. atroviride* LU132 was probably affected by the colony age, as conidium production in the LD1 regime was greater than for LD2. Transfer of colonies to the dark or to dark/light conditions at 25 d did not result in conidium production to the same level, either for the LD2 or LDL2 regimes, compared with colonies transferred at 20 d to dark or dark/light for either the LD1 or LDL1 treatments. However, *Trichoderma* species are known to require light for the biosynthesis of secondary metabolites, such as peptaibols, which are highly important as antibiotics in antagonistic activity (*e.g.* Tisch & Schmoll, 2010). Although light is important in fungal life cycles, Friedl *et al*. (2008) emphasised that *T. atroviride* conidiation is strongly carbon-source dependent, and light only enhances the extent of conidium production. From the results of the present study, the influence of light on production of *T. atroviride* LU132 conidia is apparently more dependent on colony age rather than on other factors, as previously mentioned.

Conidium production in *T. atroviride* LU132 is likely to be on a 20 d base cycle, with a second cycle occurring that is probably independent of light and other abiotic factors. It is assumed that the colonies would be aging, with starvation and metabolite accumulation occurring after 20 d of culture. This promoted the fungus to initiate conidium germination in the cultures, at least by those conidia that were physiologically prepared for germination. The second cycle of conidium production was completed after 45 - 50 d incubation (Figure 2.9).

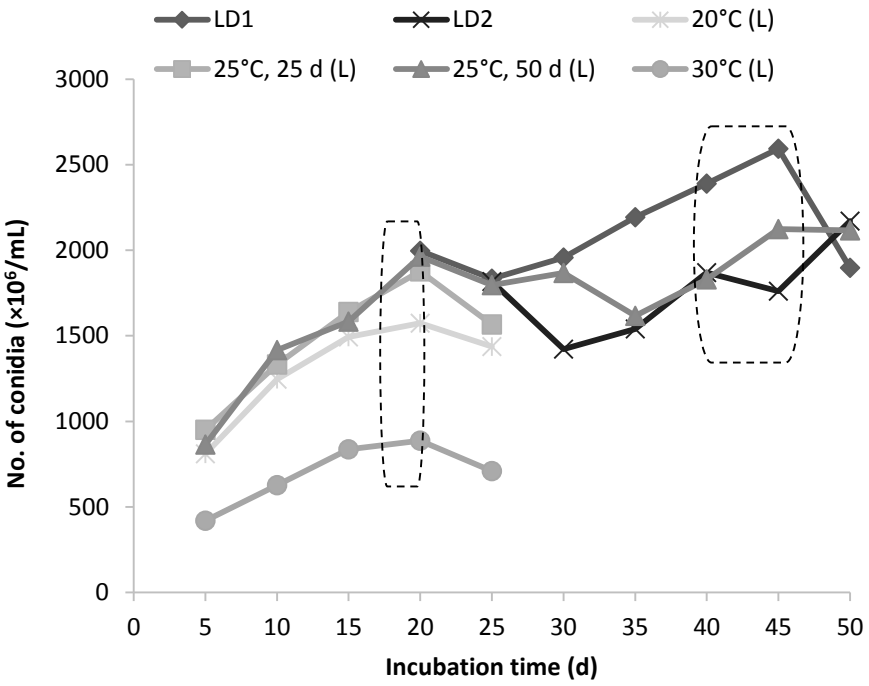

Figure 2.9 Bimodal conidium production in *Trichoderma atroviride* LU132 during 25 d in constant light (L) at different temperatures (20, 25 or 30°C) or 50 d incubation at 25°C but in different light regimes (L, LD1 or LD2). Dotted lines indicate the two peaks of conidium production in hypothetical cycles.

This is the first report suggesting that the temperature at which conidia are produced affects germination and bioactivity in *Trichoderma*. This study has demonstrated that temperatures near 25°C and incubation periods of approx. 20 d are likely to be optimum for conidium production of *T. atroviride* LU132. The study has also shown that the optimum temperature for production of this strain is not the same as the optimum for bioactivity. Optimum bioactivity resulted from conidia produced at 30°C and harvested at 15 d. Based on the bimodal conidium production cycles detected in this study, 20-d-old conidia will be mixed with fresh conidia from the second cycle of production in cultures of the strain. Ten-d-old conidia are likely to be too young to show maximum antagonism bioactivity responses. Older conidia (15 d) are less likely to include a mixture of young and old conidia, resulting in greater overall bioactivity. Conidia of *T. atroviride* LU132 obtained after 15 d incubation are likely to be of optimum bioactivity, germinability and the most suitable for use in commercial production of biological control products.

2.3 Nutrient amendments affect *Trichoderma atroviride* LU132 conidium production, germination and bioactivity

2.3.1 Introduction

The nutritional composition of growth medium can influence fungal attributes such as conidium production, germination, bioactivity, and shelf life. Carbon and nitrogen sources, and mineral supplements, have to be carefully selected to optimise cost-effective growth and conidium production of BCAs, while maintaining viability and efficacy (Jackson *et al*., 1996). An optimum balanced C:N ratio is necessary for optimum production and quality of conidia (Yu *et al*., 1998). Disaccharides have been recognised as the best carbon sources protecting microorganisms, and sucrose among disaccharides has shown quantitative and qualitative effects on the conidium production in correlation with high survival rates (Redway & Lapage, 1974; van Laere, 1989). For example, amendment of *Escherichia coli* with the non-reducing disaccharides sucrose and trehalose showed the greatest viability compared with other compatible solutes (Louis *et al*., 1994).

Microorganisms first digest preferred nutrient sources (primary), such as glucose, from carbohydrates and organic nitrogen from proteins when there is a mixture of preferred and non-preferred nutrients (secondary nutrient sources, *e.g.* sucrose or inorganic nitrogen), and repress metabolism of non-preferred nutrients (New *et al*., 2014). However, de-repression of metabolism of non-preferred nutrients will occur after the depletion of preferred nutrients. Steyaert *et al*. (2010b) demonstrated the effect of carbon/nitrogen catabolite repression in *Trichoderma* spp. in nutrient-rich media, and conversely, carbon/nitrogen catabolite de-repression under nitrogen or carbon starvation. They also showed that the primary nitrogen sources promote photoconidiation more strongly than the secondary nitrogen sources (Steyaert *et al*., 2010b). Primary nitrogen sources are preferred by fungi in comparison with secondary nitrogen sources, including complex and inorganic nitrogen sources (New *et al*., 2014). In addition, it has been proposed that light-induced conidiation is dependent on the growth medium nitrogen composition (Ellison *et al*., 1981), but nitrogen sensing is independent on expression of the blue light-regulator (blr) gene (Friedl *et al*., 2008a, b). Conidium production in *T. atroviride* is largely carbon source dependent, and preference of carbon source (carbon sensing) is under cross-regulation with photoconidiation *via* expression of *blr1* and *blr2* genes (Casas-Flores *et al*., 2006). Isolate-specific conidiation has been demonstrated in *Trichoderma* in response to primary and secondary sources of nitrogen, whereby the nitrogen catabolite repression induced photoconidiation in *T. asperellum* and *T. pleuroticola*, but *T. atroviride* did not show cross-regulation of photoconidiation with nitrogen catabolite repression (Steyaert *et al*., 2010b). However, this characteristic is not generalised for other fungi, so that in *Neurospora crassa*, nitrogen catabolism is

in cross-regulation with the “white collar pathway” to promote photoconidiation (Sokolovsky *et al*., 1992).

Previous studies have demonstrated increased intracellular accumulation of compatible solutes such as trehalose and glycine-betaine by extra-cellular addition of these compounds during production of fungal and bacterial inocula (Kets & Bonts, 1994; Kets *et al*., 1996; Bonaterra *et al*., 2005). Trehalose and polyol compounds have been shown to protect cells against adverse environmental conditions such as low water activity, nutrient starvation, and heat shock (Hallsworth & Magan, 1994, 1996; Teixido *et al*., 2005; Liu *et al*., 2009). Effects of glycine-betaine have been well studied, but mainly in bacteria, where these compounds increased cell tolerance against desiccation (Caesar & Burr, 1991; Welsh, 2000). In most cases, glycine-betaine has been reported as a species-specific compatible solute that increases tolerance to drying. Conversely, this compound didn’t protect *Escherichia coli* cells from damage during storage, but protected them during freeze-drying (Louis *et al*., 1994). Trehalose and polyols do not destabilise or inhibit fungal metabolism or enzymes even at high intracellular concentrations; hence, these compounds are known as compatible solutes (Brown, 1978; Jennings & Burke, 1990).

In Section 2.2 (above), temperature as a principal abiotic factor was tested for effects on *T. atroviride* LU132 conidium production, germination and bioactivity. The effects of colony age were also studied to determine the optimum incubation time for greatest bioactivity. The results showed that the optimum temperature for incubation was near 25°C, and optimum bioactivity occurred at 15 d incubation. These conditions were therefore applied for the further experiments described in this Chapter. For example, the effect of nutrient amendments on conidium production, germination and bioactivity of *T. atroviride* LU132 were examined at 25°C after 15 d.

2.3.2 Materials and methods

2.3.2.1 Fungal cultures

Cultures of *T. atroviride* LU132 and *Rhizoctonia solani* (R73-13b) were obtained as described in Section 2.2.1.1.

2.3.2.2 Nutrient amendments

***Carbon to nitrogen ratio and carbon amendments*:** A defined basal growth medium was used, which contained: 1 g/L of K_2HPO_4, 0.5 g/L of KCl, 0.5 g/L of $MgSO_4$, and 10 mg/L of $FeSO_4$. Carbon (C) concentrations were adjusted with sucrose (42% C, Sigma®) to 4.2, 8.4, and 16.8 g/L. Nitrogen (N) concentrations were adjusted with soy peptone (8% N, neutralised, Oxoid Ltd) to 0.03, 0.05, 0.11, 0.21, 0.42, 0.84, 1.68, and 3.36 g/L (Table 2.4). The combinations of C and N concentrations resulted

in C:N ratios ranging from 5:1 to 160:1. The solutions were buffered with phosphate buffers (KH_2PO_4 and K_2HPO_4) and required water was added to the medium for each treatment. The pH was adjusted to 5.5 with HCl or KOH. The solutions were then mixed with agar prior to sterilisation. Media and amendments were sterilised by autoclaving, except for soy peptone and sucrose solutions, which were filtered through 0.22 µm filters (Express™ Plus, Millipore Corporation). *Trichoderma atroviride* LU132 conidium preparation and inoculation were done as described in Sections 2.2.1.1 and 2.2.1.2. Petri dishes were incubated for 15 d, under constant light at 25°C.

Table 2.4 Different amounts of nitrogen (g/L) and corresponding different amounts of carbon (g/L) giving different C:N ratios in the growth media for *Trichoderma atroviride* LU132.

C:N ratio	Carbon g/L		
	4.2	**8.4**	**16.8**
5:1	0.84	1.68	3.36
10:1	0.42	0.84	1.68
20:1	0.21	0.42	0.84
40:1	0.11	0.21	0.42
80:1	0.05	0.11	0.21
160:1	0.03	0.05	0.11

***Trehalose and glycine-betaine*:** To measure the effect of exogenous sources of trehalose and glycine-betaine on conidium production, germination, and bioactivity, buffered basal medium agar was amended with a C:N 5:1 containing 8.4 g/L carbon determined from the carbon amendment experiment (above). This ratio gave the greatest conidium production in that experiment. Trehalose (D-(+)-trehalose dehydrate, Sigma®) or glycine-betaine (Sigma®) were separately added after autoclaving of the medium (as described previously), to final concentrations of 0.1, 0.6 or 1.2 mM of these compounds. The experimental control was basal medium amended with a C:N 5:1 with carbon source concentration of 20 g/L sucrose, determined in the C:N ratio experiment (above). Petri dishes (9 cm diam.) each containing 40 mL of amended agar medium were inoculated with *T. atroviride* LU132 conidium suspensions as described in Section 2.2.1.2. Petri dishes were incubated for 15 d, under constant light at 25°C.

2.3.2.3 Assessments

After 15 d, conidia produced in different nutrient conditions were harvested in SDW and counted, as described in Section 2.2.1.2. Harvested conidia were used for the germination assessments (described in Section 2.2.1.3), and bioactivity was assessed in dual culture assays (Section 2.2.1.4).

2.3.2.4 Experimental design and statistical analyses

The experiments were set up as randomised complete block designs with three replicates. In the C:N ratio experiment, each block (replicate) contained 18 treatments (six C:N ratios × three carbon concentrations). In the trehalose and glycine-betaine experiment, each block (replicate) contained seven treatments (three trehalose concentrations, three glycine-betaine concentrations and one C:N ratio as the control). Data from both experiments were subjected to analysis of variance (ANOVA), and treatment means were separated using Fisher's unprotected test of LSD. All analyses were performed using procedures in GenStat® 16th edition (VSN International Ltd). Experiments were performed twice to test the results were reproducible. The results of the two experiments did not differ significantly and the results presented are from the second set of experiments.

2.3.3 Results

2.3.3.1 Conidium production

The different C:N amendments significantly affected conidium production of *T. atroviride* LU132 ($P < 0.001$). Conidium production decreased with increasing C:N ratios from 5:1 (2.23×10^9 conidia/mL) to 160:1 (4.44×10^8 conidia/mL) in a continuous trend. A carbon content of 4.2 g/L gave a mean of 9.26×10^8 conidia/mL, 8.4 g/L gave 1.31×10^9 conidia/mL, and 16.8 g/L gave 1.45×10^9 conidia/mL. There was a significant main effect ($P = 0.001$) of carbon concentration on conidium production, with increasing carbon concentration resulting in an increased conidium production (Figure 2.10; Table 2.5). There was also a significant interaction ($P = 0.001$) between carbon concentration and C:N ratio on conidium production, with maximum conidium production (2.52×10^9 conidia/mL) obtained at C:N 5:1 with 8.4 g/L carbon, however, at higher C:N ratios of 160:1 maximum conidium production was obtained with 16.8 g/L carbon (Figure 2.10; Table 2.5). Increased carbon amendments were correlated with increasing intensity of colony coloration (dark green colour) after one week of incubation (Figure 2.10). Later, compact hyphae developed at high carbon concentrations and low C:N ratios, and colony colours were pale green or grey. After two weeks of incubation, the colonies were dark green where C:N ratio and carbon concentration were greater. However, increased colour intensity was correlated with reduced conidium yields, to a minimum of 2.3×10^8 conidia/mL in C:N 160:1 (4.2 g/L carbon). Compact hypha and severe rugosity of colony surfaces were evident in cultures with high carbon

amendments, and mostly in interaction with low C:N ratios, typically at 5:1 (Figure 2.11). Rugose colonies were characterized by wrinkled colony morphology, and conidium suspensions were in mucilage form when washed in SDW for harvesting. Figure 2.12A shows a culture after conidium harvest. Conidia obtained from rugose colonies were clumped or in chains (Figure 2.12B), which were not easily separated even after vigorous shaking.

The trehalose and glycine-betaine amendments gave significant effects on conidium production ($P < 0.001$). Conidium production increased from 2.13×10^9 to 2.64×10^9 conidia/mL with increasing concentrations of trehalose (Table 2.6). Similarly, conidium production increased from increasing glycine-betaine concentrations in the growth medium, from 1.89×10^9 to 2.97×10^9 conidia/mL. Only concentrations of trehalose (0.6 and 1.2 mM) gave more conidia in comparison to the control (basal medium of C:N ratio (5:1) and 8.4 g/L carbon).

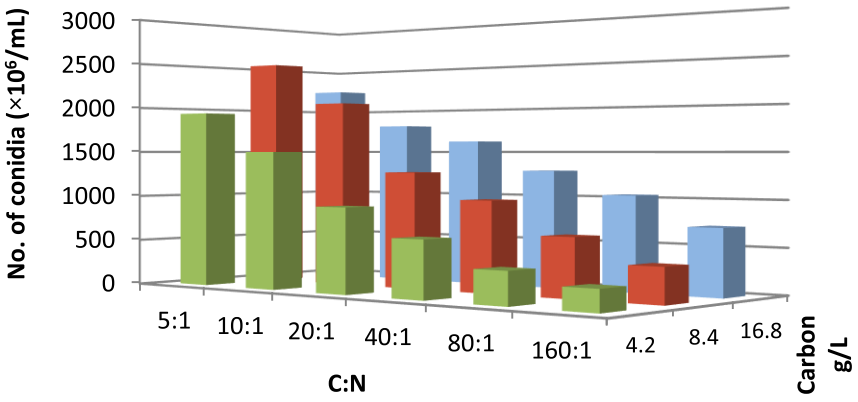

Figure 2.10 Mean numbers of *Trichoderma atroviride* LU132 conidia produced at 25°C after 15 d, from cultures amended with different amounts of carbon (g/L) and C:N ratios ($LSD_{0.05}$: 92.1).

Table 2.5 Main effect means for conidium production, germination and bioactivity of *T. atroviride* LU132 from treatments of different C:N ratios and carbon contents in growth media..

Treatment		Conidia no. (× 10^6/mL)				Average germination % (AUC)[1]				Bioactivity (inhibition %)				Bioactivity (overgrowth %)			
		Carbon g/L			Main effect mean	Carbon g/L			Main effect mean	Carbon g/L			Main effect mean	Carbon g/L			Main effect mean
		4.2	8.4	16.8		4.2	8.4	16.8		4.2	8.4	16.8		4.2	8.4	16.8	
Carbon-to-nitrogen (C:N)	**5:1**	1933	2517	2227	2226	79	74	68	74	77	71	71	73	78	78	50	69
	10:1	1500	2050	1803	1784	73	69	64	69	72	71	69	71	70	63	58	64
	20:1	920	1273	1620	1271	71	66	59	65	71	69	68	69	63	48	73	62
	40:1	620	987	1290	966	66	62	54	61	69	68	67	68	48	58	48	52
	80:1	350	637	1037	674	61	56	54	57	68	68	65	67	60	57	57	58
	160:1	230	380	723	444	56	52	49	52	66	64	61	63	55	48	77	60
Main effect mean		926	1307	1450		68	63	58		70	68	67		63	59	61	
$LSD_{0.05}$		37.6			53.2	0.5			0.7	1.1			1.6	1.7			2.4
Significance[2]		***			***	***			***	ns			***	***			***

[1] Averaging over time, based on the trapezoidal area under the curve (AUC).

[2] ***: the effects of treatments are statistically significant at $P = 0.001$; ns: not significant ($P > 0.05$).

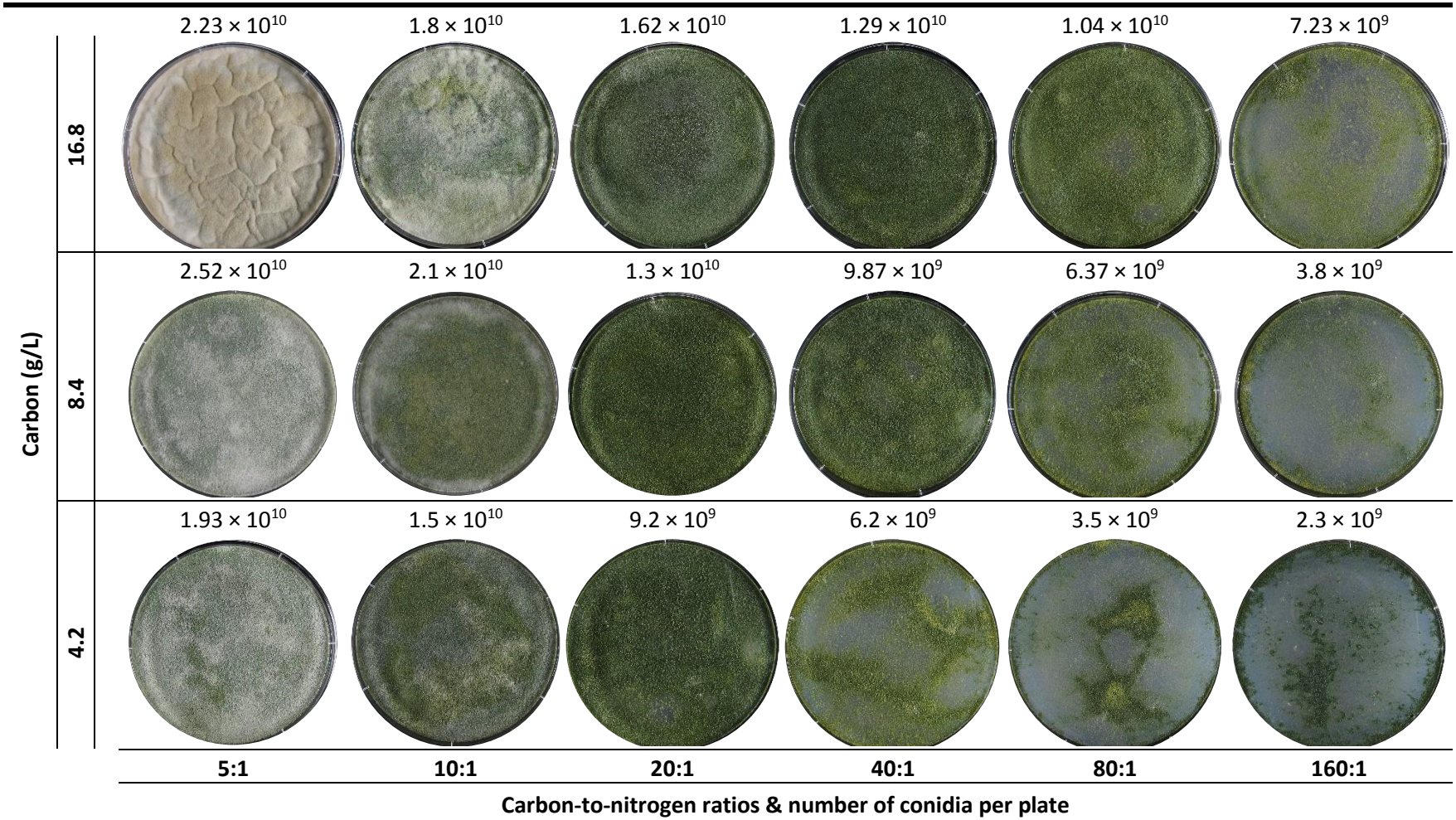

Figure 2.11 Culture morphological and colour variations, and corresponding mean numbers of conidia per plate, obtained for *Trichoderma atroviride* LU132 grown on media with different C:N ratios and carbon contents ($LSD_{0.05}$ = 9.21 × 10^8). The morphologies shown are representative of three independent replicates with similar results.

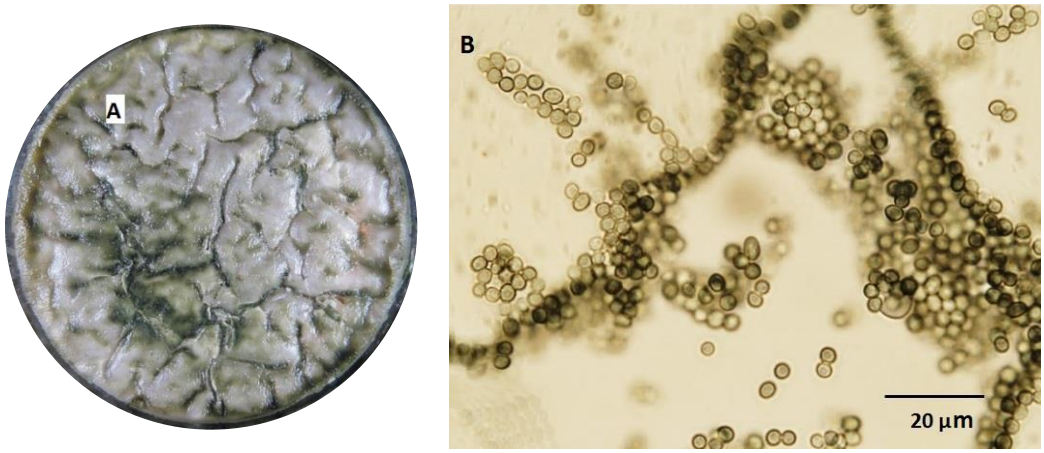

Figure 2.12 A: Rugosity of a *Trichoderma atroviride* LU132 culture after conidia harvest. B: clumped and chains of conidia in SDW, obtained from a culture where the fungus was grown at high carbon concentration (16.8 g/L) and a C:N 5:1.

Table 2.6 Main effect means for conidium production, germination and bioactivity of *Trichoderma atroviride* LU132 from treatments of different trehalose and glycine-betaine concentrations (mM/L) in growth media..

Treatment		Conidia no. (×10^6 conidia/mL)	Average germination (AUC)[1]	Bioactivity	
				Inhibition%	Overgrowth%
Trehalose (mM)	**0.1**	2130	61	67	42
	0.6	2500	72	70	55
	1.2	2643	78	75	65
Glycine-betaine (mM)	**0.1**	1893	50	71	50
	0.6	2117	61	67	62
	1.2	2397	55	63	75
Control (C:N 5:1)		2403	70	71	62
$LSD_{0.05}$		61.6	1.1	2	3
Significance[2]		***	***	***	***

[1] Averaging over time based on the trapezoidal area under the curve (AUC).
[2] ***: the effects of treatments are statistically significant at $P = 0.001$.

2.3.3.2 Conidium germination

Trichoderma atroviride LU132 conidia produced at different C:N ratios and different carbon concentrations had significantly different conidium germination percentages ($P < 0.001$). There was a continuous decline in mean germination from 74% for C:N 5:1 to 52% for C:N 160:1. There was a significant main effect ($P = 0.001$) of carbon concentration on germination, with increasing carbon concentration resulting in reduced germination percentage from a maximum of 68% at 4.2 g/L carbon to a minimum of 58% at 16.8 g/L carbon. However, in interaction with C:N ratios, the greatest conidium germination of 79% was obtained for conidia produced from a C:N 5:1 and 4.2 g/L carbon, and the least germination was 49% was for conidia from culture medium amended with C:N 160:1 and 16.8 g/L carbon (Table 2.5).

Different concentrations of trehalose and glycine-betaine also significantly affected the mean conidium germination percentages ($P < 0.001$). The trehalose amendments of 0.6 and 1.2 mM gave increased germination in comparison to the other trehalose or glycine-betaine concentrations, including the control (Table 2.6). Maximum germination of 78% was achieved with a trehalose concentration of 1.2 mM followed by 0.6 mM (72%). The least germination of 50% was recorded for glycine-betaine (0.1mM) in comparison with the control (70%).

2.3.3.3 Bioactivity

Conidia from *T. atroviride* LU132 colonies from media amended with different carbon contents or C:N ratios gave significantly different inhibition activity against *R. solani* in dual culture assays ($P < 0.001$). The main effect of interactions between carbon contents and C:N ratios was statistically significant ($P = 0.001$). When the amount of carbon was reduced from 16.8 to 4.2 g/L, the percentage inhibition of *R. solani* increased from 67% to 70%. Also, different C:N ratios increased inhibition activity, from 63% from C:N 160:1 to 73% from C:N 5:1 (Table 2.5). *Trichoderma atroviride* LU132 produced from cultures with different C:N ratios and carbon contents did not significantly affect overgrowth activity of *R. solani* ($P = 0.12$). Overall there was an increase in percentage overgrowth with increasing carbon concentration and with decreasing C:N ratio. The main effects of C:N ratios or carbon amendments were statistically significant, but both factors did not follow the same constant increasing or declining trends, similar to inhibition activity by *T. atroviride* LU132 colonies.

The trehalose and glycine-betaine amendments both significantly affected bioactivity (inhibition and overgrowth) of *T. atroviride* LU132 against *R. solani* in dual culture assays ($P < 0.001$) (Table 2.6). The greatest inhibition (75%) was achieved from conidia produced at high concentration of trehalose (1.2 mM), and the least (63%) was from high concentration of glycine-betaine (1.2 mM). However, only the greatest concentration of trehalose significantly increased inhibition activity of *T. atroviride* LU132

colonies compared with the control (71%). Trehalose amendments gave a constantly increasing inhibition trend with increasing trehalose concentrations. Conversely, increasing the glycine-betaine concentration reduced inhibition activity by *T. atroviride* LU132 test colonies. Overgrowth activity of *T. atroviride* LU132 from conidia produced at different concentrations of trehalose and glycine-betaine showed an increase that corresponded to increased concentrations of both trehalose and glycine-betaine. Only glycine-betaine (1.2 mM) gave significantly greater overgrowth (75%), compared with the experimental control (62%) (Table 2.6).

2.3.4 Discussion

Medium composition of nutrient amendments with either different C:N ratios or trehalose and glycine-betaine affected quantity and quality of *T. atriviride* LU132 conidia produced after 15 d of culturing. These differences were also evident from morphological characteristics of the *T. atroviride* LU132 cultures. Increased intensity of colony coloration was associated with increased C:N ratio in the growth medium. However, numbers of conidia reduced to a minimum when the medium C:N ratio was 160:1. This result is in accordance with those reported by Jackson & Bothast (1990), who concluded that increased carbon concentration was responsible for increased melanisation and reduced conidium production after 7 d incubation for the bioherbicide fungus, *Colletotrichum truncatum*. While incubation time in the present study with *T. atroviride* LU132 was 15 d, visual observations in the first week of incubation confirmed increasing coloration with increasing carbon amendment, but increased C:N ratio corresponded with intense coloration and reduced numbers of conidia after two weeks. Intensification of colour in fungi is linked to increased antagonistic properties and structural stability, as well as resistance to extreme environmental conditions such as water stress and UV radiation (Gorbushina *et al*., 2003). Highly coloured colonies (after 15 d incubation) in the present study gave the least conidium fitness (quantity and bioactive quality), although, dark coloured conidia might be more stable and retain viability in storage compared with lighter coloured conidia. Investigation of this is outlined in Chapter 3.

In nitrogen-limited conditions at high C:N ratios, high carbon concentration of 16.8 g/L caused the growth medium to be very viscous, which in turn may have altered the physiological pathways in *T. atroviride* LU132, resulting in production of conidium matrices probably as a result of excess sucrose as a carbon source. Although high carbon concentrations inhibited conidium production, colony growth was similar to that reported by Betina & Zajacova (1978), where high glucose concentrations inhibited conidiation in *T. viride* while there was no negative effect on colony growth. It has been postulated that the conidium matrices can reduce germinability. However, these matrices can also increase the viability of conidia during storage (Nicholson & Moraes, 1980; McRae & Stevens, 1990).

It is therefore expected that these types of conidia, obtained in the present study, would later show stability during long-term storage. Furthermore, high carbon concentrations and also in interaction with C:N ratios, typically at 5:1, resulted in compact hypha and severe colony rugosity, which in turn contributed to the mucilage in the conidium suspensions. Profuse exopolysaccharide matrix and phenotypic characteristics such as wrinkled colonies are referred to as “rogue” phenotypes, which have been linked to increased survival and stability in variable environments (Rashid *et al*., 2003). In the present study, conidia obtained from rugose colonies gave chains or clumped conidia, and this aggregation caused difficulties in conidium counting. However, as discussed above, these conidia may show stability during storage.

The present study indicated that a C:N 5:1 is optimal for conidium production, and in interaction with a carbon concentration of 8.4 g/L, these growth medium conditions gave the greatest conidium production, while higher concentrations of carbon or C:N ratios gave the least number of conidia produced. The decreased conidium production due to increased C:N ratio is in accordance with results reported by Yu *et al*. (1998) for conidium yield of the bioherbicide fungus *Colletotrichum coccodes*. Furthermore, the present results are also similar to those of Schisler *et al*. (1991), who demonstrated that conidium production of *C. truncatum*, as well as efficacy in infectivity (germination and appressorium formation) against *Sesbania exatata*, were greater when growth medium C:N ratio was reduced to 5:1.

In the present study, germination percentage of conidia produced under different nutrient amendment conditions was reduced when either C:N ratio or carbon concentration was increased. At similar nitrogen concentrations, there was a decrease in germination percentage with increasing carbon concentrations. This indicates the strong effect of carbon concentration rather than nitrogen concentration, so that increased sucrose as a carbon source reduced germination percentage, and reduced sucrose gave the greatest germination proportions. Increased germination was probably related to optimization of the contents of trehalose and some other polyols (*e.g.* glycerol and erythritol) in conidia during their production. Conidia containing these compounds germinate more rapidly when transferred to a new environment (Pedreschi *et al*., 1997; Bonaterra *et al*., 2005). The reduced germination percentage of *T. atroviride* LU132 in the present study could be related to increased conidium matrix due to extra carbon source, as an inhibiting factor for germination, as has been shown for *Colletotrichum graminicola* (Nicholson & Moraes, 1980; McRae & Stevens, 1990).

Bioactivity of *T. atroviride* LU132 conidia amended with different C:N ratios and carbon concentrations showed similar trends as for the germination results, where rapid germination and growth by *T. atroviride* LU132 conidia in dual culture plates resulted in greater inhibition activity against colony growth of *R. solani*. Similarly, as seen for germination, inhibition activity of *T. atroviride* LU132 colonies

produced at equal nitrogen concentrations decreased with increasing carbon concentration. Overgrowth activity by *T. atroviride* LU132 conidia fluctuated across nutrient amendments so no correlation with any nutrient variations could be made.

The *T. atroviride* LU132 colonies grew and developed under constant light, and soy peptone was the primary organic nitrogen source. Primary nitrogen sources promote photoconidiation in *Trichoderma* spp. more strongly than secondary nitrogen sources (Steyaert *et al*., 2010b). Sucrose as a disaccharide hydrocarbon source was used in the present study as it has been shown to have quantitative and qualitative effects on conidium production in correlation with high survival rates (van Laere, 1989).

In the presence of the preferred nutrient, catabolite repression will occur in the gene regulation pathways, and derepression of non-preferred nutrients will occur when there is a depletion of preferred nutrients. Excessive nutrient availability will also cause nutrient catabolite repression. For example, Steyaert *et al*. (2010b) demonstrated that nutrient-rich media containing carbon/nitrogen repressed carbon/nitrogen catabolite genes in *Trichoderma* spp., while under nitrogen or carbon starvation, conidiation was induced by carbon/nitrogen catabolite derepression under gene regulation. In the present study, the amount of sugar in media varied from 10 g/L (4.2 g/L carbon) to 40 g/L (16.8 g/L carbon), this last concentration being excessive for *T. atroviride* LU132. The *Trichoderma* colonies reacted to different carbon concentrations, as indicated by variations in colony morphology. Severe rugosity was observed in colonies produced on high carbon concentration media, especially with low C:N ratios, while colonies with low density occurred at high C:N ratios. Morphological variations were not related to differences in biological control activity of conidia produced. However, as discussed earlier, C:N ratios may affect conidium survival. A well balanced C:N ratios (1:5 to 1:10), but based on different carbon concentrations, supported reasonable conidium production, but with a high C:N ratio of 160:1, conidium production was least. Catabolite repression probably affected conidium production reducing *Trichoderma* development at high C:N ratios and specifically at high carbon concentrations. The main effect of carbon concentration on *T. atroviride* LU132 was apparent as production of very dense mycelial growth (from low to severe rugosity) in agar cultures, while the main effect of C:N ratios was expressed as a change from high to sparse conidium production correlated with the shift from low C:N ratio of 5:1 to high C:N ratio of 160:1.

Different concentration of trehalose and glycine-betaine in the basal agar growth medium (C:N 5:1, 8.4 g/L carbon) affected the quantity and quality of conidia produced. Trehalose at intermediate and high concentrations promoted conidium production and percentage germination, but all tested glycine-betaine concentrations gave less conidium production and germination compared with the nil glycine-betaine treatment. Inhibition and overgrowth activity of *T. atroviride* LU132 colonies was increased from conidia produced at high trehalose concentrations in comparison to the experimental

control. Previous studies have demonstrated increased intracellular accumulation of compatible solutes such as trehalose and glycine-betaine by extra-cellular addition of these compounds during production of fungal and bacterial inocula (Kets & Bonts, 1994; Kets *et al*., 1996; Bonaterra *et al*., 2005). Herzog *et al*., 1990 suggested that betaine might protect enzymatic activity of trehalase resulting in the regulation of intracellular trehalose level. In turn, betaine accumulation as nutrient reserve in cells is dependent on nitrogen availability. While ample amount of nitrogen is available, intracellular trehalose content becomes depressed or exhausted. Although trehalose and glycine-betaine absorbance by cells is related to nitrogen availability, the uptake of these compatible solutes has been referred to as isolate-specific characteristics (Louis *et al*., 1994). In the present study, the negative effects of glycine-betaine on quantity and quality of *T. atroviride* LU132 conidia was much greater than the effects of trehalose amendments. However, *T. atroviride* LU132 colonies resulting from conidia produced at high glycine-betaine concentrations gave the greatest overgrowth activity against *R. solani*. Biochemical analyses and examination of test conidia produced may reveal if the basal medium (C:N 5:1, 8.4 g/L carbon) and trehalose and glycine-betaine conditioning had inhibitory effects on absorbance of these amendments. Therefore, how much these compounds have been taken into the cells in the experiments described here should be determined using appropriate biochemical analyses. These analyses are reported in Chapter 5 of this study.

2.4 pH affects *Trichoderma atroviride* LU132 conidium production, germination and bioactivity

2.4.1 Introduction

In filamentous fungi, transcription levels of many genes whose products are required to operate either outside cells or at cell surfaces are influenced by the ambient pH (Peñalva & Arst, 2004). The environmental adaptation to pH enables these fungi to grow over wide pH ranges. *Trichoderma* spp. like many other microorganisms, can adapt to different ambient pHs by changing the pH of the surrounding environment to provide optimal conditions for the production of metabolites (Moreno-Mateos *et al*., 2007). When the ambient pH is conducive, *Trichoderma* is able to express genes involved in biosynthesis and secretion of metabolites. In addition, intracellular acidification in *T. viride* has been shown to increase cAMP levels, which in turn promote photoconidiation (Gresik *et al*., 1991). The ability to alter pH is crucial for a biological control agent to be successful in agricultural systems, for example where the addition of fertilisers are likely affect BCAs. It has been suggested that the external pH might regulate the growth, conidium production, colony morphology and enzyme activity for mycoparasitism and protein secretion by *T. harzianum* (Moreno-Mateos *et al*., 2007). The pH regulation in filamentous fungi is achieved *via* a pH-sensing response system, by gene regulation of the zinc finger transcription factor PacC (Tilburn *et al*., 1995). Under neutral to alkaline conditions, this system activates the transcription of alkaline-responsive genes, while under acidic conditions acidic responsive genes are repressed (Caddick *et al*., 1986). The pH regulatory system was first recognised in *Aspergillus nidulans* acting *via* the function of genes *pac*C, *pal*A, B, C, D, F, H and I (Caddick *et al*., 1986; Arst *et al*., 1994; Tilburn *et al*., 1995; Denison, 2000). In *T. harzianum*, *pac*1 (homologous to *pac*C in *A. nidulans*), is the transcriptional factor regulating pH. The transcriptional factor *pac*1 was also shown to regulate other genes involved in antifungal activity and ant agonism (Moreno-Mateos *et al*., 2007). In *T. harzianum*, when the *pac*1 gene was silenced, fungal growth was optimal at pH 3 (imitating acidic conditions), but did not show overgrowth activity against pathogens in dual culture assays or expression of genes involved in antagonistic activity such as the *chit*42 (chitinase) and *qid*74 (cell wall protein) genes. However, a *T. harzianum* mutant with a *pac*1 allele active at all pH's tested, grew optimally at pH 7.5 (imitating alkaline conditions) with clear overgrowth activity, but did not show expression of *pap*A (protease) and *gtt*1 (glucose permease) genes, indicating that *pac*1 negatively controlled expression of these two genes (Moreno-Mateos *et al*., 2007).

Trichoderma spp. are predominantly acid-loving fungi, and as such are less commonly used to control pathogens that grow and infect under alkaline than under acidic conditions (Kredics *et al*., 2003). *Trichoderma* spp. produce conidia most abundantly on buffered low pH growth media, and also on unbuffered media at different pH values when external stimuli such as light, cell damage and nutrient

starvation induce expression of genes involved in alteration of the intercellular pH (Gresik *et al*., 1991; Moreno-Mateos *et al*., 2007; Steyaert *et al*., 2010a). Furthermore, in *T. atroviride* there is cross-regulation between photoconidiation and the ambient pH. No regulatory link was found with nitrogen status when the influence of ambient pH on conidiation was studied in *T. pleuroticola*, *T. atroviride* and *T. hamatum* in response to light exposure and injury (Steyaert *et al*., 2010b). These results showed that photoconidiation is strictly dependent on low (acidic) pH, and, further, that *Trichoderma* colony response is likely to be species-specific.

In Section 2.2 of the present study, principal factors affecting conidium quality were studied, and optimum temperature and incubation period were chosen to test for effects of nutritional composition in growth media. It is also possible that pH may substantially affect conidium production and quality, so this possibility was examined experimentally. Standard PDB plus appropriate agar was used as colony substrate in this study, since previous research has demonstrated that nitrogen catabolite repression in photoconidiation is not induced in PDA cultures of *Trichoderma* (Steyaert *et al*., 2010b). To enable the ability of *T. atroviride* LU132 to modify the external pH to be determined, the agar media were buffered with a range of phosphate buffers (5 - 40%) prior to adjusting each to a range of pH 3.5 - 8.5.

2.4.2 Materials and methods

2.4.2.1 Fungal cultures

Cultures of *T. atroviride* LU132 and *R. solani* (R73-13b) were obtained as described in Section 2.2.1.1.

2.4.2.2 pH amendments

Different proportions (5, 10, 20 and 40%) of phosphate buffers (1 M KH_2PO_4 or K_2HPO_4) were mixed with PDB at the final volume of 1 L in distilled water. Final pHs of 3.5 4.5, 5.5, 6.5, 7.5 or 8.5 were obtained by adding appropriate amounts of HCl or KOH and agar prior to sterilisation by autoclaving. The pH of agar plates was measured prior to fungal inoculation, and again after 15 d incubation using a flat membrane pH meter (inLab® Surface, Mettler Toledo International Inc.). *Trichoderma* and *Rhizoctonia* colony preparation and inoculation were carried out as described in Sections 2.2.1.1 and 2.2.1.2. Petri dishes were incubated for 15 d under constant light at 25°C (Section 2.2).

2.4.2.3 Assessments

After 15 d incubation, conidia produced at the different pH levels were harvested in SDW and counted as described in Section 2.2.1.2. Harvested conidia from 5% buffered pH series only were used for

assays of germination (described in Sections 2.2.1.3) and bioactivity in dual culture assays (Section 2.2.1.4).

2.4.2.4 Experimental design and statistical analyses

The experiment was set up using a randomised complete block design with three replicates. Each block (replicate) contained six treatments (six different pH levels). Data from experiments were subjected to analysis of variance (ANOVA), and treatment means were separated using Fisher's unprotected test of LSD. All analyses were performed using procedures in GenStat® 16th edition (VSN International Ltd). Experiments were performed twice to test the results were reproducible. The results of the two experiments did not differ significantly and the results presented are from the second set of experiments.

2.4.3 Results

2.4.3.1 Conidium production

The pH amendments significantly affected conidium production of *T. atroviride* LU132 ($P < 0.001$), which was evident from colony form differences at 5% buffer concentration (Figure 2.13). Conidium production fluctuated at the different pH levels in 5% buffer, but there were significant increases in the number of conidia as pH increased from 3.5 to 6.5. Conidium production was least at pH 8.5 (Table 2.7). Numbers of conidia were slightly more at pH 4.5 (1.1×10^9/mL) compared with at pH 3.5 (1.03×10^9/mL), but less at pH 5.5 (7.63×10^8). The greatest numbers of conidia (1.27×10^9/mL) were produced at pH 6.5. Progressively fewer conidia developed at pH 7.5 (1.09×10^8/mL) and pH 8.5 (5.00×10^6/mL). Conidium production was reduced with increasing buffer concentrations from 5 to 40% so that at 10% buffer concentration, no conidia were produced at pH 8.5, and that was the case for most of pH treatments at 20 and 40% buffer concentrations (Table 2.7). For each respective pH the 10% buffer concentration gave similar numbers of conidia to the 5% concentration. The pH of culture media after 15 d incubation generally changed only slightly (by no more than 1 pH unit) from that in the media at the beginning of the experiment (Figure 2.14). The exception was for cultures where the original medium was at pH 8.5, and at the buffer concentrations of 5 or 10%. For these treatments, pH increased to close to pH 5.5 during the culture period. This effect did not occur for the 20 or 40% buffer concentration treatments. Insufficient conidia were produced at pH 8.5 (buffer 5%) to enable further assessment of germination and bioactivity. The effect of nutrient availability in high buffer concentration of 20% and at pH 7.5, was assessed by testing effects of different PDB concentrations (1×, 2× or 3× standard concentration). Figure 2.15 shows morphological variation in *T. atroviride* LU132 colonies at pH 7.5 and 20% buffer and the different PDB concentration treatments.

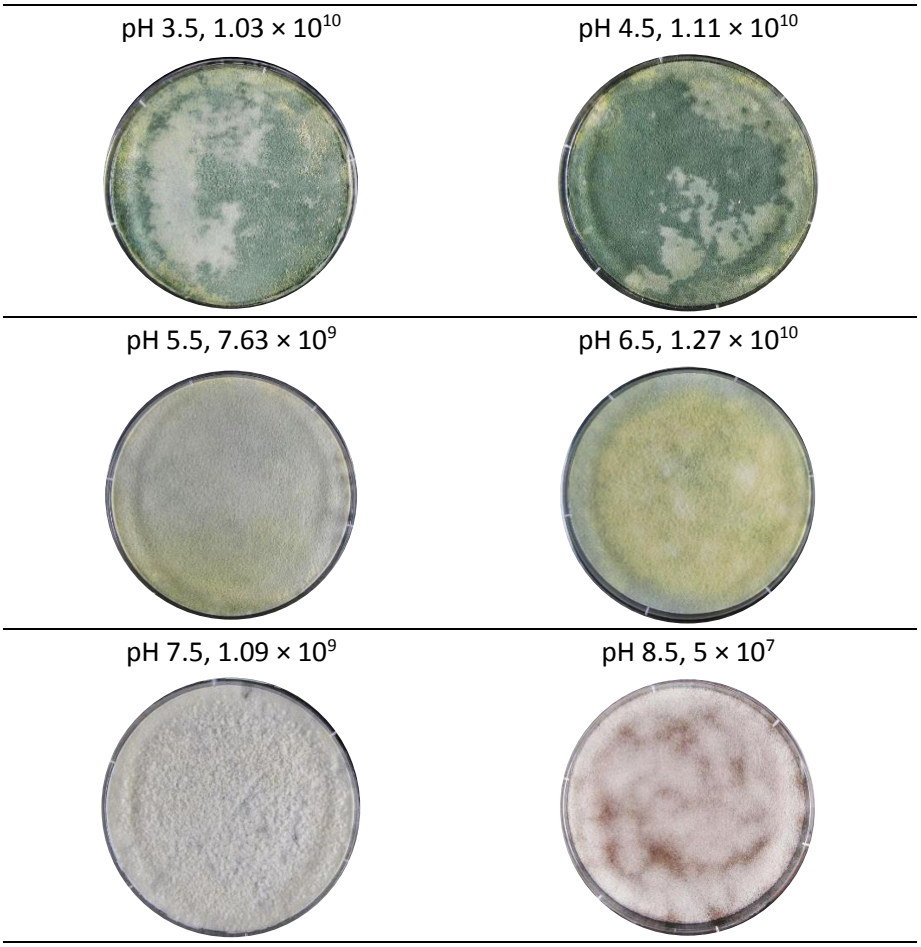

Figure 2.13 Variation in morphology of *T. atroviride* LU132 colonies in agar plates at different pH values (5% buffer). Mean numbers of conidia produced are also indicated. The Petri plates shown are representative of three replicate plates for each pH value.

Table 2.7 Main effect means for conidium production of *Trichoderma atroviride* LU132 conidia produced from different medium pHs and different buffer concentrations, after 15 d culture at 25°C.

Treatment		Conidia no. (×10^6/mL)			
		5% buffer	10% buffer	20% buffer	40% buffer
pH	**3.5**	1030	803	640	0.02
	4.5	1110	933	0	0.08
	5.5	763	587	0	0
	6.5	1273	1070	0.1	0
	7.5	109	33	0	0
	8.5	5	0	0	0
$LSD_{0.05}$		**75.1**	**67.8**	**14.5**	
Significance[1]		***	***	***	-

[1] ***: the effects of treatments are statistically significant at $P = 0.001$.

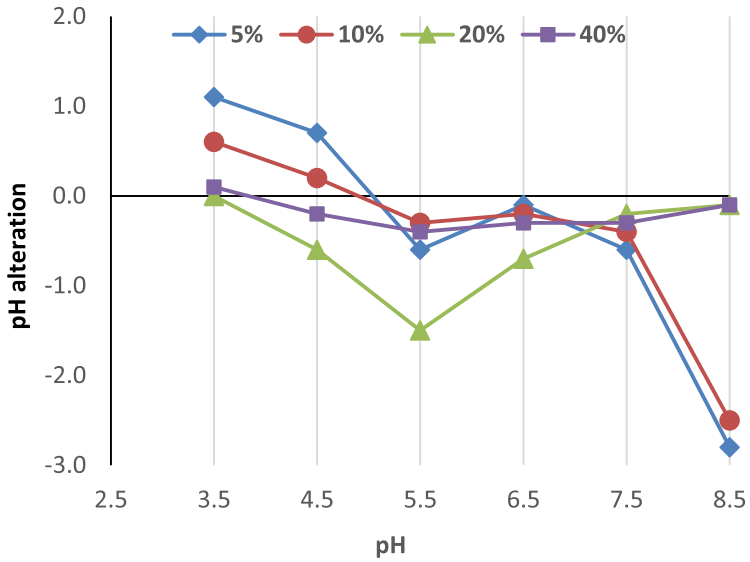

Figure 2.14 Changes in the pH (acidification) of the medium measured in agar plates after 15 d of growth of *Trichoderma atroviride* LU132 cultures, growing on media that was originally formulated with different pHs, and in different buffer concentrations (5, 10, 20 or 40%).

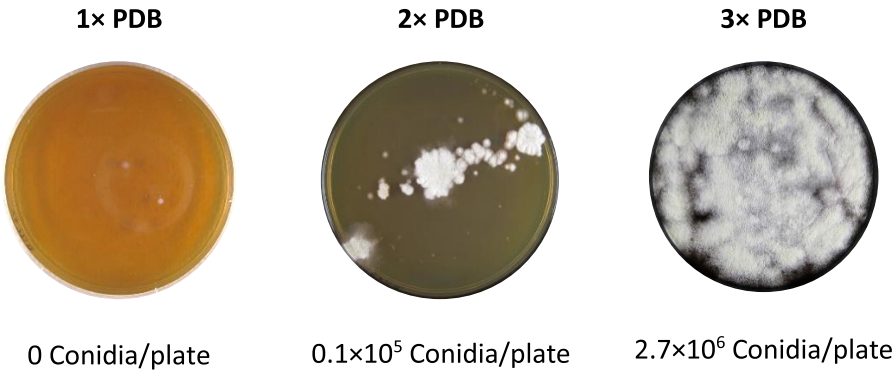

Figure 2.15 Morphological variation and the number of conidia per plate from *T. atroviride* LU132 colonies at pH 7.5 and 20% buffer and different nutrient concentrations (1×, 2× or 3× potato dextrose broth (PDB) concentrations). The morphologies shown are representative of three independent repetitions with similar results.

2.4.3.2 Conidium germination

Germination of conidia produced on media of different pHs in 5% buffer was significantly affected ($P < 0.001$). The greatest germination percentage was 86% for conidia produced at pH 7.5, and the least (63%) for conidia produced at pH 5.5 (Table 2.8).

2.4.3.3 Bioactivity

Trichoderma atroviride LU132 conidia produced on media in 5% buffer and at different pHs gave significantly different amounts of inhibition and overgrowth activity against *R. solani* in the dual culture assays ($P < 0.001$). Inhibition of R. solani was least (62%) from conidia produced at pH 4.5, and greatest (76%) from conidia produced at pH 7.5 (Table 2.8). Overgrowth activity was least (32%) from conidia produced at pH 3.5, and greatest (92%) for conidia produced at pH 4.5.

Table 2.8 Main effect means for conidium production, germination and bioactivity against *Rhizoctonia solani* (in dual culture assays) for *Trichoderma atroviride* LU132 grown on agar media at different pHs (from 5% pH buffered amendments).

Treatment		Conidia no. (×10^6 conidia/mL)	Average germination% (AUC)[1]	Bioactivity	
				Inhibition%	Overgrowth%
pH	**3.5**	1030	68	67	32
	4.5	1110	69	62	92
	5.5	763	63	69	57
	6.5	1273	68	70	67
	7.5	109	86	76	73
	8.5	5	ND[3]	ND	ND
LSD$_{0.05}$		75.1	1.5	2.4	5.3
Significance[2]		***	***	***	***

[1] Values averaged over time based on the trapezoidal area under the curve (AUC).
[2] ***: the effects of treatments are statistically significant at $P = 0.001$.
[3]ND : not determined

2.4.4 Discussion

The colonies of *Trichoderma atroviride* LU132 altered the pH of the culture media containing different buffer concentrations resulting in changes in conidium production, germinability and bioactivity. The effects of pH in *Trichoderma* species has been studied previously. For example, cellulase production from *T. reesei* was affected by medium pH (Kadam & Keutzer, 1995; Ferreira *et al*., 2009). Inhibition of pH drift in the medium culture has also been shown to affect growth and enzyme production in this fungus (Ferreira *et al*., 2009). In this study, different buffer concentrations were tested to assess the effect of minimising pH drift on conidium production as well as germination and bioactivity for conidia produced at different pHs. Across all initial pH values tested, the greatest numbers of conidia were produced in media with the lowest buffer concentration (5%), but this treatment gave the greatest pH change when the original culture medium was at pH 8.5. High conidium production at low initial pH buffer concentration was also reported by Pažout *et al*. (1982) for *Penicillium cyclopium*, where

increased buffer concentration inhibited further conidium production and decreased germinability. Since the buffer capacity and pH in culture media should be optimised to give greatest fungal productivity, in the present study conidia produced at higher buffer concentrations were not tested for germination and bioactivity because only the culture conditions which gave greatest conidium production were assessed for these characteristics.

Trichoderma atroviride LU132 colonies altered the pH level in the culture media containing different buffer concentrations. Steyaert *et al*. (2010a) studied photoconidiation of several *Trichoderma* species in ambient pH (buffered (0.4 M) or unbuffered) in PDA cultures. *Trichoderma atroviride* LU132 did not produce conidia in buffered PDA at pH values above 4.4, while conidia were produced on unbuffered PDA at pH values from 2.8 to 5.2 and alkalisation rather than the predicted acidification occurred, which is similar with shift in pH seen as in the present study. Furthermore, the pH-dependence of photoconidiation was affected by the buffering capacity of the medium, indicating that as well as *pac*1 genes, other genes are also involved in enabling *T. atroviride* LU132 to react to ambient pH and buffering capacity (pH status). This also occurred in the present study, where *T. atroviride* LU132 did not produce conidia at most pH values where high buffer concentrations were used. Similarly, *T. harzianum* has shown *pac*1 to cross-regulate other genes suggesting conidiation is probably dependent on buffering condition rather than pH levels (Moreno-Mateos *et al*., 2007). As has been outlined in Section 2.4.1, there may be differences in the role of *pac*1 in the antagonism of different phytopathogens by *Trichoderma*. This gene has a positive role in expression of genes involved in antagonistic activity such as *chit*42 (chitinase) and *gid*74 (cell wall protein) genes, while negatively affecting the expression of genes involved in overgrowth activity such as *pap*A (protease) and *gtt*1 (glucose permease) (Moreno-Mateos *et al*., 2007). In the present study, the results suggest that *pac*1 had a positive role in the expression of genes involved in the bioactivity of *T. atroviride* LU132 in dual culture assays against the *R. solani* colonies. At the higher pH from 5.5 to 7.5, greater *pac*1 expression (to alter pH) is suggested to have occurred and therefore both inhibition and overgrowth activities were increased, while down regulation of genes involved in inhibition activity at pH 4.5 would be hypothesised to lead to up regulation of genes involved in overgrowth activity at the greatest value compared with other pH values. Since genes involved in overgrowth activity were probably not suppressed at a pH range from 5.5 to 7.5, buffering status is also likely to be involved in expression of these genes.

Optimum conidium production was observed at pH 6.5. Optimum conidium fitness (maximum germination and inhibition of *R. solani* colonies) occurred at pH 7.5. Almost no change in pH from the initial value seen occurred for either of these pH values. This suggests a trade-off between optimum pH for productivity and that for bioactivity. It has been suggested that the addition of nutrients (PDB)

would promote conidiation since the ability of the fungus to alter pH has been correlated with nutrient availability (Pažout *et al*., 1982). Similarly, a marked increase in pH value has been related to depletion of carbohydrate sources in culture medium for *T. reesei* (Bailey & Tähtiharju, 2003). For *Penicillium cyclopium*, low glucose content in the culture medium caused the pH of the medium to rise due to the utilisation of organic acid, thus inhibiting further conidium development. In a medium with high glucose content conidium production was not inhibited (Pažout & Schröder, 1988). However, in the present study with *T. atroviride* LU132, the addition of nutrients at pH 7.5 and high buffer concentration (20%) improved growth compared with low nutrient concentrations, only slight conidium production occurred. This may be due to the addition of nutrients, which can cause low osmolarity resulting in disruption of the cellular hydrogen bonds causing DNA supercoiling, which in turn disrupts cellular mechanisms (Higgins *et al*., 1988; Galán & Curtiss, 1990).

Maximum germination, and inhibition activity against *R. solani* (in the dual culture assays) was measured for conidia obtained from medium at pH 7.5 (5% buffer concentration). Accumulation of low-molecular-weight polyols (such as trehalose and erythritol) in conidia have been shown to correlate with increased germination rates when these conidia were transferred to new environments (Hallsworth & Magan, 1996; Pedreschi *et al*., 1997; Bonaterra *et al*., 2005). In the present study, pH 7.5 at 5% buffer concentration possibly induced accumulation of these compounds resulting in maximum germination and bioactivity of *T. atroviride* LU132.

2.5 Water activity affects *Trichoderma atroviride* LU132 conidium production, germination and bioactivity

2.5.1 Introduction

Water is a key component of living cells, necessary for enzymatic activities of microorganisms and essential for their stability and survival (Pitt & Hocking, 2009). The availability of water for microorganisms relates to the quantity of water in culture media or substrates, and is referred to as water activity (a_w) or expressed as relative humidity (Ayerst, 1969). Water activity, was introduced by Scott (1957) as a physiochemical concept of water availability in foods in which microorganisms cause spoilage. Water movement is influenced by the amount of solid particles in substrates and also by the substrate structure, which is referred to "matric potential" (Ψ_m), and this parameter is used to measure effects of medium structure on water movement (Pitt & Hocking, 2009; Huang *et al*., 2010). However, research has shown that the effects of matric potential on microbial germination and growth are limited compared with alteration in water activity. For example, increasing agar content of culture media for *Aspergillus niger* and *Eurotium herbariorum* from 2.5% to 12.5% had limited effect on conidium germination compared with the reduction in germination recorded with an equivalent reduction in a_w (Huang *et al*., 2010).

Life is dependent on the a_w being in the range 0.999 to 0.600. Amongst living organisms, animal survival requires the a_w to be in the range of 1.000 to 0.999, while for plant survival a_w greater than 0.980 is required (Pitt & Hocking, 2009). Water activity also influences microbial growth and development and negatively controls cellular output when water availability is suboptimal (Troller, 1987). Water requirement for fungal metabolism and growth differ between and within species, and is also affected by other culture conditions (Marín *et al*., 1998; Hallsworth & Magan, 1999; Pitt & Hocking, 2009). For example, in a study by Magan & Lacey (1984) on water requirements for fungi including *Aspergillus* spp., *Penicillium* spp., *Alternaria alternata, Cladosporium herbarum* and *Verticillium lecanii*, different species were shown to have different water requirements. Generally, the minimum water activity for germination of these fungi was less than that for mycelial growth. However, the minimum water activity for biological activity varied depending on pH and temperature.

Cellular osmo-protection has been reported to be accomplished *via* increased NADPH and oxidized NAD^+ to protect structures in redox reactions through scavenging reactive oxygen species (Foyer *et al*., 1994; Takemoto *et al*., 2007). Compounds such as polyols found in the cytoplasm of fungi and which accumulate in response to external water deficit are also thought to act as 'physiological buffering agents' (Jennings & Burke, 1990). Fungi are able to counteract low water availability due to accumulation of polyols (sugar alcohols), including low weight molecules such as the straight-chain

sugars erythritol, manitol and sorbitol, and high weight molecules such as the cyclic sugars sucrose, glycerol and trehalose (Brown, 1978; Jennings, 1985). In *Aspergillus ochraceus*, accumulation of endogenous polyols in conidia was shown to be much greater than in whole colonies, when the colonies were under water stress induced by osmotic solutes or matric potential (Ramos *et al*., 1999). Reduced water availability increased the amounts and types of polyols in conidia and colonies, with trehalose predominating (90%) in conidia. Moreover, yeasts and mould fungi have been shown to have different water requirements depending on growth and development stages (Beuchat, 1983). For example, the a_w required for physiological and morphological activities in these organisms was greater for metabolite production than for colony growth, which in turn was greater than for conidium germination (Beuchat, 1983; Gervats *et al*., 1988). Similarly, *Monilinia fructicola* produced conidia at lower water availability compared with the level which was optimal for mycelial growth (Hong & Michailides, 1999). In *T. atroviride,* water activity, like other abiotic factors, is regulated *via* gene expression, and *seb*1 has been identified as the transcriptional factor regulating responses to osmotic stress (Peterbauer *et al*., 2002). However, a_w is not the only factor affecting fungal growth and development, as different abiotic factors interact during culturing (Sparringa *et al*., 2002).

In Section 2.2, the principal factors affecting conidium quality were studied, and the optimum temperature and incubation time established in those experiments were chosen to test effects of growth medium nutritional composition. Since water activity could also affect conidium production and quality, experiments were also carried out to assess these effects.

2.5.2 Materials and methods

2.5.2.1 Fungal cultures

Cultures of *T. atroviride* LU132 and *R. solani* (R73-13b) were obtained, as described in Section 2.2.1.1.

2.5.2.2 Water activity amendments

The water activity of the culturing medium (PDB) was modified by addition of calculated amounts of glycerol, to obtain different a_w values, including: 0.998, 0.995, 0.985, 0.977, 0.961 and 0.948 at 25°C. The water activities (and glycerol concentrations; g/L) were as follows: 0.998 (5), 0.995 (10), 0.985 (50), 0.977 (100), 0.961 (175) and 0.948 (250). The different media were buffered with 0.1 mM NaH_2PO_4. The water activities of cultures were measured using a dew point device (Aqua Lab CX-2, Decagon, Inc.). The adjusted media at the defined a_w values were mixed with required amounts of agar (1.5%) prior to sterilisation. *Trichoderma atroviride* LU132 inoculation was performed as described in Section 2.2.1.2. The inoculated plates for each a_w value were incubated at 25°C under constant light for 15 d in separate sealed containers over salt solutions (LiCl) at concentrations

corresponding to the a_w of the medium (Figure 2.16). Different concentrations of the salt solution resulted in the relative humidity in the sealed container being at equilibrium with the a_w of the culture media (Beuchat, 1983; Troller, 1987; Pitt & Hocking, 2009).

Figure 2.16 *Trichoderma atroviride* LU132 incubated under constant light at 25°C, in Petri plates containing media of different water potentials (a_w), in sealed containers, over saturated salt solutions (LiCl) at different a_w, corresponding to the respective media a_w values.

2.5.2.3 Assessments

After 15 d incubation, conidia produced in the different a_w cultures were harvested in SDW and counted as described in Section 2.2.1.2. Harvested conidia were used for germination assessments and bioactivity dual culture assays as described, respectively, in Sections 2.2.1.3 and 2.2.1.4.

2.5.2.4 Experimental design and statistical analyses

The experiments were set up as randomised complete block designs with three replicates. Each block (replicate) contained six treatments (six a_w levels). Data from this experiment were subjected to analysis of variance (ANOVA), and treatment means were separated using Fisher's unprotected test of least significant difference (LSD). All analyses were performed using procedures in GenStat® 16th edition (VSN International Ltd). Experiments were performed twice to test the results were

reproducible. The results of the two experiments did not differ significantly and the results presented are from the second set of experiments.

2.5.3 Results

2.5.3.1 Conidium production

Conidium production of *T. atroviride* LU132 was significantly affected by the different water activities tested ($P < 0.001$). The number of conidia produced was greatest at a_w 0.995 (9.73×10^8 conidia/mL) and the least at a_w 0.948 (3×10^4 conidia/mL) (Table 2.9). Insufficient conidia were produced at a_w 0.948 to enable further assessment in germination and bioactivity assays.

Table 2.9 Main effect means for conidium production, germination and bioactivity of *Trichoderma atroviride* LU132 grown in cultures at different water activities (a_w).

Treatment		Conidia no. ($\times 10^6$ conidia/mL)	Average Germination% (AUC)[1]	Bioactivity	
				Inhibition%	Overgrowth%
a_w	**0.998**	557	50	64	52
	0.995	973	46	66	62
	0.985	283	21	68	82
	0.977	397	48	70	72
	0.961	680	78	74	95
	0.948	0.03	ND[2]	ND	ND
$LSD_{0.05}$		34.3	1.7	2.4	4.7
Significance[3]		***	***	***	***

[1] Values averaged over time based on the trapezoidal area under the curve (AUC).
[2] ND : not determined.
[3] *** : the effects of treatments are statistically significant at $P = 0.001$.

2.5.3.2 Conidium germination

Germination percentage of *T. atroviride* LU132 conidia was significantly affected by the different water activity of the media on which they were produced ($P < 0.001$). Mean conidium germination was greatest (78%) from media at a_w 0.961, and least (21%) from a_w 0.985 (Table 2.9).

2.5.3.3 Bioactivity

Water availability in media significantly ($P < 0.001$) affected the bioactivity of *T. atroviride* LU132 conidia as indicated by both inhibition and overgrowth of *R. solani* colonies in dual culture assays. Inhibition activity against *R. solani* (Table 2.9) was least (64%) for conidia produced at a_w 0.998, and greatest (74%) for conidia produced at a_w 0.961. There was a trend of increasing inhibition with

decreasing water availability across the different water availabilities assessed. Mean overgrowth activity by *T. atroviride* LU132 against *R. solani* colonies followed a similar trend, and was the least (52%) for conidia from cultures at a_w 0.998 and the greatest (95%) for conidia from cultures at a_w 0.961.

2.5.4 Discussion

Trichoderma atroviride LU132 colonies grew and produced some conidia at all of the water activities tested. Studies of other fungi have indicated that water activity optima may differ for mycelial growth and conidium production. Hong & Michailides (1999), studying *Monilinia fructicola*, demonstrated that the water activity for optimal mycelium growth was higher than required for optimal conidium production, and temperature interacted with water activity affecting both conidium production and mycelial growth. Similarly, the optimum growth of *Fusarium* species was reported by Marín *et al*. (1995) to be affected by the interactions between water activity, pH and temperature. In the present study, the interaction between water activity and temperature and pH was not investigated. However, there is an indication that optimum conidium production occurred at a_w 0.995, and this may be due to the interaction between the a_w and other culture conditions being optimal for maximum conidium production. Whether the water activity giving optimum conidium production would vary at different temperature and pH regimes was not tested.

Addition of glycerol to decrease water activities of the growth media, and thereby altering the water availability, possibly resulted in an increase in the polyol content of *T. atroviride* LU132 conidia produced during culturing. This has been suggested from results of other studies (Eichner & Karel, 1972; Brown & Simpson, 1972; Hocking, 1986). It has been suggested that the presence of exogenous compatible solutes gives increased intracellular accumulation of these compounds during the production of fungal biological control inocula (Jennings & Burke, 1990; Welsh, 2000; Pascual *et al*., 2003). Of these the compatible solutes, trehalose has been shown to protect membranes from damage during desiccation (Crowe *et al*., 1984), and has been reported to improve desiccation tolerance and survival of conidia during storage (Jin *et al*., 1991; Pascual *et al*., 2003). However, adjusting the water activity using glycerol in the culture medium of *Epicoccum nigrum* gave accumulation of other polyols rather than trehalose (Pascual *et al*., 2003). In the present study, the greatest germination percentage was for conidia produced from *T. atroviride* LU132 colonies grown at a_w 0.961, the lowest a_w tested for germination. This is likely to be due to the accumulation of intracellular polyols, which have been associated with rapid conidium germination (Al-Hamdani & Cooke, 1987; Hallsworth & Magan, 1996; Pedreschi & Aguilera, 1997; Sterflinger, 1998). In contrast, the least germination was for conidia produced at the intermediate water activity of 0.985. This lower

germination does not appear to be directly related to water activity, as conidia produced at slightly higher (0.995) and slightly lower (0.997) water activities had significantly greater germination. It is likely that the adverse effect of other culture conditions, such as interactions between pH and temperature at this a_w 0.985, negatively affected both conidium production and quality as measured by germination. However, this did not translate to reduction in bioactivity with a trend for increased bioactivity (*R. solani* inhibition and overgrowth) with increasing glycerol content of media cultures from which the conidia were produced. Pascual *et al*. (2003) reported that increased endogenous reserves in conidia of *Epicoccum nigrum* probably resulted in dormancy, which apparently improved viability during storage. Therefore, delay in germination of conidia produced at the intermediate water activity of 0.985 could be due to a type 'dormancy' – delayed germination, but these conidia could have superior potential for survival during storage. This will be assessed further in Chapter 3.

2.6 Summary of key results

- Optimum conidium production of *T. atroviride* LU132 occurred in cultures grown at 25°C, while the optimum growth temperature for germination and bioactivity of the fungus was 30°C.
- Conidia produced after 15 d are likely to be the most suitable for use in BCA commercial production.
- A trade-off between quantity and quality of *T. atroviride* LU132 conidia occurred with respect to optimum production temperature/incubation period.
- Conidium production was a continuous process, and a scheduled dark/light regime increased conidium production.
- Bimodal patterns of conidium production were detected in *T. atroviride* LU132, in 20 d base cycle, which is likely to be dependent on colony age rather than abiotic factors.
- Conidia produced on media with low amounts of carbohydrate at C:N of 5:1 had rapid germination and high bioactivity. This ratio probably gives an optimum balance of C:N for growth of the *T. atroviride* LU132.
- Large amounts of carbohydrate possibly repressed carbon catabolism resulting in decreased conidium production, germination, and bioactivity.
- Low water activity and high pH in production gave rapid germination and high bioactivity from the resulting conidia, probably by accumulation of compatible solutes (*e.g.* trehalose and glycerol).

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Chapter 3

Biological fitness of *Trichoderma atroviride* LU132 produced in different culture conditions during long term storage

3.1 Introduction

Identification of the factors that affect conidium stability during production and storage has been shown to influence the success of biological control agents (BCAs). Several sources of stress, mainly abiotic factors such as temperature, water activity, nutritional status, pH, and relative humidity (RH), can affect viability and bioactivity of fungal conidia (Magan, 2001; Teixidó *et al*., 2005). Biomass used as BCAs must be prepared at economically acceptable cost while retaining high levels of germinability, be able to survive adverse environmental conditions, and maintain viability during long periods of storage (Hjeljord & Tronsmo, 1998). The decisive factors determining the success of a BCA as a commercial product are the shelf life stability and bioactivity after storage, as well as ecological fitness through enhanced stress tolerance, with all of these factors potentially improving BCA efficacy and performance (Edel-Hermann *et al*., 2009). For a BCA to be effective, it should be able to tolerate desiccation while retaining germination capability. For most fungal BCAs, the conidia must be dried to induce dormancy for formulation development and prolonged shelf-life, and also to protect the agent from microbial contamination (Jin & Custis, 2011). Fungal BCA formulations based upon dehydration are popular due to increased storage potential, ease of handling, and long stability (Pedreschi & Aguilera, 1997).

Stability of microorganisms during storage has been shown to be influenced by drying processes (Antheunisse & Arkestelin-Dilksman, 1979), storage conditions (Mary *et al*., 1985), and rehydration processes (Leach & Scott, 1959). Drying is not a risk-free process, and can cause rapid decreases in viability of BCAs by reducing re-growth ability (Jackson, 1997; Bonaterra *et al*., 2005). Furthermore, storage and subsequent rehydration can cause further decreases in conidium viability and bioactivity (Eleutheria *et al*., 1993). During drying, due to low water availability, the cell surfaces are exposed to reactive oxygen species which results in oxidation of nucleic acids, proteins and membrane lipids (Kohen & Nyska, 2002; Bai *et al*., 2003). Intracellular concentrations of reactive oxygen species correlate well with viability, so that elevation of reactive oxygen species in cells is usually an index of low viability (Branduardi *et al*., 2007). The concentration of malondialdehyde, a lipid peroxide, has been shown to be an index of the level of lipid oxidation for evaluation of cellular damage by oxidative stress (Turton *et al*., 1997).

The stability of *Trichoderma* conidia in terms of viability and bioactivity is heavily influenced by abiotic factors and the nutritional composition of growth media, which affect biological control efficacy and desiccation tolerance (Agosin & Acuilera, 1998). Conidium germination in other fungi is dependent on protein synthesis, as demonstrated in *Aspergillus nidulans*, *Neurospora crassa*, and *Fusarium solani* (Cochrane & Cochrane, 1970; Loo, 1976; Osherov & May, 2000). Mirkes (1974) demonstrated that dormant conidia of *Neurospora crassa* contained pre-existing pools of free ribosomes, which interact with mRNA to form polysomes in the presence of nutrient sources, for example carbon. During storage, the dormancy status is likely to be associated with substantial changes in physiology and biochemistry of conidia (Mandels, 1981). For example, dormancy in *Myrothecium verucaria* was maintained by physical separation of endogenous reserves of the substrate from metabolic enzymes, *e.g.* trehalose from trehalase (Mandels, 1963; Mandels *et al*., 1965; Mandels and Maguire, 1972). Also, insolubility and compartmentalisation of reserves of lipids in some conidia has been postulated as a mechanisms for enhancing conidium survival (Reisener, 1976).

Previous studies have shown that in several microorganisms conidium compatible solutes such as trehalose and polyols can be associated with osmotic adjustments, and can protect cell membranes and proteins from inactivation and denaturation (Brown & Simpson, 1972; Crowe *et al*., 1984; Carpenter & Crowe, 1988). Trehalose and polyol compounds have been shown to contribute to acceleration of germination, enhancement of bioactivity, and resistance to adverse environmental conditions such as desiccation, freezing, heat, osmotic or oxidative stress, nutrient starvation, dehydration, and exposure to toxic chemicals (Al-Hamdani & Cooke, 1987; Hallsworth & Magan, 1994; Hallsworth & Magan, 1995; Bonaterra *et al*., 2005).

Exposure to sublethal stress treatments has also been shown to cause accumulation of compatible solutes in beneficial microorganisms (Bonaterra *et al*., 2007; Liu *et al*., 2009). For example, Palazzini *et al*. (2009) reported that osmo-adaptation to low water activity enhanced the biological control activity of *Bacillus subtilis* and *Brevibacillus* sp. under adverse environmental conditions. Similarly, stress tolerance and biological control efficacy of the yeast antagonist *Metschnikowia fructicola* were enhanced by high temperature and oxidative stress, suggesting that the heat-shock treatment caused cross-protection to reactive oxygen species and high temperatures (Liu *et al*., 2011). Moreover, exposing the yeast *Candida oleophila* to sub-lethal levels of oxidative stress, resulted in tolerance to lethal levels of peroxide hydrogen, low pH and high temperature. This treatment also enhanced biological control activity of *C. oleophila* against infection of apple fruit by *Penicillium expansum* and *Botrytis cinerea* (Liu *et al*., 2012).

The overall objective of the experiments outlined in this Chapter was to define the intrinsic stability of *Trichoderma atroviride* LU132 conidia produced under different incubation and nutritional

conditions, and to identify the influence of storage conditions on conidium viability and long-term survival (shelf life). The stability of biological control agents at temperatures of approx. 20˚C is commonly assessed by commercial companies, as this approximates to the conditions likely to be experienced during storage. However, the packages containing biological control agents may undergo a heat shock either during storage, or in transit to the market or during application to crops (Kim *et al*., 2011). Testing at 30˚C and different relative humidities would enable the negative impacts of extreme conditions on conidium viability to be identified. Culture age and culture conditions have been shown to influence the physiological quality of inoculum and subsequently determine bioactivity potential (Hallsworth, 1996).

From results outlined in Chapter 2 Section 2.2, temperature and length of incubation period affected conidium production, germination and bioactivity of *T. atroviride* LU132. Based on the optimum temperature (25˚C) and length of incubation (15 d) determined from those studies, conidium production, germination and bioactivity were then assessed for different culture conditions of nutrients, pHs and water activities. Culture conditions which resulted in production of 15-d-old conidia with the greatest bioactivity against colony growth of *R. solani* (30°C, a_w 0.961, C:N 5:1, 1.2 mM trehalose and pH 7.5), and the least bioactivity (20°C, a_w 0.985, C:N 160:1, 1.2 mM glycine-betaine and pH 5.5) were selected to examine effects of subsequent storage conditions on conidium viability and bioactivity.

3.2 Material and methods

3.2.1 Origin of fungal cultures

Cultures of *T. atroviride* LU132 and *Rhizoctonia solani* (R73-13b) were obtained as previously described (Section 2.2.1.1).

3.2.2 Culture amendments

The different culture conditions applied are outlined in Table 3.1, to assess their effects on production of *T. atroviride* LU132 conidia and on conidium viability and bioactivity after different storage periods and conditions. For each treatment, the media were adjusted to the required nutritional composition, pH or water activity as described in Chapter 2 Section 2.2.1 for temperature treatments; Section 2.3.2 for nutrient treatments; Section 2.4.2 for pH treatments; Section 2.5.2 for water activity treatments. A total of 153 Petri plates were inoculated with *T. atroviride* LU132 as described in Section 2.2.1.2. The production experiment was set up as a completely randomized design for temperature treatment and as a randomized complete block design for nutrient amendments (C:N ratios, trehalose and glycine-betaine), pH and water activity treatments, in three replicates or blocks, each containing 51 plates.

Petri plates were incubated in conditions as described in Chapter 2, corresponding to each conidium production treatment. After 15 d incubation, one plate was randomly selected from each replicate or block of each conidium production treatment, and the conidia were harvested (as described in Section 2.2.1.2) to be assessed for the numbers of conidia, percentage germination, and bioactivity (as fresh conidia). The other 150 plates from each treatment were considered as pseudoreplicates and harvested as described in Section 2.2.1.2.

Harvested conidia from each treatment were bulked together and dried in a laminar flow cabinet overnight to be used in a storage experiment. The batch of bulked conidia for each treatment was divided between sterile blue glass microtubes (as suggested by Islam *et al*., 2007). Microtubes were stored unsealed for up to six months at 30˚C under two humidity storage conditions: 0% RH over silica gel or 50% RH achieved by placing the microtubes over a saturated salt solution (LiCl) at a concentration (8.5 M/kg) corresponding to an a_w of 0.500 (Barbosa-Cánovas *et al*., 2008).

The storage experiment was set up as a randomised complete block design in three blocks for each humidity. Each block contained five microtubes (for five storage assessment times) for each of the ten production treatment (50 microtubes per block × three blocks = 150 microtubes).

Table 3.1 Culturing conditions used for production of *Trichoderma atroviride* LU132 conidia for assessing effects on viability and bioactivity at different times during six months storage under different conditions.

Treatments[1]	Culture conditions						
	T (°C)	a_w	pH	C (g/L)	C:N	C Source[2]	Buffer[3]
20°C	20	0.995	5.6	10	10:1	Dex	-
30°C	30	0.995	5.6	10	10:1	Dex	-
a_w 0.985	25	0.985	5.5	30	30:1	Dex + Gly	Phosphate
a_w 0.961	25	0.961	5.5	80	80:1	Dex + Gly	Phosphate
C:N 5:1	25	0.998	5.5	4.2	5:1	Suc	Phosphate
C:N 160:1	25	0.993	5.5	16.8	160:1	Suc	Phosphate
1.2 mM Tre	25	0.994	5.5	8.4	5:1	Suc + Tre	Phosphate
1.2 mM GB	25	0.994	5.5	8.4	5:1	Suc + GB	Phosphate
pH 5.5	25	0.993	5.5	10	10:1	Dex	Phosphate
pH 7.5	25	0.976	7.5	10	10:1	Dex	Phosphate

[1] Tre: trehalose, GB: glycine-betaine.
[2] Dex: dextrose; Gly: glycerol; Suc: sucrose.
[3] Phosphate buffer Na_2HPO_4 (0.1 mM) in water activity test and appropriate concentrations of K_2HPO_4 /KH_2PO_4 to adjust required pH in nutrient and pH tests.

3.2.3 Assessments

The numbers of conidia, conidium germination and bioactivity were assessed as described in Chapter 2 Section 2.2.1 for fresh conidia of each treatment, and also for the dried conidia from each storage condition (0 or 50% RH), initially and then after two weeks, one, three or six months storage. These assessments were based upon the AUC for the different parameters, as described in Chapter 2 Section 2.2.1.7.

The number of conidia for each treatment at each assessment time were assessed by suspending 0.1 g of the stored conidia in 1 mL of sterile distilled water plus 0.01% Tween 80 in 1.7 mL microtubes. These were then shaken for 30 min to ensure that aggregated conidia were separated. The number of conidia in the resulting suspensions were counted using a haemocytometer (Section 2.2.1.2). The conidium suspensions were then used to determine conidium germination and bioactivity based on the AUC for the different parameters. Germination assays were carried out as described in Section 2.2.1.3, but for a period of 12 to 48 h (assessments at 12, 14, 16, 18, 20, 22, 24, 36 and 48 h). Assessments of bioactivity was carried out in dual culture assays against *Rhizoctonia solani* as previously described in Section 2.2.1.4.

Three comparisons were conducted to separate the effects of different factors, including drying effect (DE), storage effect (SE) and accumulated effects of drying and storage (DE+SE), which mostly caused reduction in numbers of conidia, germination and bioactivity. For numbers of conidia, only the storage effect was measured, since fresh and dry conidia were prepared in different ways. The reduction was measured for each storage condition (0 or 50% RH) separately and across both conditions combined, and the percentage reductions for each factor, and combination of factors, were calculated as follows:

% reduction due to drying (DE) = (F0 − D0/F0) × 100
% reduction due to storage (SE) = (D0 − D6/D0) × 100
% reduction due to combination of drying and storage (DE + SE) = (F0 − D6/F0) × 100;
where F0 is fresh conidia at time 0, D0 is dry conidia at time 0, D6 is dry conidia after six months.

3.2.4 Statistical analyses

The average means for the conidium numbers, germination and bioactivity at each storage assessment time in the two storage conditions (0% or 50% RH) were analysed separately and also combined. Data from each storage condition showed the effect of storage condition *versus* conidium treatment effect at each storage time, as well as over five assessment times, based upon the AUC of the respective data. The combined data across both storage conditions combined showed the effect of conidium production conditions *versus* length of storage at each assessment time followed by an average over

five assessment times based upon the AUC. The data were subjected to analysis of variance (ANOVA) and treatment means separated using Fisher's unprotected test of least significant difference (LSD). To compare between the culture condition factors which varied between treatments, a second analysis was conducted to determine the significance of contrasts at three levels of probability: P = 0.05, 0.01, or 0.001. For some variables (i.e. water activity), changing that variable resulted in a change in another variable. Therefore, a treatment comparison was only considered valid when only one culture condition parameter varied between the two treatments, with the remaining conditions being constant (Table 3.1). For example, the comparison of 20°C and 30°C was valid because these two treatment conditions varied only in temperature (20°C *versus* 30°C). The valid treatment comparisons included 20°C *versus* 30°C, C:N 5:1 *versus* C:N 160:1 and trehalose (1.2 mM) *versus* glycine-betaine (1.2 mM). All analyses were performed using procedures in GenStat® 16th edition (VSN International Ltd). The storage experiment was performed once.

3.3 Results

Storage conditions caused statistically significant (P < 0.001) decreases in the number, germination and bioactivity of test conidia during six months of storage, with the mean reductions in conidium number, germination and bioactivity being greater for conidia stored at 50% RH compared with 0% RH (Table 3.2). The effect of drying on the decrease in the number of conidia is shown in Table 3.3. The effects of drying, storage and combination effects of drying and storage on the reduction in the germination are shown in Table 3.4, and on the bioactivity in Table 3.5. These effects were measured in each of the storage conditions of (0% RH and 50% RH), and also across both storage conditions combined. Full data sets at each assessment time for each storage condition and both conditions combined are presented in Section 3.7 (Appendices).

Table 3.2 Mean percentage reductions in the number, germination and bioactivity of *Trichoderma atroviride* LU132 conidia at storage condition of 30°C and 0 or 50% RH after six month storage across all culture production conditions.

Assessments	% Reduction	
	0% RH	50% RH
No. of conidia	59%	61%
Germination	91%	97%
Bioactivity	35%	42%

3.3.1 Numbers of conidia

For all culture condition treatments, storage for six months resulted in reductions in numbers of conidia (Table 3.3). When conidia were stored at 0% RH, culture condition treatments of 20°C gave 45% reduction in numbers of conidia, a_w 0.961 gave 44% reduction, C:N 5:1 gave 40% reduction and C:N 160:1 gave 44% reduction. These reductions after six months storage were significantly less compared with all the other culture treatments. The greatest percentage reduction in conidium numbers at 0% RH was for conidia produced at 30°C (79% reduction) and on medium containing 1.2 mM glycine-betaine (78% reduction), and these reductions were significantly greater than for all other treatments. When stored at 50% RH, the same culture conditions (20°C, a_w 0.961, C:N ratio 5:1 and 160:1) as for 0% RH resulted in significantly lower reductions in conidium numbers (50 - 54%) after six months storage compared with all other treatments. The greatest reduction in numbers of conidia at 50% RH was for culture conditions 30°C (74% reduction), a_w 0.961 (63%), 1.2 mM trehalose (61%), 1.2 mM glycine-betaine (67%), pH 5.5 (69%) and pH 7.5 (63%). There was a significant ($P < 0.001$) effect of culture conditions on the percentage reduction in conidium numbers after six months storage across both storage conditions combined (0 and 50% RH). The greatest reduction in conidium numbers was for conidia produced at 30°C (77%) and 1.2 mM glycine-betaine (73%), while conidia produced from cultures incubated at 20°C (50% reduction), or with C:N 5:1 (46%), C:N 160:1 (47%) and a_w 0.961 (49%) had significantly less reductions in conidium numbers after six months storage, compared with conidia produced under all other culture conditions.

Regarding the valid comparison treatments, the mean number of conidia produced from the temperature treatments were significantly different ($P < 0.05$) under both storage conditions (0 or 50% RH). The C:N ratio treatments gave significant differences ($P < 0.05$) at 0% RH and with the greatest difference when stored in 50% RH. However, there was no significant difference between these treatments across both storage conditions of 0 and 50% RH. The numbers of conidia at each of the five storage assessment times in each storage condition (0% or 50% RH) and across both conditions combined are shown in Appendix 3.1.

3.3.2 Conidium germination

There was a statistically significant effect ($P < 0.001$) of culture conditions on percentage germination at each of the different storage conditions (0 and 50% RH), and also across both storage conditions combined (Tables 3.4). Conidium production conditions resulted in the greatest percentage germination at 0% RH for conidia produced at C:N 5:1 (38% germination), and also where the data for the two storage conditions were combined (32% germination). These germination proportions were

significantly (P = 0.001) greater than for all the other growth medium treatments, while at 50% RH, conidia produced at C:N 160:1 (29%) gave the greatest average germination.

Table 3.3 Mean numbers (×10^7/mL) of *Trichoderma atroviride* LU132 conidia from different culture production treatments (see Table 3.1), and reduction (%) after storage for six months (SE)[1], under storage conditions of 0% RH, 50% RH and across both storage conditions combined (0 and 50% RH). Data is based on AUC[2] values.

Treatment[3]	0% RH		50% RH		(0 + 50)% RH	
	Conidia no.	%SE	Conidia no.	%SE	Conidia no.	%SE
20°C	1015	45	885	54	950	50
30°C	568	79	678	74	623	77
a_w 0.985	758	65	835	63	797	64
a_w 0.961	1001	44	904	53	953	49
C:N 5:1	1161	40	971	52	1066	46
C:N 160:1	1130	44	1059	50	1094	47
1.2 mM Tre	741	62	736	61	739	62
1.2 mM GB	552	78	670	67	611	73
pH 5.5	891	64	754	69	822	67
pH 7.5	767	64	867	63	817	64
$LSD_{0.05}$	27	5.6	32.2	5.5	75.6	5.7
Overall mean	858	59	836	61	847	60
Significance of contrasts between valid comparisons[4]						
20°C *vs* 30°C	***	***	***	***	***	***
C:N 5:1 *vs* C:N 160:1	*	ns	***	ns	ns	ns
1.2 mM Tre *vs* 1.2 mM GB	***	***	***	*	**	***

[1] SE: storage effect as % reduction.
[2] AUC: area under the curve.
[3] Tre: trehalose, GB: glycine-betaine.
[4] *, **, ***: the effects of treatments are statistically significant at P = 0.05, 0.01 or 0.001, respectively; ns: not significant (P > 0.05).

For all culture condition treatments, storage for six months resulted in reduced germination due to effects of drying, storage or combined effects of drying and storage. Drying, as indicated by the drying effect (DE), caused the greatest reduction in average germination with conidia produced at pH 7.5 (56% reduction) before storage at time 0, while the least reduction in average germination occurred for conidia produced at a_w 0.985 (−43%) and 1.2 mM glycine-betaine (−41%) followed by 20°C (−35%). These negative reductions can be translated as positive proportion change (increase) in germination compared with corresponding values of average germination when conidia were fresh. For example, the 20°C treatment gave 55% actual germination when conidia were fresh and there was 35% increase in proportion of germination after drying, which means 55 × 35/100 ≈ 19% increase in actual germination. Therefore, average percentage germination of dried conidia is calculated as 55 + 19 = 74. Storage at 0% RH for six months resulted in the greatest reduction (96 - 100%) in germination for

conidia produced at 20°C, a_w 0.961, 1.2 mM trehalose, 1.2 mM glycine-betaine, pH 5.5 and pH 7.5, and the least reduction by C:N 5:1 (71%). In storage at 50% RH, the least reduction in germination of conidia occurred with conidium production treatments of C:N 5:1 (85%) and C:N 160:1 (83%), which were significantly less ($P < 0.001$) compared with all other culture conditions (99 - 100%).

Accumulation of drying and storage effects (DE+SE) caused reductions in average proportions of conidium germination. The least reduction was recorded for conidia produced with C:N 5:1 (35% reduction) at 0% RH storage, followed by 55% at 50% RH storage and 79% across both storage conditions combined. These values were significantly ($P < 0.001$) less than all the other growth medium treatments.

Regarding the valid comparison treatments, there were significant differences ($P < 0.01$) between percentage germination of each valid production treatment at each storage condition and also both storage conditions combined (Table 3.4). There was less reduction in germination for conidia from the 30°C growth treatment compared with 20°C. Conidia produced at 20°C gave the least average germination (6 - 8%) and significantly less ($P = 0.001$) than average germination for conidia produced at 30°C (23 - 34%), at different storage conditions and combined. After storage at 0% RH, the average germination of conidia produced with C:N 5:1 (38%) was greater ($P = 0.001$) than average germination with C:N 160:1 (23%). Significant differences ($P = 0.01$) in average germination occurred between C:N 5:1 (32%) and C:N 5:1 (26%) across both storage conditions combined. In contrast, at 50% RH, germination of conidia produced from a C:N ratio of 160:1 (29%) was greater ($P = 0.001$) compared with those produced with C:N of 5:1 (26%). Trehalose amendment gave greater ($P < 0.01$) average germination (19 - 25%) compared with glycine-betaine (14 - 17%) in each of both storage conditions and for both conditions combined.

There was a delay in germination initiation for stored conidia compared with fresh conidia. No germination was observed after 12 h incubation for the dried conidia immediately after harvest (0 month), whereas for fresh conidia, germination had been initiated for most treatments after 12 h incubation or earlier (Figure 3.1). The maximum germination progressively decreased with increasing storage time, across both storage conditions combined for all treatments. Some culture conditions gave greater negative impacts on germination than others. For example, although dried conidia produced at 20°C and C:N 5:1 initially (0 month) had the same percentage germination (93%), after one month storage there was no germination of conidia produced at 20°C, while germination was still 70% for conidia produced at C:N 5:1. The percentage germination of conidia at each of the five storage assessment times in each storage condition (0% or 50% RH) or across both conditions combined are shown in Appendix 3.2.

Table 3.4 The mean germination (%) of *Trichoderma atroviride* LU132 conidia from different culture production treatments (see Table 3.1) as fresh or after storage for six months based on AUC[1] values and also reduction[2] (%) after drying (DE), storage (SE) and combined (DE+SE) under storage condition of 0% RH, 50% RH and across both storage conditions combined (0 and 50% RH). Data is based on AUC[2] values for 12-22 h germination assessment.

Treatment[3]	%Ger[4] (Fresh)	%DE	0% RH			50% RH			0% + 50% RH		
			%Ger	%SE	%(DE+SE)	%Ger	%SE	%(DE+SE)	%Ger	%SE	%(DE+SE)
20°C	55	−35	8	100	100	6	100	100	7	100	100
30°C	74	5	34	81	68	23	99	99	28	90	91
a_w 0.985	21	−43	10	84	74	9	100	100	9	92	89
a_w 0.961	78	13	24	96	95	14	100	100	19	98	98
C:N 5:1	79	7	38	71	35	26	85	55	32	78	79
C:N 160:1	49	35	23	80	86	29	83	86	26	82	90
1.2 mM Tre	78	6	25	96	95	19	100	100	22	98	98
1.2 mM GB	55	−41	17	100	100	14	100	100	16	100	100
pH 5.5	63	17	10	100	100	10	100	100	10	100	100
pH 7.5	86	56	10	97	96	10	100	99	10	98	100
$LSD_{0.05}$	1.8	2.2	0.6	4.6	5.8	0.8	1.2	1.8	4.4	6.2	6.6
Overall mean	64	2	20	91	85	16	97	94	18	94	94
Significance of contrasts between valid comparisons[5]											
20°C *vs* 30°C	***	***	***	***	***	***	ns	ns	***	***	**
C:N 5:1 *vs* C:N 160:1	***	***	***	***	***	***	*	***	**	ns	**
1.2 mM Tre *vs* 1.2 mM GB	***	***	***	ns	ns	***	ns	ns	**	ns	ns

[1] AUC: area under the curve.

[2] DE: % reduction due to drying effect, SE: % reduction due to storage effect after six months, DE + SE: % reduction due to combination of drying and storage effects.

[3] Tre: trehalose, GB: glycine-betaine.

[4] Ger: germination.

[5] *, **, ***: the effects of treatments are statistically significant at $P = 0.05$, 0.01 or 0.001, respectively; ns: not significant ($P > 0.05$).

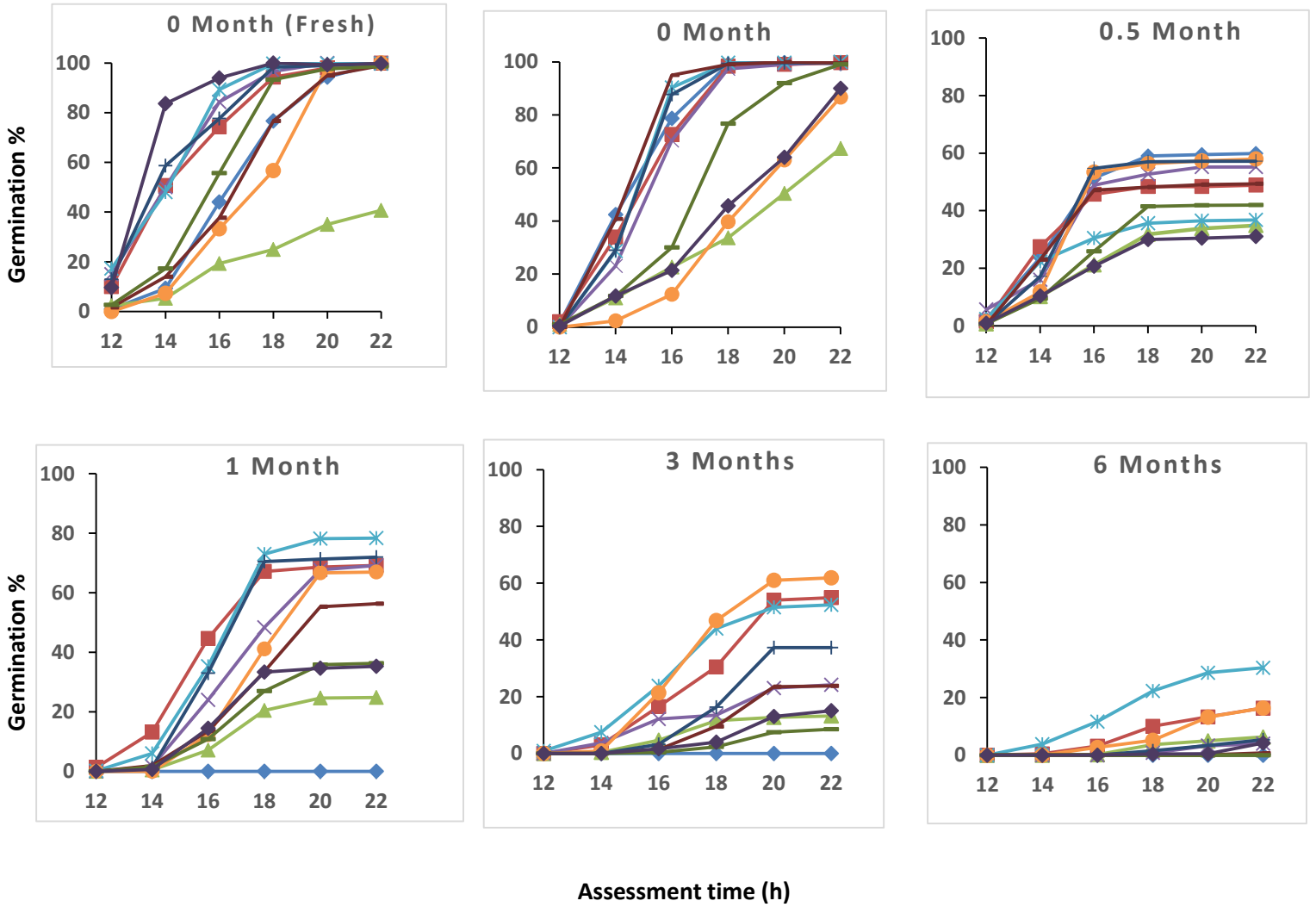

Figure 3.1 Mean percentage germination after 12 to 22 h incubation for *Trichoderma atroviride* LU132 conidia either immediately after harvest (Fresh) or after drying and storage across both 0 and 50% RH combined at different times (0 to six months) for the different culture conditions (see Table 3.1). The culture conditions for conidium production were: temperature (20 or 30°C), a_w (0.985 or 0.961), C:N (5:1 or 160:1), trehalose (1.2 mM) or, glycine-betaine (1.2 mM) and pH (5.5 or 7.5).

3.3.3 Bioactivity

The bioactivity of *T. atroviride* LU132 test conidia decreased during storage and there were statistically significant effects ($P < 0.001$) of the different culture conditions on the percentage reduction in bioactivity across both storage conditions combined (Table 3.5). Conidium production conditions resulting in the greatest bioactivity (68%) was recorded for conidia produced at 30°C when stored at 0% RH, while the least bioactivity (25%) occurred for conidia produced at 20°C when stored at 50% RH.

Drying affected bioactivity of conidia from different culture conditions, with the greatest reduction (%DE) occurring for conidia produced at 20°C (23% reduction), and the least reduction (12%) was recorded for C:N 160:1 (−12%). This resulted as an overall elevation in proportion of bioactivity compared with fresh (61%) conidia (61 × 12/100 ≈ 7%).

The greatest negative effect of storage on the bioactivity of conidia occurred for conidia produced at 20°C (100% reduction), which was greater than any of the other growth treatments after six months storage at 0% RH. The least negative effects were recorded for conidia produced from the 30°C (13% reduction), a_w 0.985 (18%), C:N 5:1 (15%) and C:N 160:1 (10%) treatments, during storage at 0% RH. Under storage conditions of 50% RH and across both storage conditions combined, similar treatments showed the least negative effects of storage on bioactivity during six months of storage, except that the a_w 0.985 treatment gave greater reduction in bioactivity than the other treatments as 33% reduction at 50% RH and 25% reduction in bioactivity across both storage conditions combined.

Accumulation of drying and storage effects (DE+SE) caused statistically significant ($P < 0.001$) reductions in average bioactivity of *T. atroviride* LU132 colonies after six months of storage under different storage conditions. The least reductions in bioactivity due to drying and storage effects were recorded for treatments of 30°C (12% reduction), C:N 5:1 (11%) and C:N 160:1 (13%) at 0% RH, which were significantly less than for all the other treatments. Similarly, the least reductions in bioactivity (after storage at 50% RH) occurred for the growth treatments of 30°C (16% reduction), C:N 5:1 (17%) and C:N 160:1 (12%). This was also the case across both storage conditions combined (0 and 50% RH), where the 30°C (19% reduction), C:N 5:1 (20%) and C:N 160:1 (−1%) treatments gave the least reductions in bioactivity, which is translated as proportion increase in bioactivity of dry conidia compared with fresh conidia.

Regarding the valid comparison treatments, there was less reduction in bioactivity of the colonies from conidia produced from the 30°C growth treatment compared with the 20°C treatment. Conidia produced at 20°C gave no bioactivity (100% reduction), where the reduction was significantly ($P = 0.001$) greater than average reduction in bioactivity of colonies from the 30°C growth treatment (13 -

15%), at different storage conditions and combined. the storage treatment of 0% RH, the C:N 5:1 ratio production treatment (15% reduction) *versus* C:N 160:1 (10%) treatment gave no significant difference in percentage reduction in bioactivity and similarly across both storage conditions combined for the C:N 5:1 (17%) *versus* C:N 160:1 (10%) treatments. However, the C:N 5:1 treatment (19%) *versus* C:N 160:1 (10%) showed significant difference ($P = 0.05$) when stored at 50% RH. There was no significant difference in reduction of bioactivity after six months storage between the valid comparison treatments of 1.2 mM trehalose (36% reduction) *versus* 1.2 mM glycine-betaine (33%) at storage condition of 0% RH, 1.2 mM trehalose (46%) *versus* 1.2 mM glycine-betaine (44%) at storage condition of 50% RH, 1.2 mM trehalose (41%) *versus* 1.2 mM glycine-betaine (38%), across both storage conditions combined (0 and 50% RH). The percentage bioactivity of *T. atroviride LU132* colonies against *R. solani* colony growth at each of the five storage assessment times in each of the two storage conditions, and across both conditions combined, is shown in Appendix 3.3.

Table 3.5 Mean bioactivities (%) of conidia from *Trichoderma atroviride* LU132 colonies from different culture production treatments (see Table 3.1), against *Rhizoctonia solani* colnoy growth. Conidia were either fresh or stored for six months. Data presented are based on AUC[1] values and also reduction[2] (%) after drying (DE), storage (SE) and combined (DE+SE), under storage conditions of 0% RH, 50% RH and across both storage conditions combined (0 and 50% RH).

Treatment[3]	%Bio[4] (Fresh)	%DE	0% RH			50% RH			0% + 50% RH		
			%Bio	%SE	%(DE+SE)	%Bio	%SE	%(DE+SE)	%Bio	%SE	%(DE+SE)
20°C	74	23	30	100	100	25	100	100	28	100	100
30°C	77	6	68	13	12	64	15	16	66	14	19
a_w 0.985	68	12	59	18	28	53	33	32	56	25	34
a_w 0.961	74	19	56	27	36	52	39	45	54	33	45
C:N 5:1	77	4	65	15	11	64	19	17	65	17	20
C:N 160:1	61	−12	63	10	13	63	10	12	63	10	−1
1.2 mM Tre	75	7	57	36	40	52	46	45	55	41	45
1.2 mM GB	63	5	56	33	42	48	44	50	52	38	42
pH 5.5	69	9	50	53	58	41	61	63	46	57	61
pH 7.5	76	16	53	50	53	48	57	59	51	54	61
$LSD_{0.05}$	1.8	2.8	2.1	8.7	8.7	1.7	8.2	8.7	3.7	7.5	6.6
Overall mean	71	9	56	35	39	51	42	44	53	39	43
Significance of contrasts between valid comparisons[5]											
20°C *vs* 30°C	**	***	***	***	***	***	***	***	***	***	***
C:N 5:1 *vs* C:N 160:1	***	***	ns	ns	ns	ns	*	ns	ns	ns	***
1.2 mM Tre *vs* 1.2 mM GB	***	ns	ns	ns	ns	***	ns	ns	ns	ns	ns

[1] AUC: area under the curve for 22 h germination assessment.

[2] DE: % reduction due to drying effect, SE: % reduction due to storage effect over five assessment times, DE + SE: % reduction due to combination of drying and storage effects.

[3] Tre: trehalose, GB: glycine-betaine.

[4] Bio: bioactivity.

[5] *, **, ***: the effects of treatments are statistically significant at $P = 0.05$, 0.01 or 0.001, respectively; ns: not significant ($P > 0.05$).

3.4 Discussion

Results from the storage experiments described here showed that culture conditions during conidium production are important factors influencing numbers of conidia, germination and bioactivity of *T. atroviride* LU132. Furthermore, drying and storage conditions had considerable impacts on conidium fitness in this fungus.

The conidia produced at different temperatures (20 or 30°C), as one of the valid comparisons, showed highly significant differences in conidium fitness. High temperature of 30°C during production induced conidia to germinate rapidly and inhibit *R. solani* colony growth to a greater extent than the conidia produced at 20°C. This indicates that the lower temperature during conidium production did not produce conidia capable of resisting unfavourable storage conditions. Conidia that can resist unfavourable conditions may have physical attributes (*e.g.* strengthened cell walls, impervious cell membranes) or have accumulated beneficial compounds (*e.g.* trehalose), to allow them to resist drying and unfavourable storage conditions. The higher temperature of 30°C during conidium production may have caused water shortage and possibly enhanced conidium cell walls or membranes, and could also have induced accumulation of compatible solutes such as trehalose in the conidia. These factors may have been involved in acceleration of germination rate, enhanced bioactivity of conidia and tolerance to the adverse environmental conditions (Pedreschi *et al*., 1997; Bonaterra *et al*., 2005). In the present study, water shortage in the culture media during conidium production probably occurred following incubation at the high temperature (30°C), which may have exposed the conidia to oxidation. This type of culture conditioning may also have positively affected cell walls and membranes, and elevated polyol compounds, resulting in thermo-, osmo-, and oxidation-adapted conidia with reasonable bioactivity and viability. This is in spite of numbers of conidia being produced at the higher temperature being relatively low.

In experiments described in Chapter 2, of the C:N ratios tested, the ratio 160:1 gave the least bioactivity, and this result was similar with that obtained for fresh conidia tested initially in the storage experiment. Conidia from the C:N 160:1 treatment, however, when stored for six months, gave greater bioactivity with no significant difference from the with C:N 5:1 treatment at both of the relative humidity storage conditions (0 or 50% RH), and also across both of these conditions combined. *Trichoderma* conidia produced from the C:N 160:1 treatment had greater bioactivity than from all of the other treatments except 30°C. Furthermore, no significant differences in bioactivity occurred between the C:N 5:1 and C:N 160:1 treatments. This indicates that C:N ratio was not important as a factor affecting survival and bioactivity of *T. atroviride* LU132 during storage. Other studies have demonstrated that high levels of hydrocarbons in culture growing media can increase the viability of

conidia during storage. For example, Moore & Higgins (1997) showed that high C:N ratio in culture medium for the entomopathogenic fungus *Metarhizium flavoviride* gave greater viability during storage than for low C:N ratio. In contrast, Montazeri & Greaves (2002) showed that low C:N ratio for production of *Colletotrichum truncatum* gave conidia with more desiccation tolerance than those produced at high C:N ratio. These variable results indicate that this factor has variable effects for different fungi, and may differently affect different *Trichoderma* species. This response should be verified for *T. atroviride*, and should be carefully assessed when other species are considered.

The growth medium C:N ratio treatment of 5:1 gave high conidium production, germination and bioactivity for fresh *T. atroviride* LU132 conidia (see Chapter 2), and also stability and bioactivity after storage. Carbohydrate type and concentration, pH, and water availability have elsewhere been shown to be key factors determining the trehalose content of conidia during culturing conditions (Hallsworth & Magan, 1994). Trehalose and sucrose have also been shown to protect membranes and proteins in bacteria during drying (Leslie *et al*., 1995). In the present study, the 5:1 C:N ratio treatment probably provided an effective balance of C to N, and resulted in optimized polyol content (*e.g.* glycerol and erythritol) during conidium production. Storage at 30°C was not excessive for survival of conidia produced at the 5:1 C:N treatment, possibly due to the conidia having strong cell walls and membranes, and elevated contents of compatible solutes.

Conidia produced in cultures supplemented with 1.2 mM trehalose and 1.2 mM glycine-betaine had lower conidium fitness compared with the conidia produced at different C:N ratios. Although, the overall C:N ratio in the base medium for trehalose and glycine-betaine was the same as that in the 5:1 C:N treatment, these media contained 8.4 g/L of carbon, while the 5:1 C:N medium contained 4.2 g/L carbon. Other researchers have shown that addition of compatible solutes such as trehalose and glycine-betaine increases the intracellular accumulation of these compounds during the production of BCA inocula (Kets & Bonts, 1994; Kets *et al*., 1996; Pedreschi *et al*., 1997; Bonaterra *et al*., 2005). In the present study, there were no positive effects caused by either trehalose or glycine-betaine treatments on the quantity and quality of the conidia to withstand unfavourable environmental conditions during storage, with the exception that glycine-betaine addition increased germination after drying (%DE) compared with the fresh conidia (Table 3.4). This indicated that excessive sucrose as a carbon source possibly inhibited absorption of trehalose and glycine-betaine into the fungal cells during growth and development. These compounds did not improve conidium stability and bioactivity, possibly because they did not affect cell wall or membrane integrity, or accumulation of trehalose, polyols or glycine-betaine, so temperature of 30°C during storage was above the extreme threshold and therefore detrimental for these conidia.

In this study, conidia produced under different culture conditions were exposed to stresses of drying and storage, and these resulted in substantial decreases in the number, germination and bioactivity of conidia during six months of storage. Dry conditions (0% RH) were more suitable for storage of the conidia than storage at 50% RH. This indicates that drying induced water content during storage that was more optimal for maintenance of viability after long-term storage (Moore *et al*., 1995). It has also been reported that low water activity in the conidia of *Neurospora crassa* reduced the metabolic activity of dry conidia and maintained viability for long-term of storage (Fahey *et al*., 1978). In another example, the conidia of *Coniothyrium minitans* showed delayed germination after drying, but maximum germination was achieved similar to non-dried conidia (Jones *et al*., 2004).

In the present study, conidia were dried in a laminar flow cabinet under airflow for 24 h at room temperature. This time of dehydration was reasonably rapid, and could have affected conidium fitness. The rate of drying has been known to be a factor affecting the longevity of BCAs based fungal conidia. Viability of microorganisms during storage is generally influenced by drying processes (Antheunisse & Arkestelin-Dilksman, 1979), storage conditions (Mary et al., 1985), and the rehydration processes (Leach & Scott, 1959). Rapid drying has been shown to decrease conidium survival compared with slow drying (Hong *et al*., 2000). Furthermore, viability may be maintained by gradual drying processes compared with rapid drying (Friesen *et al*., 2005).

Rehydration of test conidia in the present study was done in water immediately at each assessment time. This rapid rehydration could be another factor affecting conidium fitness. The impact of rehydration has been studied in *Metarhizium anisopliae* by Moore *et al*. (1997). They found that rapid rehydration of conidia, where dried conidia were pre-soaked in water, gave much lower germination percentages than when the conidia were initially rehydrated over a saturated water atmosphere prior to placing in water. Similarly, Magalhães & Boucias (2004) showed that gradually rehydrated conidia of *Metarhizium anisopliae* gave greater germination percentages than those rehydrated more rapidly. In the present study, rapid drying, along with desiccation due to high temperature during storage (30°C) resulted in considerable reductions in the number of conidia, germination percentage and bioactivity against *R. solani*.

Dehydration, thermal stress and oxidation are principal factors which irreversibly affect conidium fitness, due to their ability to damage cellular membranes and structural integrity. These effects manifest as vesicle fusion, redistribution of inter-membrane particles, and phase transitions of phospholipid bilayers (Crowe *et al*., 1984; Aguilera & Karel, 1997; Ananta et al., 2005; Morgan *et al*., 2006; Fernandez-Sandoval *et al*., 2012). However, temperature has been reported to be more important than dehydration and oxidation for effects on longevity and bioactivity of *Beauveria brongniartii* and *Metarhizium anisopliae* during storage (Horaczek & Viernstein, 2004). Furthermore,

temperature fluctuations were reported to cause changes in membrane fluidity by causing protein denaturation, enzyme inactivation, and DNA and RNA breakage in *Trichoderma harzianum* (Fernandez-Sandoval *et al*., 2012).

In the present study, the conidia were exposed to headspace oxygen in desiccator containers. Miller *et al.* (1997) showed that oxygen is a principal requirement for stability of bioactivity of *Metarhizium anisopliae* conidia during long-term storage at room temperature. However, the headspace oxygen in the present study might not have been sufficient to support adequate metabolic activity of conidia during the storage period. These results indicate that suitable packaging, permeable to normal atmosphere gases, low permeability to moisture, and impermeable to external microorganisms, are likely to be required to give optimum conditions for *T. atroviride* LU132 conidia during storage.

In this study there was some delay in the initiation of germination of test conidia which increased over the storage period from 0 to six months (Figure 3.1). Unfavourable environmental conditions such as high temperature or exposure to UV radiation could cause irreversible physiological or genetic changes to retard conidium germination (Brags *et al*., 2006; Hsia *et al*., 2014). Kiewnick (2006) studied germ-tube elongation of *Paecilomyces lilacinus* under different temperatures, demonstrating that the germination percentage decreased with increasing temperature, and germination time doubled. In the present study, in addition to high storage temperature of 30°C, conidia could enter into dormancy as an immediate or gradual response to the stresses of drying. Delayed germination is likely to result in less effect of biocontrol activity of *Trichoderma*.

The results of this study have shown that environmental conditions are not independent factors as they are likely to be associated with changes in other factors. For example, during conidium production at 30°C, other factors probably influenced *T. atroviride* LU132, such as desiccation, oxidation and, possibly, rapid alteration in pH. Therefore, the conidia produced in different culture conditions may not have been presenting the direct effects of individual growth condition factors such as temperature, water activity, or pH. Extreme environmental conditions (*e.g.* temperature, a_w and pH) may improve the quality of conidia. However, with regarding the number of conidia for biocontrol inoculum production, using suitably balanced nutrient sources, particularly regarding the C:N ratio of growth media, is likely to give better conidium fitness than other adaptations aimed to alleviate extreme environmental conditions.

3.5 Summary of key results

- Conidium fitness of *T. atroviride* LU132 was maintained in dry conditions (0% RH) to a greater extent than in non-dry conditions, probably through reduced metabolic activity of conidia during long-term storage.

- *Trichoderma* conidia to be stored are affected by dehydration, temperature and other factors such as oxidation, before and during storage, and also by rehydration after storage.
- No significant effects on bioactivity were detected between the C:N 5:1 and C:N 160:1 conidium production treatments, indicating that C:N ratio in culture medium of *T. atroviride* LU132 was not important as a factor affecting conidium survival.
- Excessive sucrose in growth media probably inhibited absorption of trehalose and glycine-betaine, and affected conidium fitness.

3.6 References

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3.7 Appendices

Appendix 3.1 Mean number of *T. atroviride* LU132 conidia (×10^7/mL) produced from different culture production treatments (see Table 3.1) at each storage assessment time (0, 0.5, 1, 3 and six months) when stored at 30°C and 0% RH, 50% RH and across both storage conditions combined (0 and 50% RH).

Treatment[1]	Assessment time (month) at 0% RH					50% RH					0 and 50% RH				
	0	0.5	1	3	6	0	0.5	1	3	6	0	0.5	1	3	6
20°C	1530	1443	1233	873	840	1530	1330	1090	740	700	1530	1387	1162	807	770
30°C	1803	940	743	397	377	1803	1053	940	487	467	1803	997	842	442	422
a_w 0.985	1657	1143	963	600	573	1657	1297	1147	637	613	1657	1220	1055	618	593
a_w 0.961	1443	1443	1283	847	803	1443	1427	1270	700	677	1443	1435	1277	773	740
C:N 5:1	1670	1460	1333	1057	1007	1670	1353	1180	820	803	1670	1407	1257	938	905
C:N 160:1	1750	1527	1333	980	973	1750	1473	1320	887	873	1750	1500	1327	933	923
1.2 mM Tre	1517	1040	960	597	570	1517	1010	867	630	583	1517	1025	913	613	577
1.2 mM GB	1670	900	753	377	373	1670	927	750	553	547	1670	913	752	465	460
pH 5.5	1820	1403	1233	670	647	1820	1117	1003	567	560	1820	1260	1118	618	603
pH 7.5	1727	1143	903	617	617	1727	1347	1180	660	647	1727	1245	1042	638	632
$LSD_{0.05}$	111.1	64.2	72.9	33.4	85.2	111.1	64.5	70.3	39.6	65.8	111.1	97.4	112.1	84.4	88.7
Overall mean	1659	1244	1074	702	678	1659	1233	1075	668	647	1659	1239	1074	685	663
Significance of contrasts between valid comparisons[2]															
20°C *vs* 30°C	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
C:N 5:1 *vs* C:N 160:1	ns	*	ns	***	ns	ns	**	ns	**	*	ns	ns	ns	ns	ns
1.2 mM Tre *vs* 1.2 mM GB	**	***	***	***	***	**	*	**	***	ns	**	*	**	***	*

[1] Tre: trehalose, GB: glycine-betaine.

[2] *, **, ***: the effects of treatments are statistically significant at $P = 0.05$, 0.01 or 0.001, respectively; ns: not significant ($P > 0.05$).

Appendix 3.2 Mean percentage germination of *T. atroviride* LU132 conidia produced from different culture production treatments (see Table 3.1) at each storage assessment time (0, 0.5, 1, 3 and six months) when stored at 30°C and 0% RH, 50% RH and across both storage conditions combined (0 and 50% RH). Data is based on AUC (as described in Chapter 2, Section 2.2.1.7) averaged over 12 - 48 h germination assessments at each storage assessment time.

Treatment[1]	Assessment time (month) at 0% RH					50% RH					0 and 50% RH				
	0	**0.5**	**1**	**3**	**6**	**0**	**0.5**	**1**	**3**	**6**	**0**	**0.5**	**1**	**3**	**6**
20°C	93	70	0	0	0	93	42	0	0	0	93	56	0	0	0
30°C	91	51	73	48	26	91	42	54	47	4	91	47	63	47	15
a_w 0.985	62	28	21	13	11	62	38	24	13	1	62	33	23	13	6
a_w 0.961	91	69	62	25	8	91	34	58	18	3	91	52	60	22	6
C:N 5:1	93	42	73	60	33	93	30	67	35	24	93	36	70	47	28
C:N 160:1	75	58	53	47	21	75	50	62	63	15	75	54	57	55	18
1.2 mM Tre	92	71	65	38	10	92	37	64	26	0	92	54	65	32	5
1.2 mM GB	93	58	36	29	3	93	36	61	13	0	93	47	48	21	2
pH 5.5	86	47	36	5	1	86	31	28	14	0	86	39	32	9	1
pH 7.5	76	36	32	13	10	76	23	31	18	6	76	30	32	16	8
$LSD_{0.05}$	1.0	1.0	2.0	1.9	3.5	1.0	2.8	2.8	2.6	1.3	1	1.6	1.3	1.6	1.8
Overall mean	85	53	45	28	12	85	36	45	25	5	85	45	45	26	9
Significance of contrasts between valid comparisons[2]															
20°C *vs* 30°C	**	***	***	***	***	*	ns	***	***	***	*	ns	***	***	***
C:N 5:1 *vs* C:N 160:1	***	***	***	***	***	***	***	***	***	***	***	*	***	ns	***
1.2 mM Tre *vs* 1.2 mM GB	ns	***	***	***	***	ns	ns	**	***	**	ns	ns	***	**	ns

[1] Tre: trehalose, GB: glycine-betaine.

[2] *, **, ***: The effects of treatments are statistically significant at $P = 0.05$, 0.01 or 0.001, respectively; ns: not significant ($P > 0.05$).

Appendix 3.3 Mean inhibition proportions (%) of *Trichoderma atroviride* LU132 colonies from different culture production treatments (see Table 3.1) against *Rhizoctonia solani* colony growth at each storage assessment time (0, 0.5, 1, 3 and six months) when stored at 30°C and 0% RH, 50% RH and across both storage conditions combined (0 and 50% RH).

Treatment[1]	Assessment time (month) at 0% RH					50% RH					0 and 50% RH				
	0	0.5	1	3	6	0	0.5	1	3	6	0	0.5	1	3	6
20°C	57	69	47	29	0	57	71	44	18	0	57	70	46	24	0
30°C	73	72	70	69	63	73	73	64	63	62	73	73	67	66	63
a_w 0.985	59	68	69	57	49	59	59	62	53	40	59	63	66	55	44
a_w 0.961	61	69	64	55	44	61	68	64	51	37	61	69	64	53	41
C:N 5:1	74	71	59	67	63	74	72	65	64	60	74	72	62	66	62
C:N 160:1	68	71	59	65	61	68	69	64	61	61	68	70	61	63	61
1.2 mM Tre	69	74	64	57	44	69	68	66	49	38	69	71	65	53	41
1.2 mM GB	59	69	65	57	40	59	67	66	43	33	59	68	65	50	37
pH 5.5	63	70	67	49	29	63	66	54	37	24	63	68	61	43	27
pH 7.5	63	67	64	56	32	63	67	61	49	27	63	67	63	53	29
$LSD_{0.05}$	2.2	4.8	2.4	3.7	5.1	2.2	2.6	3.2	3.4	5.0	2.2	3.4	4.0	5.8	4.6
Overall mean	65	70	63	56	43	65	68	61	49	38	65	69	62	53	40
Significance of contrasts between valid comparisons[2]															
20°C *vs* 30°C	***	ns	***	***	***	***	ns	***	***	***	***	ns	***	***	***
C:N 5:1 *vs* C:N 160:1	***	ns	ns	ns	***	**	ns	ns	ns	ns	***	ns	ns	ns	ns
1.2 mM Tre *vs* 1.2 mM GB	***	ns	ns	ns	ns	***	ns	ns	**	ns	***	*	ns	ns	ns

[1] Tre: trehalose, GB: glycine-betaine.

[2] *, **, ***: The effects of treatments are statistically significant at $P = 0.05$, 0.01 or 0.001, respectively; ns: not significant ($P > 0.05$).

Chapter 4

Biological control activity of *Trichoderma atroviride* LU132 against *Rhizoctonia solani* in ryegrass; effects of different culturing conditions

4.1 Introduction

Trichoderma spp. are well recognized as biological control agents (BCAs) of soilborne plant pathogens, and have also been shown to promote plant growth in the absence of pathogens (Harman & Björkman, 1998; Harman, 2000; Harman *et al*., 2004b; Bae *et al*., 2009). *Trichoderma* fungi antagonise a wide range of soil-borne phytopathogenic organisms through competition for space and nutrients (Verma *et al*., 2007a), parasitism, and antibiosis (Harman, 2006; Mukherjee *et al*., 2008). *Trichoderma* spp. firstly use nutrients in soil but also need to reach sources of nutrients such as exudates from plant root systems (Whipps, 2001), which enables them to colonise root/soil interfaces and also endophytically enter roots and provide protection to host plants from pathogen infection (Harman, 2011). Rhizosphere colonisation has been widely studied but mostly for isolates that are known BCAs or plant growth promoters. Cripps-Guazzone (2014) showed that rhizosphere competence was widespread within the *Trichoderma* species she tested. From 11 *Trichoderma* species, 82% of 22 isolates were rhizosphere competent on sweetcorn in non-sterile soil. Further, Cripps-Guazzone showed that rhizosphere competence is probably specific between a particular *Trichoderma* isolate and plant species. Cripps-Guazzone also showed that *T. atroviride* LU132 was rhizosphere competent on ryegrass. Some *Trichoderma* species have been reported to live inside host plants, developing intra- and/or inter-cellular associations, and spend all or part of their life cycles entirely within plant tissues without causing symptoms of disease (Vega Fernando *et al*., 2010). These fungi can bring many benefits to their host plants, including improved photosynthetic efficiency, increased nutrient and water up-take, and tolerance to abiotic stresses (Singh *et al*., 2011).

Rhizoctonia solani is a soil-borne plant pathogen that is responsible for yield losses in a broad range of agricultural and horticultural crops (Adams, 1988). This fungus has detrimental effects on perennial ryegrass seedlings causing damping-off and root rot diseases (Smiley et al., 1993). *Trichoderma* spp. have been shown to be suppressive of diseases caused by *R. solani* in a wide range of crops grown under greenhouse conditions (Lewis & Lumsden, 2001). *Trichoderma atroviride* LU132 was shown to supress *R. solani* infection on ryegrass, with this biological control agent also shown to promote the growth of ryegrass in the absence of the pathogen (Kandula *et al.*, 2007). However, effects of inoculum

production conditions on conidium quality, both the ability of *Trichoderma* conidia to colonise and to maintain populations in host rhizospheres, and ability to promote plant growth and suppress soil-borne disease, have not been investigated. The study by Lynch *et al*. (1991) illustrated that a threshold level of *Trichoderma* was needed to give effective control of the *R. solani* and *Pythium ultimum* initiated damping-off in lettuce. However, Hohmann *et al*. (2011) showed that increased *Trichoderma* inoculum and rhizosphere colonisation may not always be beneficial. Conidium quality in *Trichoderma* may influence rhizosphere colonisation, since conidia are able germinate, grow and multiply in the rhizosphere, and this ability is potentially influenced by the incubation conditions and media composition during their production.

Most of the studies on biological control activity in *Trichoderma* spp. have focused on mycoparasitism of fungal plant pathogens. Application of *Trichoderma*-based BCAs into the soil can reduce disease severity and improve the growth of treated plants (Harman & Björkman, 1998; Bae *et al*., 2009). For example, *Trichoderma* can improve the growth of lettuce and suppress *R. solani* and *P. ultimum* damping-off (Lynch *et al*., 1991). It has been postulated that *Trichoderma* spp. promote plant growth in a manner similar to mycorrhizae through penetration of root tissue (Yedidia *et al*., 1999).

Trichoderma spp. are common soil saprophytes, opportunistic and avirulent plant symbionts, and are well-recognized potential BCAs of soilborne plant pathogens (Harman, 2000). They produce metabolites that enhance plant growth and resistance to biotic and abiotic stresses through rhizosphere competency and endophytic colonisation, and these responses are strain-specific characteristics mainly correlated with biological control activity (Hoyos-Carvajal *et al*., 2009b). However, for *Trichoderma stromaticum*, a mycoparasite of the cacao witches' broom pathogen *Moniliophthora perniciosa*, endophytic colonisation of shoots and roots induced resistance and growth promotion, but were not responsible for suppression of the pathogen (De Souza *et al*., 2008). Furthermore, four *Trichoderma* spp. showed colonization of above ground tissues of cacao trees (Bailey *et al*., 2009). They were mostly re-isolated from stems (xylem and bark) and apical meristems rather than leaves. Glandular trichomes were also colonized by *Trichoderma* spp., where they formed appressorium-like bodies during stem colonization. In another example, *T. atroviride* was reported as an endophyte in the roots of the medicinal herb *Salvia miltiorrhiza* (Ming *et al*., 2012).

In the previous sections of the present study, *T. atroviride* LU132 fresh or dry conidia obtained from different culture growing conditions were assessed (during six months of storage) for quantity and quality (two measures; germination and subsequent bioactivity against *Rhizoctonia solani*). The aim of the experiments described here was to explore the effects of culture growing conditions on the biological control activity of *T. atroviride* LU132 conidia for protection of ryegrass against the soilborne

pathogen, *Rhizoctonia solani* in pot experiments. This research also attempted to detect any plant growth promotion effects in controlled soil environment experiments.

In Chapter 3 of the present study, it was found that *T. atroviride* LU132 conidia produced at high temperature of 30°C or at a C to N ratio of 5:1 maintained optimum viability and bioactivity after storage. Interaction effects of temperatures (20 or 30°C) and sugars (dextrose, 4.2 g/L or sucrose, 4.2 g/L at a constant C:N ratio of 5:1) during conidium production on the subsequent bioactivity of *T. atroviride* LU132 conidia were detected in pot experiments in the presence of *R. solani*. Here, the effects of *T. atroviride* LU132 conidium growth conditions on ryegrass plant growth parameters, and rhizosphere and endophytic colonization in presence or absence of *R. solani* were examined. The previous experiments (Chapters 2 and 3) were performed under controlled conditions, and *T. atroviride* LU132 colonies directly inhibited *R. solani* growth and development on dual inoculated agar plates. To enable the direct effect of *T. atroviride* LU132 on both the pathogen and ryegrass host plants to be evaluated, pasteurised potting mix was used as the growing media which limited microbial competition.

4.2 Plant growth promotion experiment

4.2.1 Materials and methods

4.2.1.1 Fungal cultures and inoculum preparation

Cultures of *T. atroviride* LU132 and *R. solani* (strain R73-13b) were obtained as previously described in Section 2.2.1.1. *Rhizoctonia solani* was inoculated from the main stock culture onto PDA and incubated at 22°C for 7 d and used to inoculate the substrate for mass production. A modified method of Paula Junior *et al*. (2007) was used for production of *R. solani* inoculum. Dry parboiled rice grains (Uncle Ben's®) were immersed in water for 24 h then washed and drained. In a 250 mL Erlenmeyer flask, 50 g of the parboiled rice was autoclaved twice for 20 min at 121°C and 205 kPa and cooled overnight at room temperature. Each flask received two mycelial agar discs (5 mm diam.) of *R. solani* cut from the outer margin of a colony growing on a PDA plate. The flasks were then incubated in the dark at 22°C for 7 d. The evenly colonized grains were then dried in a laminar flow cabinet at room temperature for 48 h.

For production of *T. atroviride* LU132 colonies, a defined basal medium containing a specific amount of carbon and nitrogen was used, which contained: 1 g/L of K_2HPO_4, 500 mg/L of KCL, 500 mg/L of $MgSO_4$, and 10 mg/L of $FeSO_4$. Carbon concentration was adjusted with dextrose (40% C) or sucrose (42% C) to 4.2 g/L. Nitrogen concentration was adjusted with soy peptone (8% N) to 0.84 g/L. The combination of C and N concentration resulted in a C:N ratio of 5:1, the ratio determined from

experiments outlined in Chapter 3 to be suitable for producing high quality conidia. Preparation of media cultures and inoculation with *T. atroviride* LU132 conidia were carried out as described in Section 2.3.2.2. Inoculated plates were incubated at 20°C or 30°C in incubators for 15 d under constant light. Treatments included conidia produced at 20°C, amended with dextrose (20°C-Dex) or sucrose (20°C-Suc), and conidia produced at 30°C, amended with dextrose (30°C-Dex) or sucrose (30°C-Suc). Ten replicate Petri plates for each of these treatments were set up. Conidia for the subsequent experiments were harvested as described in Section 2.2.1.2 and diluted in water to a concentration of 2×10^7 conidia/mL based on haemocytometer counts.

4.2.1.2 Pot experiment

A potting mix containing 600 L Southland peat and 400 L sterilized pumice was used for this experiment, with the following fertilizer additions: 1.0 kg Osmocote Exact Mini™ (16% N, 3.5% P, 9.1% K), 4.0 kg dolomite lime, and 1.0 kg Hydroflo® per m^3. The potting mix was first pasteurised in autoclave bags at 81°C, 184 kPa for 1 h. The potting mix was either left un-inoculated (as experimental controls) or inoculated with *R. solani* (Rs) at three concentrations (Rs1 = 0.04%, Rs2 = 0.4% and Rs3 = 4% w/w) and incubated at 22°C for 24 h. For each of the *T. atroviride* LU132 conidium production treatments, conidium suspension (2×10^7 conidia/mL) were applied to separate batches of non-inoculated or *R. solani*-inoculated potting mix to obtain a final inoculum concentration of 2×10^6 conidia/g of potting mix. The inoculated potting mix was mixed thoroughly, moisture content was determined gravimetrically and adjusted to 40% of the maximum water-holding capacity (Wk_{max}). Twenty seeds of perennial ryegrass (*Lolium perenne* cv. Trojan, free of endophyte and fungicide) were sown to a depth of 1 cm in each 8 cm diam. sterilised plastic pot (0.5 L capacity) containing 450 g potting mix, either pre-inoculated with *T. atroviride* LU132 conidia, with *R. solani* mycelial fragments on rice grains, both *T. atroviride* LU132 test conidia and *R. solani* mycelial fragments, or non-inoculated. Potting mix inoculated with *T. atroviride* LU132 and non-inoculated (negative controls) received sterilized rice grains at the same rate as used pots inoculated with *R. solani*. Ten replicates were set up for each treatment. Pots were kept in a glasshouse with mean temperature of 22°C (±2°C) and watered daily.

4.2.1.3 Assessments

The effects of *T. atroviride* and/or *R. solani* treatments on ryegrass seedling emergence were determined by counting the number of emerged seedlings at 5, 6, 7, 10 and 15 d after sowing. Numbers of tillers from three plants per pot were counted after 15, 20 and 35 d after sowing. Seedling emergence and number of tillers were averaged over time based upon the AUC for the recorded data. To determine the effect of *T. atroviride* LU132 conidium treatments on ryegrass growth, the ryegrass

plants were harvested at 42 d after sowing. Shoot height was measured from the surface of the potting mix, then the plants were removed, washed under running tap water, separated into roots and shoots, and these were weighed separately. Ryegrass roots were scanned using a scanner (WinRhizo, Regent Instrument Inc.) to determine the length and the number of lateral roots of each plant. Root and shoot samples were dried in an oven at 65°C for 2 d, and the dry weights were then recorded.

4.2.1.4 Experimental design and statistical analyses

This experiment was set up as a randomised complete block design in ten blocks (replicates). In each block there were 20 pots, one for each of the 20 treatments, including: one pot for each of the four *T. atroviride* LU132 conidium production treatments, one pot pre-inoculated with each of the three inoculation rates of *R. solani*, one pot for each of the three *R. solani* inoculum rates and four *T. atroviride* LU132 conidia treatments combinations, and one pot as a negative control (non-inoculated). Data for ryegrass growth parameters were statistically analysed using analysis of variance (ANOVA) in GenStat® 16th Edition (VSN International Ltd). Differences between treatment means (multiple comparisons) were determined using unprotected least significant differences (LSDs) at $P = 0.05$. The experiment was performed once.

4.2.2 Results

The growth conditions used to produce *T. atroviride* LU132 conidia varied in their effect on ryegrass growth and ability of the conidia to reduce the effects of *R. solani* when subsequently applied in the pot experiment. The effects of *T. atroviride* LU132 treatments against *R. solani* were evident from the differences between plants in the presence or absence of the biological control agent (Figure 4.1). There was a significant effect ($P < 0.001$) of *T. atroviride* LU132 production treatments on all plant growth parameters in the presence or absence of different concentrations of *R. solani* inoculum (Table 4.1). Conidia produced from colonies grown at 30°C with sucrose had no significant effects on seedling emergence, numbers of tillers or total plant fresh or dry weights, but significantly reduced root length and the number of lateral roots compared with the non-inoculated control (Table 4.1). In contrast, conidia from colonies produced at 20°C and sucrose significantly increased all plant parameters apart from total dry weight. *Trichoderma atroviride* LU132 conidia from colonies from 30°C and dextrose had no effects on most plant growth parameters apart from increasing seedling emergence and total fresh weight. Plants inoculated with different concentrations of the pathogen gave significant differences in growth parameters compared with non-inoculated plants, and also for plants inoculated with individual *T. atroviride* LU132 conidium production treatments. At the lowest concentration of the pathogen (Rs1), seedling emergence, root length and number of lateral roots were reduced compared with the non-inoculated controls, while number of tillers, and plant fresh and dry weights

were not significantly different from the non-inoculated plants. *Trichoderma atroviride* LU132 conidium treatments in the presence of the lowest pathogen concentration (Rs1) increased seedling emergence compared with the pots inoculated with Rs2 and Rs3 concentrations, but was not significantly different from the non-inoculated pots. Increasing *R. solani* inoculum concentration significantly reduced all growth parameters compared with the non-inoculated plants. Plants co-inoculated with *T. atroviride* LU132 treatments and a high concentration of the pathogen (Rs3) had increased parameters compared with those inoculated only with Rs3.

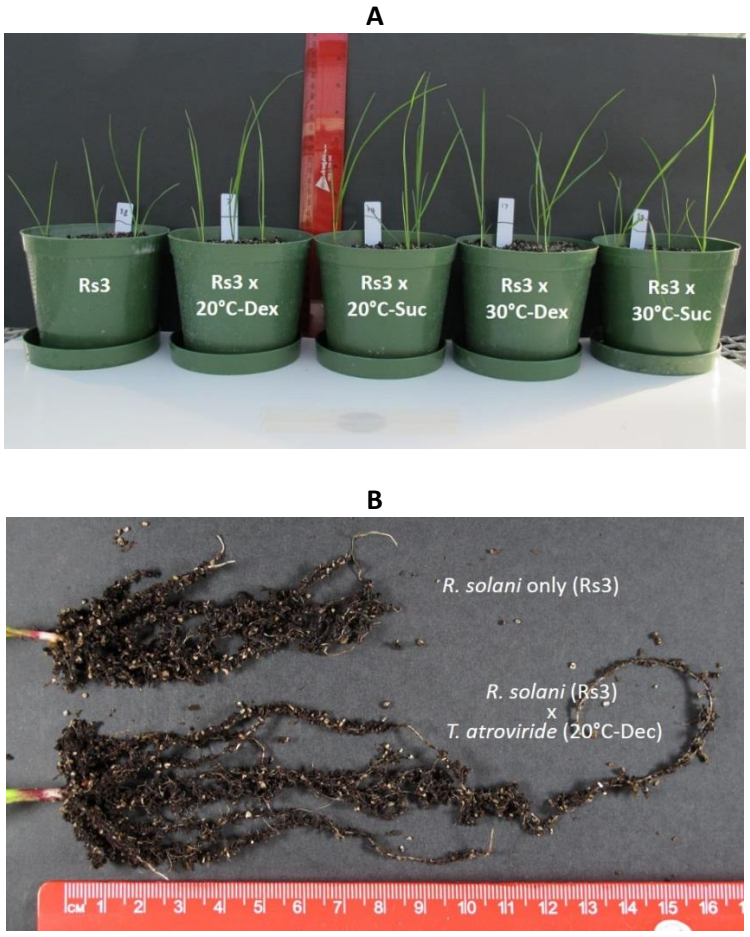


Figure 4.1 Ryegrass plants (A: shoots and B: roots) inoculated with high concentrations of *Rhizoctonia solani* (Rs3) inocula (4% w/w) per pot in the presence or absence of *Trichoderma atroviride* LU132 treatments 28 d after sowing. *Trichoderma atroviride* conidia for potting mix inoculations were produced at 20°C or 30°C on media amended with sucrose (20°C-Suc & 30°C-Suc) or dextrose (20°C-Dex & 30°C-Dex), at C:N ratios of 5:1.

Table 4.1 Mean seedling emergence and growth parameters of ryegrass plants inoculated with or without *Trichoderma atroviride* LU132 conidia produced under different incubation temperatures (20°C or 30°C) and media emended with dextrose (Dex) or sucrose (Suc), and inoculated with different concentrations of *Rhizoctonia solani* (Rs).

Treatment[1]	Seedling emergence[2] %	No. of tillers[3]	Total fresh weight[4]/g	Total dry weight[4]/g	Root length[5] (cm)	No. of lateral roots[5]
Nil	83	20	7.9	0.76	1397	8712
20°C-Dex	88	22	9.2	0.83	1224	6924
20°C-Suc	89	23	8.8	0.84	1526	10241
30°C-Dex	88	20	9.2	0.84	1443	8494
30°C-Suc	87	23	9.4	0.86	1259	7652
Rs1	65	19	7.1	0.73	1133	6453
Rs2	56	17	5.6	0.59	1020	6169
Rs3	43	10	0.8	0.09	297	1570
Rs1 × 20°C-Dex	79	18	7.2	0.67	1147	6761
Rs1 × 20°C-Suc	81	23	7.9	0.76	1269	7286
Rs1 × 30°C-Dex	82	21	7.7	0.72	1265	6563
Rs1 × 30°C-Suc	80	20	7.7	0.74	1336	7406
Rs2 × 20°C-Dex	65	19	8.1	0.73	1199	6931
Rs2 × 20°C-Suc	63	18	7.2	0.69	1095	6464
Rs2 × 30°C-Dex	67	17	7.4	0.70	1121	6502
Rs2 × 30°C-Suc	61	16	6.9	0.63	1161	7133
Rs3 × 20°C-Dex	53	12	2.9	0.27	559	3121
Rs3 × 20°C-Suc	76	16	2.9	0.28	523	2894
Rs3 × 30°C-Dex	81	16	2.7	0.28	504	2925
Rs3 × 30°C-Suc	60	13	2.7	0.26	440	2617
$LSD_{0.05}$	**4**	**0.7**	**0.8**	**0.08**	**116.5**	**935**

[1] Rs1 = 0.04%, Rs2 = 0.4% and Rs3 = 4% w/w of rice grains inoculated with *Rhizoctonia solani* added to potting mix (PM); *T. atroviride* LU132 conidia for PM inoculation were produced at 20°C or 30°C amended with sucrose (20°C-Suc & 30°C-Suc) or dextrose (20°C-Dex & 30°C-Dex) at C:N ratio of 5:1.

[2] Average seedling emergence (from up to 20 plants) over 5, 6, 7, 10 and 15 DAS using AUC.

[3] Average number of tillers (from up to 20 plants) over 15, 20 and 35 DAS using AUC.

[4] Total weight of roots and shoots (mean of three plants).

[5] Root length and number of lateral roots (mean of three plants).

4.3 Rhizosphere colonisation experiment

4.3.1 Materials and methods

4.3.1.1 Fungal cultures and inoculum preparation

Cultures of *T. atroviride* LU132 and *Rhizoctonia solani* (R73-13b) were obtained following the methods described in Chapter 2 Section 2.2.1.1. *Trichoderma atroviride* LU132 colonies produced at 20°C or 30°C and on media amended with dextrose or sucrose (20°C-Dex, 20°C-Suc, 30°C-Dex, 30°C-Suc) were used in this study. Inoculum preparation for *T. atroviride* LU132 and *R. solani* were the same as described in Section 4.2.1.1. *Rhizoctonia solani* at the concentration of 0.4% w/w (see above) was used as pathogen inoculum in this study. The *T. atroviride* LU132 inoculated pots (no *R. solani*), and the non-inoculated negative control pots received sterilised rice grains at the same rate (0.4%) as for pots inoculated with *R. solani*. For the *R. solani* only and non-inoculated controls, SDW was used instead of *T. atroviride* LU132 conidium suspensions.

4.3.1.2 Pot experiment

Twenty seeds of perennial ryegrass were sown to a depth of 1 cm in each 8 cm diam. sterilised plastic pot (0.5 L capacity) containing 450 g potting mix. The growing conditions in the glasshouse, planting of ryegrass seeds, potting mix preparation, and watering regime were the same as described above (Section 4.2.1.2). Seedlings in pots were thinned to three after 7 d and again after 14 d. This was achieved by dividing each pot into three sectors (at an angle of 120° each), then randomly removing seedlings in each sector (taking no account of seedling size to not bias results).

4.3.1.2 Assessments

At different intervals (0, 7, 14, 21, 28, 35, 42 and 49 d after sowing seeds) the *T. atroviride* LU132 population in the ryegrass rhizosphere and bulk potting mix were investigated. Dry weight of potting mix was determined gravimetrically for each pot separately. At each assessment time, three plants per pot were carefully removed and shaken gently. To collect the rhizosphere potting mix, roots with adhering potting mix were suspended in 10 mL of sterile distilled water (SDW plus 0.5% Triton X100) and shaken on a flash shaker (Stuart® Bibbs Scientific Ltd) for 30 min at 500 oscillations/min at room temperature. The remaining potting mix (defined as bulk soil) from each pot was mixed thoroughly, and a 1 g sample was suspended in 10 mL SDW plus Triton X100 and treated as previously described for rhizosphere potting mix. Serial ten-fold dilutions were carried out to 10^{-6}. For population estimation, the serial dilution plating method was used to quantify and monitor *T. atroviride* LU132 (Elad *et al*., 1981). For plating, 100 mL aliquots of each dilution from 10^{-4} to 10^{-6} were spread onto triplicate Petri plates containing *Trichoderma* selective medium (TSM; Appendix A), for each rhizosphere or bulk potting mix per pot. The recipe for TSM was a modified version of the medium

initially developed by Elad *et al*. (1981), modified by Elad & Chet (1983) and modified again by Askew & Laing (1993). Plates were incubated in the dark at 20°C for 14 d. *Trichoderma* colonies were counted from plates containing 30–100 colonies. Dry weight of potting mix was determined gravimetrically for each pot separately. Data were expressed as the number of colony forming units (CFUs)/g of dry potting mix.

4.3.1.3 Experimental design and statistical analyses

This experiment was set up as a randomised complete block design in four blocks (replicates) containing 80 pots per block (ten treatments × eight assessment times). Treatments in each block were as follows: one pot for each of the four *T. atroviride* LU132 conidium production treatments, one pot pre-inoculated with *R. solani* (0.4% w/w), one pot for each of the four *T. atroviride* LU132 conidium production treatments into potting mix pre-inoculated with *R. solani* (0.4% w/w), and one non-inoculated pot as a negative control. *Trichoderma* CFU data were $\log_{10}$ transformed prior to analysis to satisfy the assumption of normality for ANOVA. Data were then statistically analysed using ANOVA in GenStat® 16th Edition. Differences between treatment means were determined using unprotected LSDs at $P = 0.05$. The experiment was performed once.

4.3.2 Results

The *Trichoderma* populations recovered from the non-inoculated (control) bulk and rhizosphere potting mix were very low (< 10^3 CFU/g potting mix, 49 d after sowing), and markedly less than populations where the *T. atroviride* LU132 conidium treatments had been applied. The species of *Trichoderma* in these populations were not identified. The non-inoculated controls were therefore omitted from the statistical analyses to avoid violating the assumption of homogeneity of variances across treatments. The *Trichoderma* population increased significantly with time ($P < 0.001$), in both the rhizosphere and bulk potting mix. Log_{10} and back-transformed population data are presented in Appendices 4.1 and 4.2. The *Trichoderma* CFUs/g of dry potting mix over time in both the rhizosphere and bulk potting mix followed similar trends, either inoculated individually or in interaction with *R. solani* (Figure 4.2). The mean number of CFUs in the rhizosphere soil 7 d after sowing was significantly greater for plants treated with conidia from *T. atroviride* LU132 from colonies produced at 20°C with dextrose, 30°C with dextrose or 30°C with sucrose when inoculated into potting mix alone compared with when co-inoculated with the pathogen, *R. solani* (Appendix 4.1). The numbers of CFUs in the rhizosphere soil from 14 to 49 d after sowing were significantly greater in non *R. solani* inoculated treatments than treatments co-inoculated with *R. solani*. Furthermore, the overall mean CFUs were greater in the rhizosphere soil compared with bulk potting mix for all *T. atroviride* LU132 conidium production conditions (See Appendices 4.1 & 4.2). In both rhizosphere and bulk potting mix, the mean

number of *Trichoderma* CFUs/g dry potting mix in the pots inoculated with conidia produced at 30°C with sucrose was greater than for the other *T. atroviride* LU132 conidium production treatments, up to 14 d after sowing. Thereafter, the populations were constantly less than those for the other *T. atroviride* LU132 production treatments up to 49 d after sowing. In the presence or absence of the pathogen, two peaks in *Trichoderma* populations were observed, occurring at 14 d and up to a maximum at 35 d after sowing. *Trichoderma* isolates characteristic of *T. atroviride* LU132 recovered after 14 d on TSM from the 20°C-Dex and 30°C-Suc treatments produced faster growing colonies, with these colonies maturing more quickly (as evidenced by dark green conidium colour) compared with those recovered from the 20°C-Suc and 30°C-Dex treatments, at all assessment times of 7, 14, 21, 28, 35, 42 or 49 d after sowing (Figure 4.3).

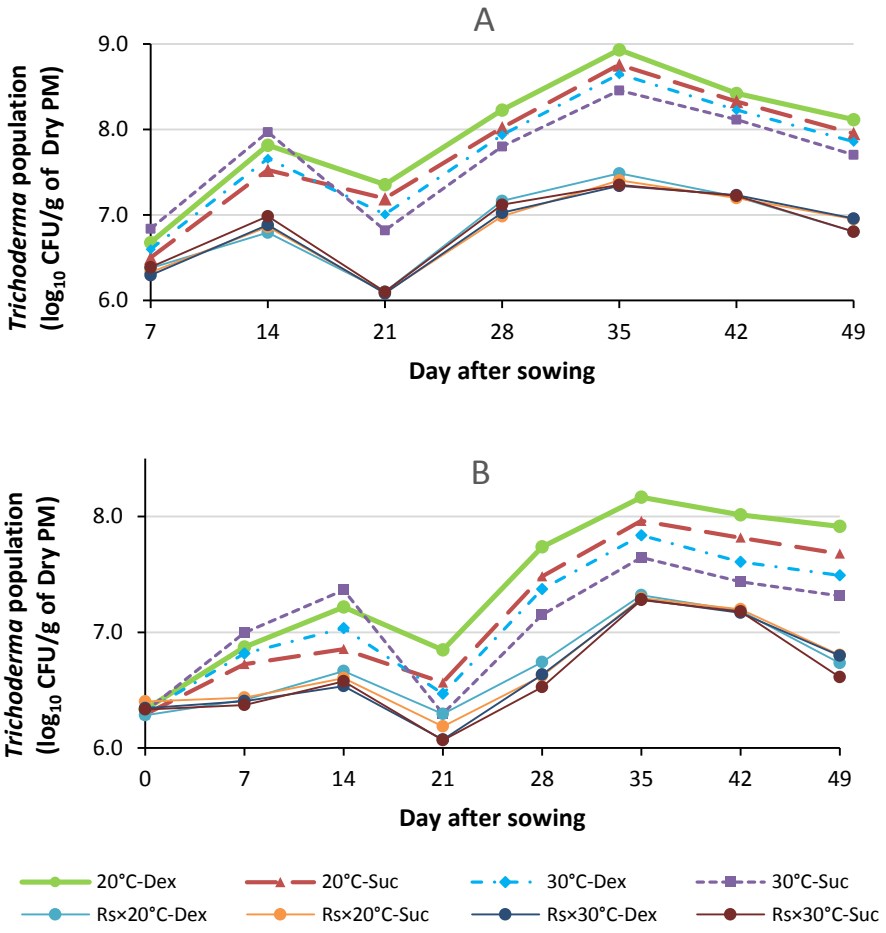


Figure 4.2 Mean *Trichoderma atroviride* LU132 populations recovered from A) the rhizosphere of ryegrass plants and B) the bulk potting mix (PM) over time in the presence or absence of *Rhizoctonia solani* (Rs). *T. atroviride* LU132 conidia for inoculation were produced at 20°C or 30°C on media amended with sucrose (Suc) or dextrose (Dex) at C:N ratio of 5:1. Rs inoculation was at a ratio of 0.4 g/L (w/w) potting mix. $LSD_{0.05}$ = 0.15 for rhizosphere soil, and = 0.14 for bulk soil.

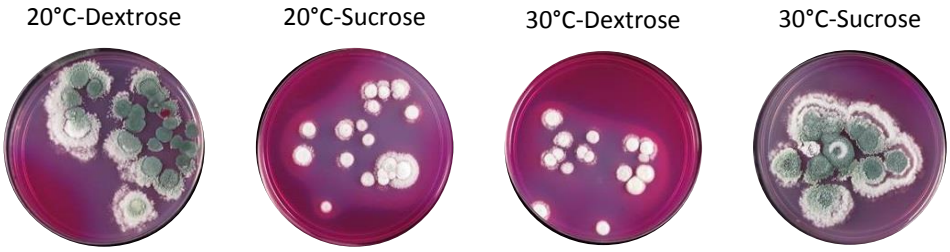


Figure 4.3 Appearance of 14-d-old *Trichoderma* colonies characteristic of *Trichoderma atroviride* LU132 on TSM recovered from potting mix amended with conidia produced from colonies incubated at 20°C or 30°C and on media amended with either dextrose or sucrose.

4.4 Endophytic colonisation assessment

4.4.1 Materials and methods

4.4.1.1 Plant material

Ryegrass plants (non-inoculated experimental controls or inoculated with *T. atroviride* LU132 conidia from colonies grown under different environmental conditions) grown and harvested for the *Trichoderma* colonisation study (Section 4.3) were used to assess endophytic colonization by *T. atroviride* LU132.

4.4.1.2 Assessments

At different intervals (0, 7, 14, 21 or 28 d after sowing), three plants for each of the *T. atroviride* LU132 conidium production treatments and the non-inoculated controls were washed carefully under running water. Surface sterilization of the plant tissues was done in a laminar flow cabinet. Plant tissues including roots, stems and leaves (3 cm from leaf bases) were cut into 3 cm long segments then immersed in 15 g/L sodium hypochlorite in water for 4 min (leaves) or 5 min (stems and roots), followed by a 1 min wash in 70% ethanol and three 1 min washes in SDW (McKinoon, 2012). To check the efficacy of the surface sterilisation method, 100 µL of the water from the last washing of each sample was spread onto TSM plates. The surface sterilised plant segments were dried on sterile filter paper in a laminar flow cabinet and cut into 0.5 cm long segments. The surface sterilised segments of roots, stems or leaves (ten pieces from each per plate) were transferred onto Petri plates containing TSM. Plates were sealed and incubated in the dark at 20°C for two weeks. *Trichoderma* colonies which grew from the plant segments on these plates were presumed to be endophytic (Marshall *et al*., 1999). *Trichoderma* colonies recovered from plant samples on TSM plates were purified by single conidium isolation. From each treatment, single colonies were selected for molecular identification.

To photograph the presence of endophytes in plant tissues, plant roots, stems and leaves were stained using rose Bengal (0.5% in 5% ethanol) or trypan blue (0.05% in lactophenol), and a minimum of three microscope slides (up to ten) were prepared from each plant part for each treatment.

4.4.1.3 Molecular identification

Fungal genomic DNA was isolated from 3-d-old growing mycelial colonies, obtained from the plant material, and also a 3-d-old *T. atroviride* LU132 colony sub-cultured from the main stock culture stored at −80°C as an identification control. The fungal genomic DNA was extracted using a genom'ic DNA isolation kit (QIAGENE® Gentra Puregene) following manufacturer's instructions. The extracted DNA was used to amplify the translation-elongation factor 1-α (*tef*1) gene which was then sequenced. *Tef*1

was selected because this gene contains introns which gives greater resolution at species level than the internal transcribed spacers (Chaverri & Samuels, 2003). The amplification was performed as follows: a 0.9 kb fragment of the 5′ end of the translation elongation factor 1-α (*tef*) gene (eEF1α1) containing three introns was amplified using the primer pair *tef*71f (5′– C AAA ATG GGT AAG GAG GAS AAG AC) and *tef*997R (CA GTA CCG GCR GCR ATR ATS AG – 3′) and the following 'touchdown' amplification protocol: 4 min initial denaturation at 95°C, four cycles each of 1 min at 95°C, 90 s at 70°C, and 90 s at 72°C, followed by 26 cycles with the annealing temperature decreasing by 0.5˚C per cycle from 68˚C to 55˚C, followed by 12 cycles with annealing at 55˚C, and with a final extension period of 7 min at 72°C (Hoyos-Carvajal *et al*., 2009a). The following internal sequencing primers were employed for *tef*1: *tef*85f (5′– AG GAC AAG ACT CAC ATC AAC G) and *tef*954r (AGT ACC AGT GAT CAT GTT CTT G – 3′) (Hoyos-Carvajal *et al*., 2009a). Sequencing reactions were prepared using the ABI Prism® BigDye™ Terminator reaction kit (v2.0, Applied Biosystems) in 5 µL volume and 1/8 dilution using 1/2 BigDye (BioCan Scientific). The cycle sequencing reaction contained the following mix: 1.0 µL BigDye, 1.0 µL 1/2 BigDye, 0.4 µL of 5 µM primer, 1.6 µL SDW, 1.0 µL (10-40 ng) PCR template, and employed the following amplification protocol: 40 cycles each of 30 s denaturation at 96°C, 15 s annealing at 50°C, and 3 min extension at 60°C. Purified PCR products of *tef1* were subjected to automatic sequencing using an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Sequences were trimmed manually by deletion of some base pairs from the beginning and the end of sequences (to reduce noise) and compared with the sequences of the positive control and original isolate held in the Bio-Protection Research Centre database (Lincoln University, New Zealand), using Standard Nucleotide BLAST on the NCBI web site.

4.4.1.4 Seedling culture and microscope examination of plants

Ryegrass seeds were surface sterilised as described above (Section 4.4.1.2) but immersion time in sodium hypochlorite solution bleach was increased to a maximum of 10 min. To confirm the effectiveness of the surface sterilisation, 100 µL of final water washing from the seed surface sterilisation was inoculated onto TSM in Petri plates. Sterile tissue culture containers (500 mL capacity) with screw caps were filled with 100 mL water agar (1%, Scharlau) then inoculated with 100 µL fresh conidium suspension of *T. atroviride* LU132 (20°C-Dex) at 2×10^5/mL, and mixed thoroughly with a sterile spatula. Non-inoculated controls were set up whereby 100 µL of SDW was mixed as described for the inoculated treatments. The surface sterilised seeds (20 per container) were placed on the surface of the non-inoculated or inoculated agar containers. Three containers each were set up for the non-inoculated or *T. atroviride* LU132-inoculated treatments. The containers were sealed and incubated at 22°C under a 12 h light/dark regime for four weeks. Plants were then harvested and roots, stem sheaths and leaves were stained with rose Bengal in ethanol or trypan blue in lactophenol.

At least three microscopic slides (up to ten) were prepared from each plant part (root, stem or leaf) and the presence of endophytes in plant tissues was assessed microscopically, and photomicrographs were taken where appropriate.

4.4.2 Results

No fungi grew on TSM from the final washes in the surface sterilisation process, which confirmed the efficiency of the sterilisation method. *Trichoderma* colonies characteristic of *T. atroviride* LU132 were recovered on TSM agar plates from surface sterilised segments of roots (14 d after sowing), stems (21 d after sowing) and leaves (28 d after sowing) of inoculated plants from the four *T. atroviride* LU132 production treatments from pot samples. No *Trichoderma* colonies were observed to grow endophytically from non-inoculated plant samples.

The primer pair *tef*71f and *tef*997R amplified a 926 bp region of the genomic DNA from pure *Trichoderma* cultures characteristic of *T. atroviride* LU132, isolated from surface sterilised plant segments on TSM agar plates (Figure 4.4). Sequencing of the *tef*1 gene revealed a high level of similarity (100%) at the species level with the positive identification control (*Trichoderma atroviride* LU132).

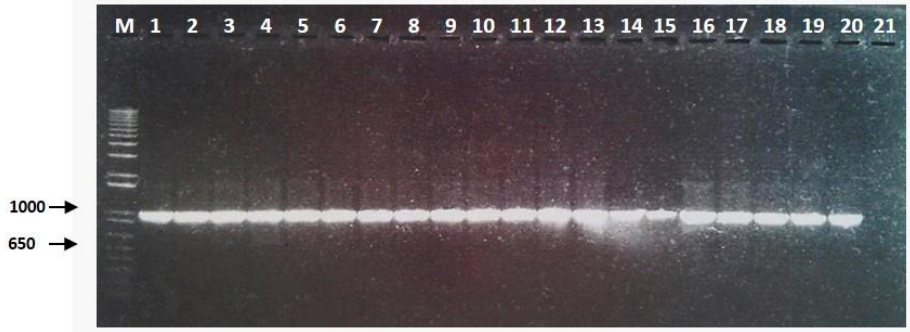
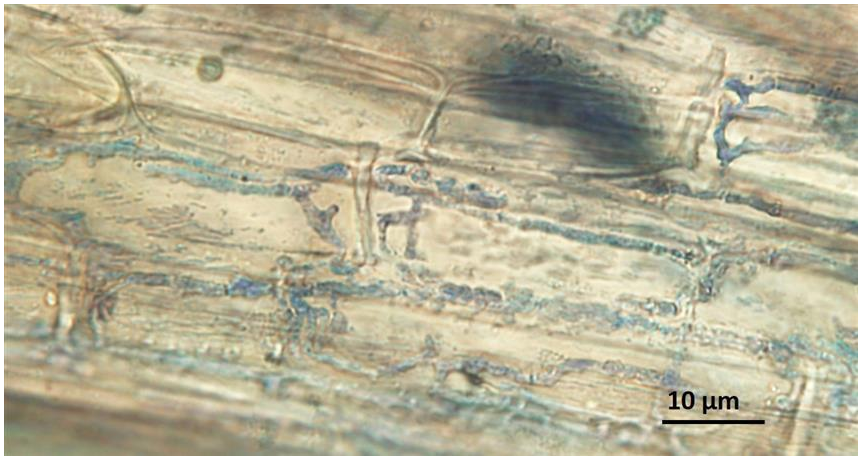


Figure 4.4 PCR amplification of genomic DNA of *Trichoderma* isolates using the *tef*71f and *tef*997R primer set. Lanes: M: molecular size marker (1 Kb Plus DNA Ladder, Invitrogen™, Life Technologies™), 1 - 6: isolates from ryegrass roots, 7 - 12: isolates from stems, 13 - 19: isolates from leaves, 20: positive *T. atroviride* LU132 control (obtained from the Biocontrol Microbial Culture Collection, Bio-Protection Research Centre, Lincoln University, New Zealand), 21: negative control (pure water). Amplified fragments were analysed by electrophoresis in a 1% agarose gel. The expected length of amplified fragments was 926 bp.

Microscope examination revealed fungal structures in root and stem sheath tissues of plants grown in pots (Figure 4.5) and on agar in tissue culture containers (Figure 4.6). No signs of fungi were seen in the non-inoculated plants (negative controls). Fungal hyphae were aligned both along and across host cells, and were frequently branched, lobed and formed infection bodies (haustorium-like structures, Figure 4.5C) inside the cells of plant tissues. Typical *Trichoderma* conidia and conidiophores were observed in some stem sheath samples from tissue culture plants (Figure 4.6B and C).

A



B

C

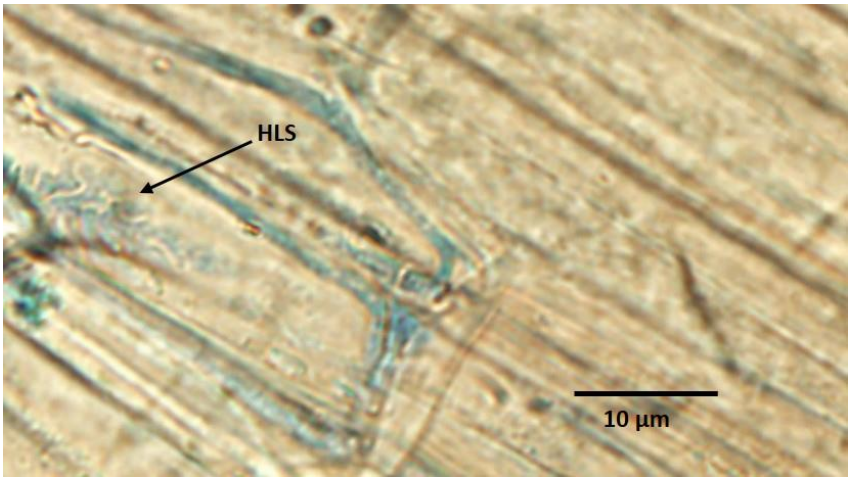


Figure 4.5 Micrographs of ryegrass plant tissued from seedlings grown in potting mix inoculated with *Trichoderma atroviride* LU132, 21 d after sowing. A: Fungal mycelium observed in a ryegrass root. B: Fungal mycelium observed in a ryegrass stem sheath. C: Haustorium-like structures (HLS) in a ryegrass stem sheath.

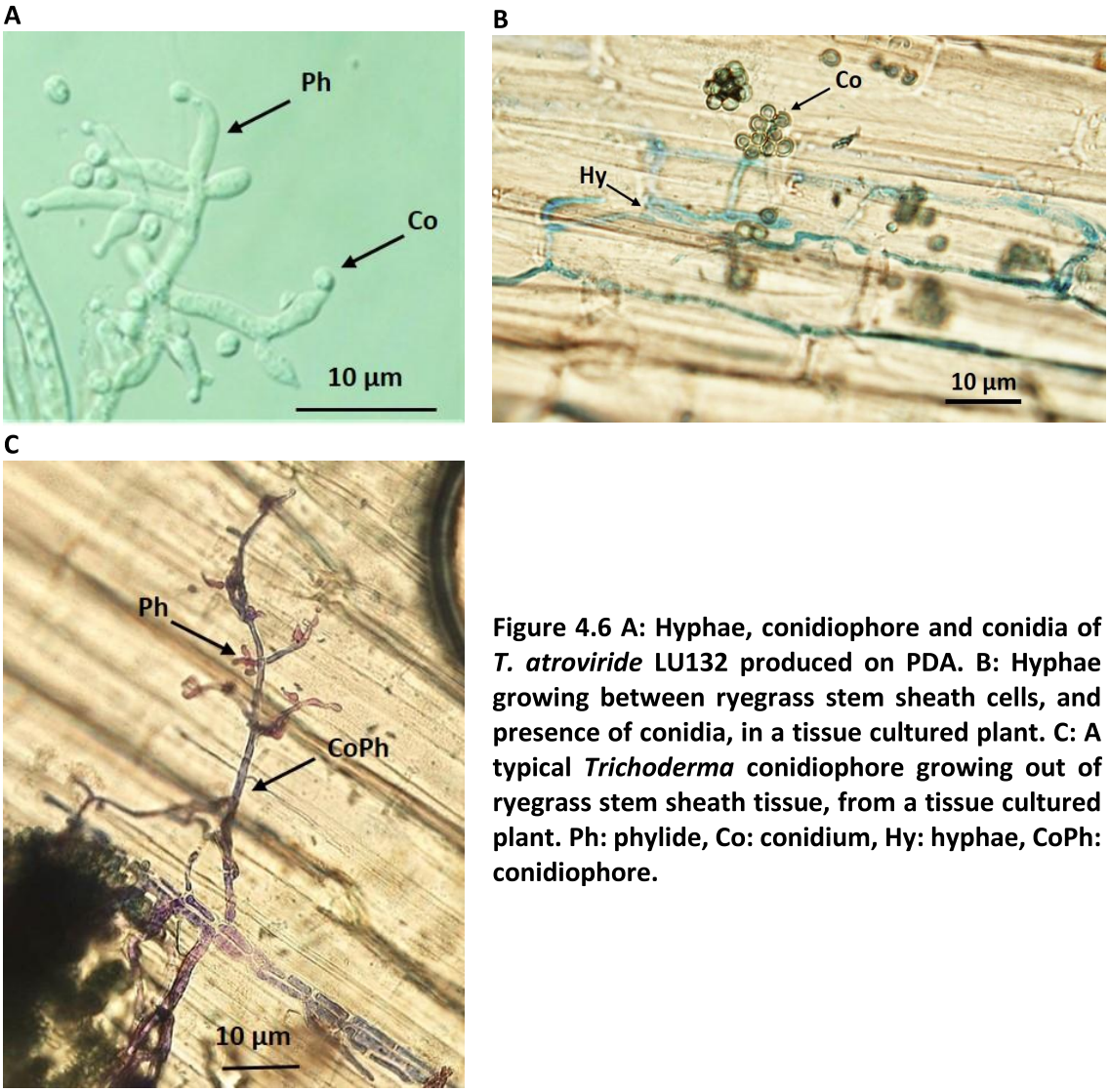


Figure 4.6 A: Hyphae, conidiophore and conidia of *T. atroviride* LU132 produced on PDA. B: Hyphae growing between ryegrass stem sheath cells, and presence of conidia, in a tissue cultured plant. C: A typical *Trichoderma* conidiophore growing out of ryegrass stem sheath tissue, from a tissue cultured plant. Ph: phylide, Co: conidium, Hy: hyphae, CoPh: conidiophore.

4.5 Discussion

Trichoderma atroviride LU132 from all four of the conidium production treatments were able to colonise ryegrass root systems, increase plant dry matter and also protect the plants from *R. solani* as a soil-borne pathogen. In this study, inoculum of *T. atroviride* LU132 was applied at 2×10^6 conidia/g of potting mix with populations in the rhizosphere potting mix being maintained above 10^6 conidia/g. It was suggested by Adams (1990) that effective disease control by *Trichoderma* requires inoculum densities of greater than 10^5 CFU/g of soil. The potting mix used in the pot experiments in this study was pasteurised, so probably contained limited microbial populations, and competition could have occurred between these and either the inoculated *Trichoderma* or the *R. solani*. This would explain the very small *Trichoderma* populations recorded in the non-inoculated potting mix, which may have been stimulated by addition of sterile rice grains. However, very low *Trichoderma* populations in non-inoculated potting mix might also indicate that the *Trichoderma* population which invaded the potting

mix did colonise the rhizosphere. Conidium production treatments gave greater populations compared with non-inoculated potting mix, but this was not always translated into ryegrass growth promotion in the absence of the pathogen.

Considering the conidium production treatments, in the absence of the pathogen, conidia produced at 20°C with sucrose were the most effective at increasing ryegrass growth. However these conidia were not the most rhizosphere competent, especially during initial stages of rhizosphere colonisation. Conidium production treatments at 20°C with dextrose and 30°C with sucrose were the least effective, and also decreased mean root lengths and numbers of lateral roots of the ryegrass plants. These treatments resulted in the greatest initial populations (first 14 d), suggesting that large *Trichoderma* populations in the rhizosphere probably inhibited plant growth in the absence of the pathogen, especially in the early stages of plant growth. These results are similar to those by Heil (2002) and Kazan & Manners (2012), who indicated that suppression of host growth by *Trichoderma* resulted from costs associated with up-regulation of host defence-related genes. In the presence of *R. solani*, the benefits of large *Trichoderma* populations probably outweighed the cost to the plants of supporting the large population, although these two treatments gave reduced ryegrass seedling emergence.

Trichoderma applications with conidia from different conidium production treatments promoted ryegrass seedling emergence and growth parameters, except for the conidia produced in the 20°C-Dex and 30°C-Suc treatments, which reduced ryegrass root lengths and numbers of lateral roots. Ousley *et al*. (1993) demonstrated the inhibitory effects of several *Trichoderma* strains on seedling emergence and growth of lettuce (*Latuca sativa* L.) in non-sterilised potting compost. Inhibitory effects of *Trichoderma* application on plant yields have been correlated with production of metabolites, which could act as auxin-like compounds as was recorded for 6-pentyl-α-pyrone produced by *T. harzianum* at concentrations over 10^{-4} M (Cutler *et al*., 1986; Cutler *et al*., 1989).

Trichoderma atroviride LU132 treatments interacted with different concentrations of *R. solani* inocula and significant differences occurred for some ryegrass host parameters, such as root length. At low *R. solani* inoculum concentrations, *T. atroviride* LU132 conidium treatments increased plant parameters including seedling emergence and total fresh weight compared with the pathogen only (Rs1) treatment at the corresponding inoculum concentration, but in many cases these parameters were not different from the non-inoculated controls. However, although all *T. atroviride* LU132 conidium treatments increased ryegrass growth at the high *R. solani* inoculum concentrations compared with the pathogen only treatment at the same concentration, these effects were not comparable to the non-inoculated treatment (control). At lower concentrations of *R. solani*, however, the amounts of disease suppression were statistically significant. As with other BCAs, *Trichoderma* has been shown to

provide variable biological control efficacy at high pathogen pressures resulting from large amounts of pathogen inoculum (Agbenin, 2011). McLean *et al*. (2012) studied the effects of pathogen pressure caused by *Sclerotium cepivorum* on biological control efficacy of *T. atroviride* LU132 for control of onion white rot. Their results indicated that this BCA was only effective at decreasing disease incidence where onion white rot incidence levels were low.

Trichoderma atroviride LU132 protected ryegrass from the negative effects of *R. solani* at early stages of plant growth, including during seedling emergence. This suggests that *T. atroviride* LU132 suppressed *R. solani* growth and development but was not able to provide the same level of ryegrass growth promotion as occurred in the absence of the pathogen. Similarly, the results outlined in Chapter 2 of the present study showed that *T. atroviride* LU132 outcompeted *R. solani* on dual plate assays. It has been suggested that *Trichoderma* metabolites contribute fungistatically to supress the growth and development of *R. solani* in soil or in agar dual cultures, which are proposed to be differentially modulated by NADPH oxidase (Dr Artemio Mendoza-Mendoza, 2014; personal communication).

In the present study, *T. atroviride* LU132 treatments increased ryegrass biomass in the presence and absence of the pathogen, demonstrating the beneficial effects of this isolate on plant performance. This was in agreement with the results of Kandula *et al.* (2007), who showed that *T. atroviride* LU132 promoted growth of ryegrass in the absence of the pathogen. However, these results were in contrast with those of Hohmann *et al*. (2011) who reported no growth promotional effects of *T. atroviride* LU132 in *Pinus radiata* seedlings, suggesting possible host-specific behaviour for this strain. Similarly, Cripps-Guazzone (2014) showed that *T. atroviride* LU132 has host-specific rhizosphere competency, being rhizosphere competent on sweetcorn, ryegrass, cauliflower and clover, but not on onion.

Regliński *et al*. (2012) reported positive effects from several *T. atroviride* isolates on growth parameters of *Pinus radiata*. *Trichoderma* fungus induced systemic resistance in the plant resulting in reduction of dieback incidence caused by *Diplodia pinea*. Induced resistance was not examined in the present study, however, *T. atroviride* LU132 in other plant systems has been shown to promote plant growth but has not been shown to induce systemic resistance. Maag *et al*. (2013) showed that while *T. atroviride* LU132 colonised the roots of oilseed rape (*Brassica napus*) and increased the total plant biomass, the accompanying increase in levels of jasmonic acid were due to the feeding behaviour of *Plutella xylostella* caterpillars rather than from *Trichoderma* induction.

With *T. atroviride* LU132 conidium production treatments, the increase in root length was correlated with increased numbers of lateral roots, whereas some treatments (20°C-Dex and 30°C-Suc) reducing root length also reduced numbers of lateral roots. The increase in the number of lateral roots has been

related to auxin or auxin-like compounds (Casimiro *et al*., 2001). For example, *T. atroviride* and *T. virens* promoted *Arabidopsis* seedling growth through an auxin-dependent mechanism, which was in accordance with proliferation of lateral roots (Contreras-Cornejo *et al*., 2009). However, production of these compounds beyond certain levels may become toxic to plants and have inhibitory effects on root development (Cutler *et al*., 1986; Cutler *et al*., 1989). In the present study, therefore, excess production of hormones such as auxin-like compounds could have been inhibitory to root length and the numbers of lateral roots, which was possibly correlated with large rhizosphere *Trichoderma* populations from conidia produced from the 20°C-Dex and 30°C-Suc production treatments.

Trichoderma populations in rhizosphere soil were greater than those in bulk potting mix. This could be due to more nutrients being available in plant rhizospheres (from root exudates) than in bulk potting mix. Root exudates could have provided organic carbon, resulting in increased rhizosphere microbial populations (Foster, 1986). For example, it has been documented that as much as 40% of plant products from photosynthesis are released as root exudates (Bais *et al*., 2006).

Trichoderma populations increased at 14 d then declined, then increased again. This suggests a bimodal population cycle, possibly resulting from a bimodal conidium production cycle similar to that described in Chapter 2, but following a different time frame. Although depletion of nutrients can stimulate conidium production (Horwitz *et al*., 1985; Betina, 1995), aging-induced conidiation initiated by gene regulation has been postulated as the main stimulation for conidium production rather than nutrient status (Chovanec *et al*., 2001). However, in a study by Hohmann *et al*. (2012), the decline in population of *Trichoderma* was suggested to be associated with depletion of nutrients in the soil, suggesting that immature roots at early stages of root colonisation do not provide sufficient nutrients to stimulate *Trichoderma* populations. Furthermore, population growth is likely to be related to the composition of root exudates, which cause changes in the soil structure and soil microbial activity. With reference to the bi-phasic *Trichoderma* growth cycle indicated from results in Chapter 2, the decline in the numbers of CFUs in rhizosphere and bulk potting mix after 14 d could be due to a shift from hyphal growth to conidium proliferation. Bae and Knudsen (2005) suggested that conidium production from *T. harzianum* hyphae was initiated when soil conditions were favourable, but increased numbers of conidia reduced biological control efficacy.

Trichoderma was recovered from the plant parts sampled in the pot experiment onto TSM agar cultures, and was then verified by PCR. No *Trichoderma* colonies were produced from the final surface sterilisation wash, confirming effectiveness of the sterilisation method used. Microscopic observation of endophytic colonisation in ryegrass from axenic agar cultures revealed hyphae and reproductive structures characteristic of *Trichoderma* similar to those from the pot experiment plants. Furthermore, no endophytic colonisation was observed in non-inoculated plants both from axenic

agar cultures and the pot experiment. *Trichoderma* penetration of plant tissue to establish avirulent symbiotic interactions with host plants has been previously reported (Harman *et al*., 2004b; Hohmann *et al*., 2012). With regarding the balance between plant growth and defence proposed by Kazan and Manners (2012), *Trichoderma* may be able to colonise the plants endophytically *via* inactivation of plant defence systems triggered by gibberellic acid (Hermosa *et al*., 2012). Therefore, root colonisation in the present study could cause growth promotion effects by *Trichoderma* and then suppression of host defence systems allowing for successful *Trichoderma* entry. Dr Artemio Mendoza-Mendoza (2014, personal communication) studied the endophytic colonisation of *Arabidopsis thaliana* by *Trichoderma* spp., and has found that *Trichoderma* spp. probably produce volatile and diffusible molecules which remotely promote plant growth before direct contact with plants. These metabolites synchronously allow successful endophytic entry *via* inactivation of plant defence systems. Dr Mendoza-Mendoza's research showed that *Trichoderma* spp. produce auxins dependent of tryptophan, which are able to degrade indole acetic acid of the host plant through suppression of auxin responsive genes in primary roots.

Sucrose as a hydrocarbon source during *T. atroviride* LU132 conidium production has been shown to result in superior conidium fitness, particularly enhancing conidium survival and bioactivity after storage (see Chapter 3). However, in the present study, 21 d after sowing, conidia produced at 20°C gave greater rhizosphere colonisation than conidia produced at 30°C, and then within each conidium production temperature (20°C or 30°C), conidia produced with dextrose (20°C-Dex or 30°C-Dex) gave greater colonisation than those produced using sucrose. This effect lasted for a period of 35 d (from 14 to 49 d after sowing). Dextrose as a carbon source in media amended to a C:N ratio of 5:1 is therefore likely to be a preferable carbon source to provide adequate physiological metabolism for *T. atroviride* LU132. *Trichoderma atroviride* LU132 conidia produced from medium with sucrose as the carbon source and incubation at 30°C (30°C-Suc) gave greater populations 14 d after sowing, both in rhizosphere and bulk potting mix. This treatment compared with culture media amended with dextrose at 30°C (30°C-Dex) showed the greatest *Trichoderma* colonisation in potting mix for the first 14 d after sowing ryegrass plants. However since the *Trichoderma* population levels for the sucrose were not maintained to 49 d after sowing this indicates that sucrose was probably quickly exhausted as an energy source. These findings suggest that conidia produced on media containing sucrose probably had stimulated germination and initial growth, with sucrose suggested to be metabolically optimal for conidium production of *T. atroviride* LU132 at temperatures from 25°C (suggested from previous experiments in this study) to close to 30°C. Dextrose, on the other hand, is suggested to be optimal at lower temperatures close to 20°C. In the presence of plant roots or other microorganisms in soil, the nutrient resources of conidia produced on media containing sucrose at 30°C might be

exhausted or become unavailable compared with conidia produced on media containing dextrose and at 20°C. Although, disaccharides (*e.g.* sucrose in the present study) have been recognised as the best carbon sources protecting microorganisms from environmental stresses and providing high survival rates (Redway & Lapage, 1974; van Laere, 1989), in the present study, the monosaccharide dextrose during conidium production resulted in superior host plant colonisation in pot experiments.

Accumulation of compatible solutes in *Trichoderma* conidia during culture conditions, discussed previously in Chapters 2 and 3 (Jennings & Burke, 1990; Welsh, 2000; Pascual *et al*., 2003), suggested that the greatest optimisation of these compounds occurred in conidium production media amended with dextrose at a C:N ratio of 5:1 and at an incubation temperature of 20°C. Although it has been suggested that at higher temperatures hydrocarbon metabolism will increase (Bossert & Bartha, 1984; Leahy & Colwell, 1990), physiological metabolism at higher temperatures (as for *T. atroviride* LU132 in the present study) could also involve complex compounds such as fatty acids, which may not be as readily available sources of energy compared with simple compounds.

The experiments described in this Chapter have shown that manipulation of conidium production culture conditions can eco-physiologically affect conidium quality, to improve the fitness of *T. atroviride* LU132 with regarding colonisation of the host plant root systems, and, in this case, protect ryegrass against *R. solani*. Medium composition has been shown to greatly affect the physiology and vigour of fungal conidia produced under these conditions, including conidium production, germination, bioactivity, and shelf life (Darby & Mandels, 1955; Jackson *et al*., 1996). The optimum C:N ratio of 5:1 in the present study (as determined in previous Chapters) with different hydrocarbon sources gave differences in ability of *Trichoderma* to colonise ryegrass rhizospheres. Dextrose (specifically, 20°C-Dex) resulted in greater colonisation both of ryegrass rhizosphere and bulk potting mix in the absence of *R. solani*.

The experiments outlined here have provided new knowledge on how manipulation of culture conditions of *T. atroviride* LU132 can influence conidium fitness as a basis for optimising commercial production of the fungus as a BCA. However, variations in *T. atroviride* LU132 bioactivity related to composition of production media should be further investigated, to provide knowledge of the underlying biochemical and conidium morphological characteristics affected by differences in conidium production conditions. Studies of some of these characteristics are outlined in Chapter 5.

4.6 Summary of key results

- The results of pot experiments showed that *T. atroviride* LU132 colonies produced from particular conidium production treatments reduced the effect of *R. solani*.

- The bimodal population cycle in *T. atroviride* LU132 (described in Chapter 2) recurred in pot experiments (recorded at CFU levels), in a manner similar to that observed previously in agar plates.
- *Trichoderma atroviride* LU132 increased some growth parameters of ryegrass plants, but not root length and numbers of lateral roots
- *Trichoderma atroviride* LU132 established endophytic colonisation and potential symbiotic interaction with ryegrass plants.
- Conidia produced on media containing dextrose colonised the bulk and rhizosphere potting mix where ryegrass plants were growing to a higher level compared to conidia produced with sucrose.
- Sucrose in conidium production medium may have stimulated germination and initial fungal growth for the first 14 d of *Trichoderma* life cycle compared with dextrose.
- Sucrose was metabolically optimal for conidium production of *T. atroviride* LU132 at temperatures close to 30°C, while dextrose was optimal at lower temperature close to 20°C.
- Conidia produced at 30°C may undergo physiological conversion of nutrient reserves to complex compounds such as fatty acids, which may not be easily available sources of energy compared with hydrocarbons.

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4.8 Appendices

Appendix 4.1 Log_{10} and back-transformed *Trichoderma* population data (CFU/ g dried potting mix) originating from *T. atroviride* LU132 conidia produced in different culture conditions. Populations were assessed at different days after sowing (7, 14, 21, 28, 35, 42 and 49), and recovered from rhizospheres of ryegrass planted in potting mix (PM) in the presence or absence of *Rhizoctonia solani*.

***Trichoderma* population (CFU/g dried potting mix) over time recovered from rhizosphere of ryegrass planted in potting mix inoculated with *T. atroviride LU132* conidia produced under different culture conditions in the presence or absence of *Rhizoctonia solani*.**

DAS[1]	Treatment[2]							
	20°C-Dex	20°C-Suc	30°C-Dex	30°C-Suc	Rs×20°C-Dex	Rs×20°C-Suc	Rs×30°C-Dex	Rs×30°C-Suc
7	6.7 (4.8×10^6)[3]	6.5 (3.2×10^6)	6.6 (4×10^6)	6.8 (6.9×10^6)	6.4 (2.4×10^6)	6.3 (2.1×10^6)	6.3 (2×10^6)	6.4 (2.5×10^6)
14	7.8 (6.6×10^7)	7.5 (3.4×10^7)	7.7 (4.6×10^7)	8.0 (9.3×10^7)	6.8 (6.2×10^6)	6.9 (7.3×10^6)	6.9 (7.6×10^6)	7.0 (9.7×10^6)
21	7.4 (2.3×10^7)	7.2 (1.6×10^7)	7.0 (1×10^7)	6.8 (6.6×10^6)	6.1 (1.3×10^6)	6.1 (1.2×10^6)	6.1 (1.2×10^6)	6.1 (1.3×10^6)
28	8.2 (1.7×10^8)	8 (1.1×10^8)	7.9 (8.6×10^7)	7.8 (6.3×10^7)	7.2 (1.5×10^7)	7.0 (9.7×10^6)	7.0 (1.1×10^7)	7.1 (1.3×10^7)
35	8.9 (8.5×10^8)	8.8 (5.7×10^8)	8.6 (4.5×10^8)	8.5 (2.9×10^8)	7.5 (3.1×10^7)	7.4 (2.5×10^7)	7.3 (2.2×10^7)	7.4 (2.3×10^7)
42	8.4 (2.7×10^8)	8.3 (2.1×10^8)	8.2 (1.7×10^8)	8.1 (1.3×10^8)	7.2 (1.6×10^7)	7.2 (1.6×10^7)	7.2 (1.7×10^7)	7.2 (1.7×10^7)
49	8.1 (1.3×10^8)	8.0 (9×10^7)	7.9 (7.2×10^7)	7.7 (5.1×10^7)	6.8 (6.4×10^6)	7.0 (8.9×10^6)	7.0 (9.1×10^6)	6.8 (6.4×10^6)
Overall mean	7.9 (8.6×10^7)	7.8 (5.7×10^7)	7.7 (5.1×10^7)	7.7 (4.7×10^7)	6.9 (7.1×10^6)	6.8 (6.8×10^6)	6.8 (6.8×10^6)	6.9 (7.2×10^6)
$LSD_{0.05}$ (LS Ratio) = 0.15 (1.4)[4]								

[1] Day after sowing.
[2] *Trichoderma* conidia were produced at 20°C or 30°C amended with sucrose (Suc) or dextrose (Dex) at C:N ratio of 5:1 inoculation in potting mix in presence or absence of *Rhizoctonia solani* (Rs) at ratio of 0.4% w/w of potting mix.
[3] The values in the table represent log_{10} and numbers in brackets are back-transformed data to give CFUs of *Trichoderma* per gram of dry potting mix recovered from rhizospheres.
[4] The LSD of 0.15 is for comparing any two log_{10} means, and the LS Ratio of 1.4 is for comparing any two back-transformed means.

Appendix 4.2 Log_{10} and back-transformed *Trichoderma* population data (CFU/ g dried potting mix) originating from *T. atroviride* LU132 conidia produced in different culture conditions. Populations were assessed at different days after sowing (0, 7, 14, 21, 28, 35, 42 and 49), recovered from bulk potting mix (PM) in the presence or absence of *Rhizoctonia solani*.

***Trichoderma* populations over time recovered from bulk potting mix inoculated with *T. atroviride* LU132 conidia produced under different culture conditions planted with ryegrass in the presence or absence of *Rhizoctonia solani*.**

DAS[1]	Treatment[2]							
	20°C-Dex	20°C-Suc	30°C-Dex	30°C-Suc	Rs×20°C-Dex	Rs×20°C-Suc	Rs×30°C-Dex	Rs×30°C-Suc
0	6.3 (2.1×10^6)[3]	6.3 (2×10^6)	6.3 (2×10^6)	6.3 (2.1×10^6)	6.3 (1.9×10^6)	6.4 (2.5×10^6)	6.3 (2.2×10^6)	6.3 (2.2×10^6)
7	6.9 (7.4×10^6)	6.7 (5.3×10^6)	6.8 (6.6×10^6)	7.0 (9.9×10^6)	6.4 (2.6×10^6)	6.4 (2.7×10^6)	6.4 (2.5×10^6)	6.4 (2.3×10^6)
14	7.2 (1.6×10^7)	6.9 (7.2×10^6)	7.0 (1.1×10^7)	7.4 (2.3×10^7)	6.7 (4.6×10^6)	6.6 (4×10^6)	6.5 (3.4×10^6)	6.6 (3.8×10^6)
21	6.8 (7×10^6)	6.6 (3.7×10^6)	6.5 (2.9×10^6)	6.3 (2×10^6)	6.3 (2×10^6)	6.2 (1.5×10^6)	6.1 (1.2×10^6)	6.1 (1.2×10^6)
28	7.7 (5.5×10^7)	7.5 (3×10^7)	7.4 (2.4×10^7)	7.2 (1.4×10^7)	6.7 (5.5×10^6)	6.6 (4.2×10^6)	6.6 (4.3×10^6)	6.5 (3.4×10^6)
35	8.2 (1.5×10^8)	8.0 (9.2×10^7)	7.8 (6.9×10^7)	7.6 (4.4×10^7)	7.3 (2.1×10^7)	7.3 (2.5×10^7)	7.3 (1.9×10^7)	7.3 (1.9×10^7)
42	8.0 (1×10^8)	7.8 (6.6×10^7)	7.6 (4.1×10^7)	7.4 (2.7×10^7)	7.2 (1.5×10^7)	7.2 (1.6×10^7)	7.2 (1.5×10^7)	7.2 (1.5×10^7)
49	7.9 (8.2×10^7)	7.7 (4.8×10^7)	7.5 (3.1×10^7)	7.3 (2.1×10^7)	6.7 (5.4×10^6)	6.8 (6.4×10^6)	6.8 (6.3×10^6)	6.6 (4.1×10^6)
Overall mean	7.4 (2.4×10^7)	7.2 (1.5×10^7)	7.1 (1.3×10^7)	7.1 (2.1×10^7)	6.7 (5.1×10^6)	6.7 (4.9×10^6)	6.7 (4.5×10^6)	6.6 (4.2×10^6)

$LSD_{0.05}$ (LS Ratio) = 0.14 (1.4)[4]

[1] Day after sowing.

[2] *Trichoderma* conidia were produced at 20°C or 30°C amended with sucrose (Suc) or dextrose (Dex) at C:N ratio of 5:1 inoculation in potting mix in presence or absence of *Rhizoctonia solani* (Rs) at ratio of 0.4% w/w of potting mix.

[3] The values in the table represent log_{10} and numbers in brackets are back-transformed data to give CFUs of *Trichoderma* colony per gram of dry potting mix recovered from bulk potting mix.

[4] The LSD of 0.15 is for comparing any two log_{10} means, and the LS Ratio of 1.4 is for comparing any two back-transformed means.

Chapter 5

Relationships between *Trichoderma atroviride* LU132 conidium "fitness" and conidium biochemical and ultrastructural characteristics

5.1 Introduction

Culture conditions and nutrient composition influence the physiological quality of inoculum and subsequent bioactivity potential of *Trichoderma* conidia for biological control programmes (Hallsworth & Magan, 1996; Agosin & Aguilera, 1998).The lipid profile, protein content in the cell walls and trehalose content of conidia are all factors that have been shown to influence the stability and bioactivity of fungi during storage (Agosin & Aguilera, 1998). Further, the polyunsaturated fatty acid contents of conidia have also been reported to affect the oxidative stability of cell membranes.

Some sugar alcohols, including arabitol, mannitol, glycerol, and erythritol, are accumulated intracellularly in fungi as osmoadaptation strategies to maintain osmotic balance and protect cellular macromolecules and enzymes against a variety of stresses such as heat, free radicals and dehydration. These compounds are known as compatible solutes ("osmolytes"), which do not influence cellular metabolism (Brown, 1978; Empadinhas & da Costa, 2008). Trehalose as an osmolyte contributes to increased bioactivity and stress tolerance (Hallsworth & Magan, 1994; Hallsworth & Magan, 1995). Levels of intracellular accumulation of trehalose have been attributed to the survival and degree of stress resistance (Hottiger *et al*., 1987; Hounsa *et al*., 1998). Accumulation of trehalose has been suggested to be induced by stress and does not normally increase in fungal conidia under steady-state culture conditions (Pedreschi *et al*., 1997). Furthermore, it has been proposed that synthesis of trehalose depends on the presence of mannitol as one of the most common polyols in microorganisms, so that a mixture of trehalose and mannitol is reported to be needed for stress tolerance (Dijksterhuis *et al*., 2006). Accumulation of osmolytes such as arabitol and mannitol in yeasts has been shown to be an osmoadaptation strategy supporting osmotic equilibrium to stabilise cellular metabolism (Brown, 1978). Accumulation of arabitol coincides with that of glycerol *via* gene regulation for production of plasma membrane channel proteins, such as the *Fps1p* gene in *Saccharomyces cerevisiae* (Luyten *et al*., 1995; Hohmann *et al*., 2000). The membrane channel proteins retain or release compatible solutes, water and other small solute molecules for osmolarity balance. For example, arabitol and glycerol are retained intracellularly during hyper-osmotic stress, but are rapidly released during hypo-osmotic stress. Tang *et al*. (2005) used *Zygosaccharomyces rouxii Fps1p* gene

mutants in studies of hypo- and hyper-osmotic shock, and showed that the concentrations of arabitol and glycerol were significantly greater in the mutants than in the wild type of the fungus.

Fatty acids are involved in cellular mechanisms, as key components of membranes, nutrient sources and transport of energy, and also play roles as gene regulators (Rustan & Drevon, 2005). Fatty acids predominantly found in organisms range from C14 to C20 molecules, but most key fatty acids contain 16 or 18 carbon atoms (Weete, 1980). Palmitic acid (16:0) can be converted to stearic acid (18:0) and other 18 carbon fatty acid isomers *via* elongation and desaturation reactions (Rustan & Drevon, 2005; Ando *et al*., 2009). *Trichoderma* spp., for example, have been reported to convert palmitic acid to stearic acid *via* elongation and to oleic acid (18:1 *c*9), and then to α–linolenic acid through desaturation (18:3 *c*9, 12, 15). *Trichoderma* spp. have been used as catalysts for fatty acid biotransformation for industrial production of polyunsaturated fatty acids, of which the conjugated forms of these compounds have been known for their biologically beneficial effects on human and animal health (Needleman *et al*., 1986; Ando *et al*., 2009). Accurate determination of the fatty acid content of conidia is crucial for optimisation of culture conditions, and for monitoring the efficiency of conidium production processes (Laurens *et al*., 2012).

Generally, low C:N ratios are associated with protein metabolism in microorganisms, while a low rate of protein synthesis as the result of a high C:N ratio is linked with high lipid production (Weete, 1980). In a study by Jackson (1997), the nutritional requirements for *Colletotrichum truncatum* inoculum production were optimised based on a C:N ratio of 10:1 to give effective biological control activity against the deleterious weed, *Sesbania exaltata*. Assessment of the conidium composition showed that conidia produced in a media containing C:N ratios of 30:1 or 80:1 consumed amino acids prior to glucose while those produced at 10:1 gave balanced utilisation of nitrogen and carbon sources. The effectiveness of biological control activity by conidia produced at C:N of 10:1 was related to the conidia containing more protein and less lipid compared with conidium production treatments at C:N of 30:1 or 80:1. The increased lipid contents were correlated with the presence of lipid droplets in the conidia and was attributed to the conversion of excessive carbon to lipids. Generally, lipid production is related to increasing glucose content in the culture media, as demonstrated by Prill *et al*. (1935) and Ward *et al*. (1935). However, when the utilisation of nitrogen and carbon sources is not balanced due to an excess of sugars, many fungi convert excess carbohydrates to lipids (Prill *et al*., 1935; Chesters & Peberdy, 1965; Weete, 1980). The ability to convert sugars to lipids in fungi has been termed “fat coefficient” or “lipid yield” which is dependent on the medium composition, culture conditions and is fungal species-specific. However, based upon the type of carbon source, the order of conversion is greater for glucose than sucrose (Ward *et al*., 1935). Growth temperature or medium pH do not influence the accumulation of lipids in fungi, but temperature could affect the degree of lipid

unsaturation (Weete, 1980). For example, high temperature of ≥ 25°C during the growth of *Phytophthora* spp. caused accumulation of saturated (palmitic acid, 16:0) or low weight unsaturated (linoleic acid, 18:2 *c*9, 12) fatty acids, while temperatures ≤ 20°C prompted accumulation of unsaturated (oleic acid 18:1 9) or high weight polyunsaturated (eicosapentaenoic acid, 20:5 *c*5, 8, 11, 14, 17) fatty acids (Duan *et al*., 2011).

Ultrastructural studies of conidia have been widely used for taxonomic differentiation, as well as determination of conidium germinability and dormancy (Bartnicki-Garcia, 1968; Hawker *et al*., 1970). Transmission electron microscopy (TEM) has been widely used to differentiate conidium ultrastructure. Further, biochemical differences can be indicated from differences in electron transparency and these attributes were illustrated for *Botrytis cinerea* by Gull & Trinci (1971). Maturation of *T. viride* was related to structural changes such as increased conidium size, number of mitochondria and conidium wall thickness, while electron dense bodies and endoplasmic reticulum disappeared (Rosen *et al*., 1974). Components similar to electron dense bodies in *T. viride* have been observed in *B. cinerea* (Buckley *et al*., 1966) and *Penicillium megasporum* (Remsen *et al*., 1967), which are broken down to smaller particles with their density gradually decreasing during conidium germination. Differences in the ultrastructure of conidia (*e.g.* in external cell walls) have been linked to differences in conidium survival and successful establishment of biological control agents (BCAs; Munoz *et al*., 1995). Despite much research on the structural changes that occur in conidia during germination, maturation and dormancy, there is little information regarding the effects of growth medium composition on these structural changes or the relationship between these structural changes and conidium fitness.

In previous chapters, the variations of bioactivity of *T. atroviride* LU132 conidia produced under different culture conditions were suggested to be possibly related to biochemical contents and/or ultrastructural characteristics. The present chapter explores biochemical and ultrastructural changes of *T. atroviride* LU132, to determine key characteristics of conidia that vary in bioactivity as indicated in previous experiments of this study.

5.2 Content and composition of sugars in *Trichoderma atroviride* LU132 conidia

5.2.1 Materials and methods

Trichoderma atroviride LU132 conidia for analyses of sugars were produced based on treatments indicated in Chapter 3 Section 3.2.2 and Chapter 4 Section 4.3.1. *Trichoderma atroviride* LU132 cultures were grown in Petri plates containing the appropriate media and at either 20°C or 30°C, with three replicates per treatment and completely randomised. Culture conditions for the four conidium production treatments used in Chapter 4 are shown in Table 5.1 with the treatments used in Chapter 3 outlined in Section 3.2.2. Conidia stored for six months at 0% RH and 30°C as described in Chapter 3 were also included.

Sugars from test conidia from each replicate plate were extracted and quantified using high performance liquid chromatography (HPLC), based on a modified method from that described by Hallsworth & Magan (1994). Freeze dried conidia (10 mg) were added to microtubes and then ground three times in a homogenizer (Mini-Mill Pulverisette 23, Fritsch) at 50 oscillations/s for 1 min, with ice chilling before and after grinding. Ultra-pure water (1.2 mL; HPLC grade) was added to the microtubes and shaken vigorously. The samples were then heated in a water bath at 80°C for 10 min and shaken every 2 min during this period. The microtubes were then centrifuged at 16,100 g for 10 min and 1 mL of the supernatant was collected and mixed with 0.5 mL *n*-hexane (HPLC grade) in new 2 mL capacity microtubes. The tubes were shaken for 1 min and supernatant was then removed and the remaining hexane in the water phase was evaporated in a laminar flow cabinet for 30 min. The water phase containing sugars in the microtubes was collected and passed through 0.22 µm filters to remove any debris left in the solution. The volume of water was replenished to 2 mL by adding ultra-pure water, and the tubes were stored in a freezer at −20°C until used for analysis. Each sample was four fold diluted with ultra-pure water before injection into the HPLC. The chromatograph was a Shimadzu LC System (Shimadzu® Corporation) equipped with an auto-sampler (SIL-10AF, Shimadzu® Corporation) and a detector (3300 ELSD, Alltech®). Each sample (20 µL) was injected into a column with dimensions 250 × 3 mm (Imtakt®, Unison UK-Amino). Extracted sugars were diluted with 82% acetonitrile and 18% ultra-pure water to match the ratio of mobile phase with flow rate of 0.7 mL/min, and the temperature of the column for sugar discrimination was adjusted to 60°C. D-trehalose (Sigma-Aldrich, 99.5%), arabitol (Sigma-Aldrich, 99.5%) and mannitol (BDH, 99%), were used as standards. Trehalose, arabitol and mannitol in the samples were separated and quantified, and peak areas compared with the standards. All data were processed with a high performance liquid chromatography workstation (LCsolution Software, Shimadzu® Corporation).

Data from sugar analyses were subjected to analysis of variance (ANOVA), and treatment means were separated using Fisher's unprotected test of least significant difference (LSD at *P*= 0.05). Significance of contrasts were explored between valid treatment comparisons based upon culture conditions, as previously outlined in Chapter 3 Section 3.2.4. All statistical analyses were performed using procedures in GenStat® 16th edition (VSN International Ltd).This experiment was performed once.

Table 5.1 Culturing conditions used for production of *Trichoderma atroviride* LU132 conidia that were assessed for fatty acid and sugar contents, and ultrastructure analysis as freshly produced conidia and after six months storage.

Treatments[1]	Culture conditions						
	T (°C)	a_w	pH	C (g/L)	C:N	C source	Buffer[2]
20°C-Dex	20	0.998	5.5	4.2	5:1	Dextrose	Phosphate
20°C-Suc	20	0.998	5.5	4.2	5:1	Sucrose	Phosphate
30°C-Dex	30	0.998	5.5	4.2	5:1	Dextrose	Phosphate
30°C-Suc	30	0.998	5.5	4.2	5:1	Sucrose	Phosphate

[1] Conidia produced at C:N (5:1) at 20°C or 30°C, amended with dextrose (20°C-Dex or 30°C-Dex) or sucrose (20°C-Suc or 30°C-Suc).
[2] Phosphate buffer; K_2HPO_4/KH_2PO_4.

5.2.2 Results

Statistically significant differences (*P*< 0.001) were detected in the total sugars extracted from *T. atroviride* LU132 conidia produced under different culture conditions (Table 5.2). Presentation of results of sugar analyses focuses on valid comparison treatments determined in Chapter 3, and also the conidium production treatment at 20°C with dextrose (20°C-Dex), which showed the greatest bioactivity in the pot experiments in Chapter 4 *versus* 20°C-Dex conidia stored for six months at 30°C and 0% RH (Table 5.2 and Appendix 5.1). The results for all the treatments are presented in Appendix 5.2. A summary of the results for germination and bioactivity initially and after storage for the different conidium production treatments determined in Chapters 3 and 4 are shown in Appendix 5.3. Of the sugars, mannitol was in the greatest mean intracellular concentration (100 mg/g dry conidia), while less trehalose (mean of 43 mg/g dry conidia) and arabitol (11 mg/g dry conidia) were detected (Table 5.2 and Appendix 5.1). Regarding valid comparisons of culturing condition treatments, all significantly (*P*< 0.001) affected trehalose and arabitol accumulation. None of the treatment comparisons significantly affected mannitol accumulation. The least trehalose accumulation was detected in conidia produced at 20°C (13 mg/g dry conidia) and correlates with the least conidium survival and bioactivity after six months storage (Appendix 5.3). The greatest trehalose accumulation (62 mg/g dry conidia) occurred for conidium production treatment at C:N ratio of 5:1, followed (in diminishing

order) by C:N ratio 160:1 or pH 7.5 treatments (55 mg/g dry conidia), 30°C (54 mg/g dry conidia), 1.2 mM Trehalose (53 mg/g dry conidia), 30°C-Suc (50 mg/g dry conidia) (Appendices 5.1 and 5.2). HPLC chromatographs for sugars analyses are presented in Appendix 5.4 for those treatments used for conidium production treatments in Chapter 4 and for conidia produced at 20°C-Dex as fresh and after six months storage (20°C-Dex, 6 mo) to determine the effects of storage period.

Table 5.2 Mean amounts of three sugars (mg/g of dry conidia) in conidia of *Trichoderma atroviride* LU132 produced from different culture conditions (see Table 3.1), as fresh conidia or after storage for six months (6 mo).

Treatment[1]	Arabitol	Mannitol	Trehalose
20°C[2]	13	121	13
30°C	11	130	54
C:N 5:1	8	92	62
C:N 160:1	17	94	55
1.2 mM Tre	10	95	53
1.2 mM GB	9	92	37
20°C-Dex	7	95	38
20°C-Dex, 6 mo	9	83	29
$LSD_{0.05}$	1.3	24.6	1.2
Overall mean	11	100	43
Significance of contrasts between valid comparisons[3]			
20°C *vs* 30°C	***	**ns**	***
C:N (5:1) *vs* C:N (160:1)	***	**ns**	***
1.2 mM Tre *vs* 1.2 mM GB	***	**ns**	***
20°C-Dex *vs* 20°C-Dex, 6 mo	***	**ns**	***

[1] Tre: trehalose, GB: glycine-betaine; 20°C-Dex: conidia produced at 20°C or 30°C, amended with dextrose (Dex).

[2] 20°C or 30°C conidia produced on PDA at 20°C or 30°C.

[3] ***: the effects of treatments are statistically significant at $P = 0.001$; ns: not significant ($P > 0.05$).

5.3 Content and composition of fatty acids in *Trichoderma atroviride* LU132 conidia

5.3.1 Materials and methods

Trichoderma atroviride LU132 conidia were produced as described in Section 5.2.1, and lipids were extracted from the conidia using a modified method described by Folch *et al*. (1957) and Lepage & Roy (1986). Freeze dried conidia (10 mg) plus a ball-bearing (5 mm diameter), in individual 2 mL capacity centrifuge tubes were cooled in a freezer at −20°C. The conidia were then ground three times in a homogenizer (Mini-Mill Pulverisette 23, Fritsch) at 50 oscillations/s for 1 min each time, while chilled with ice after during each grinding. Hexane (HPLC grade) (1.2 mL) was added to each tube, and the tubes were then transferred to a −20°C freezer. Every 30 min, tubes were shaken three times for 1 min each with a homogenizer, and were then transferred back to the −20°C freezer. Supernatant from each tube was collected and passed through a 0.22 µm filter and then mixed with 18% (w/w) KCl. Each sample was shaken for 2 min then centrifuged at 16,100 g for 5 min. Supernatant (1 mL) was taken and 1 mL hexane added to reach a final volume of 2 mL. Samples (main stocks) were then kept in a freezer at −20°C for later methyl-esterification.

Methyl-esterification was carried out using a modified method described by Chambers & Clamp (1971). Methanol (200 µL) and sodium-methoxide (in 25% w/v methanol, 100 µL) were added to 100 µL of the main stock sample. Samples were transferred to micro glass tubes (5 mL capacity) and incubated in a water bath at 55°C for 15 min and shaken regularly. Methanolic HCl (100 µL, 3 M) was added to each sample maintained at 55°C for a further 60 min with regular shaking. Samples were topped up with 600 µL hexane, shaken and centrifuged for 5 min at 16,100 g. From each tube, 500 µL supernatant was collected and transferred to a 2 mL tube. The final samples were kept in a freezer at −20°C before analysis. For gas chromatography analysis, 125 µL of the main stock solution was collected and put in 250 µL inserts into 2 mL glass vials to fit the gas liquid chromatograph.

The samples were analysed using a gas chromatograph (GC-2010, Shimadzu®), equipped with an auto sampler (AOC-20i, Shimadzu®) to inject 1 µL of a standard or sample into a GC capillary column of 100 m × 0.25 mm and 0.2 µm film thickness (Wcot Fuse Silica, Varian CP7420, Crawford Scientific). Helium was used as the carrier gas and samples were detected using a flame ionisation detector at 250°C. A wide range of standards (C4 to C26, saturated or unsaturated) were injected before injection of the test samples (Appendix 5.5). The total area acquired from standard set GLC411 was used for calculation of the total fatty acids (µg/g dry conidia), and the rest of the standards were employed for fatty acid identification. To measure the percentage of lipid unsaturation, the following equation was used to calculate unsaturation based upon the percentage of carbon bonds in lipid structures (Weete,

1980): Lipid unsaturation % = 1 × (% monoenes)/100 + 2 × (% dienes)/100 + 3 × (% trienes) + 4 … /100. Data from fatty acid analyses were subjected to analysis using the procedure described in Section 5.2.1. This experiment was performed once.

5.3.2 Results

Presentation of results of fatty acids analysis focuses on conidium production treatments previously described in Section 5.2.2 (Table 5.3 and Appendix 5.7). A summary of fatty acid analysess for conidium production treatments in Chapters 3 and 4 are shown in Appendices 5.6 and 5.7. The fatty acid profile for the different conidium production treatments showed occurrence of a wide range of fatty acids, ranging from 16 to 23 carbon atoms. Frequent fatty acids detected by GC were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 *c*9) and linoleic acid (18:2 *c*9, 12). Linoleic acid was the most abundant (overall mean of 26 µg/g dry conidia), and stearic acid (18:0) was the least abundant (8 µg/g dry conidia) recorded for for the different conidium production treatments (Table 5.3). Some fatty acids were also detected in trace amounts (Appendix 5.8) including: margaric acid (17:0 iso), α-linolenic acid (18:3 *c*9, 12, 15), nonadecylic acid (19:0), eicosadienoic acid (20:2 *c*11, 14), eicosapentaenoic acid (EPA or timnodonic acid) (20:5, *c*5, 8, 11, 14, 17), heneicosylic acid (21:0), and tricosylic acid (23:0). The conidium production treatment of 20°C gave the greatest proportion of fatty acids (66 µg/g of dry conidia), while a C:N ratio of 5:1 gave the least fatty acid production (12 µg/g dry conidia). Calculation of mean unsaturation percentage showed the greatest unsaturation for 20°C-Dex treatment (60%) from the defined medium and 30°C treatment (59%) from PDA medium, followed by the treatments of C:N 5:1 (39%) and a_w 0.985 (33% unsaturation), which were significantly different from the mean unsaturation percentage for the other conidium production treatments. The stored conidia had significantly greater total fatty acid content compared with 15-d- old conidia ($P < 0.001$) with similar conidium production conditions, except for the conidia for the 20°C-Suc treatment (48 µg/g of dry conidia), which gave more than twice the accumulated fatty acid content compared with conidia from the 20°C-Suc after 6 month storage (23 µg/g of dry conidia). The mean unsaturation percentage increased significantly ($P < 0.001$) in stored conidia compared with 15-d-old conidia with similar conidium production conditions, except for in the 20°C-Dex treatment (60% mean unsaturation) which gave more than three times the amount compared with the conidia from the 20°C-Dex after six months storage (17% mean unsaturation). Reduced unsaturation was evident in four conidium production treatments of 20°C-Dex, 20°C-Suc, 30°C-Dex and 30°C-Suc, when stored for six months, for oleic acid (18:1 *c*9) and linoleic acid (18:2 *c*9, 12). These differences were statistically significant ($P < 0.001$). The differences in mean percentage unsaturation and the most abundant fatty acids between valid comparisons are shown in Appendix 5.7. Chromatographs for fatty acid analyses are presented in Appendix 5.9 for those treatments indicated for conidium production treatments in

Chapter 4, and also for conidia produced at 20°C-Dex as fresh conidia or stored for six months, to compare the effect of storage.

Table 5.3 Mean total amounts, the main fatty acids, (µg/g of dry conidia), and the mean percentage unsaturation, in conidia of *Trichoderma atroviride* LU132 produced from different culture conditions (see Table 3.1) as fresh or conidia after six months (6 mo) storage.

Treatment[1]	Total FA	Mean % unsaturation	C16:0	C18:0	C18:1 *c*9	C18:2 *c*9, 12
20°C[2]	66	19	17	6	33	32
30°C	35	59	18	6	37	33
C:N 5:1	12	39	20	8	19	32
C:N 160:1	48	25	22	6	32	33
1.2 mM Tre	15	26	25	13	12	30
1.2 mM GB	25	21	20	9	13	27
20°C-Dex	20	60	28	9	24	38
20°C-Dex, 6 mo	27	17	18	9	12	18
$LSD_{0.05}$	**0.5**	**3**	**0.6**	**0.4**	**0.4**	**0.4**
Overall mean			20	8	21	26
Significance of contrasts between valid comparisons[2]						
20°C *vs* 30°C	***	***	**	ns	***	**
C:N (5:1) *vs* C:N (160:1)	***	***	***	**	***	**
1.2 mM Tre *vs* 1.2 mM GB	***	***	***	***	ns	***
20°C-Dex *vs* 20°C-Dex, 6 mo	***	***	***	ns	***	***

[1] Tre: trehalose, GB: glycine-betaine; 20°C-Dex: conidia produced at C:N (5:1) at 20°C and amended with dextrose (Dex). 20°C or 30°C conidia produced on PDA at 20°C or 30°C.

[2] **, ***: the effects of treatments are statistically significant at $P = 0.01$ or 0.001, respectively; ns: not significant ($P > 0.05$).

5.4 Ultrastructure of *Trichoderma atroviride* LU132 conidia

5.4.1 Materials and methods

Samples of conidia for TEM (Appendix A) assessments were selected and prepared as described in Section 5.2.1. Conidium suspensions were prepared in sterile distilled water (SDW) at a concentration of 2×10^9 (conidia/ mL), or for conidia stored for six months (treatments 20°C-Dex, 6 mo; 20°C-Suc, 6 mo; 30°C-Dex, 6 mo; and 30°C-Suc, 6 mo) at 0.1 g/mL. Conidium suspensions in 1.7 mL microtubes were centrifuged at low speed (100 g for 1 min). The supernatant was discarded and substituted with fixative. The fixative solution was made from freshly depolymerised paraformaldehyde 2% (v/v) and glutaraldehyde 2.5% (v/v), both at TEM grade (Sigma-Aldrich), in 0.1 M phosphate buffer (pH 7.2). Samples were each agitated with a pipette tip to break up the conidium mass, and were then left under vacuum for 10 min to remove air bubbles. After standing for 24 h at 4°C, samples were washed three times with phosphate buffer (pH 7.2), and post-fixation was performed in 1% (w/v) aqueous osmium tetroxide. The samples were dehydrated in an ethanol series (50, 60, 70, 80, 90, 95 and 100%) for 15 min at each step, and then embedded in LR white resin (London Resin). Embedded samples were sectioned using a Leica UCT ultramicrotome (Leica Microscopy Systems Ltd) at a thickness of 110 nm. Sections were placed on grids and stained with 1% (w/v) aqueous uranyl acetate followed by aqueous lead citrate (Roland & Vian, 1991). Sections on grids were examined using a transmission electron microscope (model JEM-1200EXII, JEOL). Each sample was blocked as coated grids in five replicates.

5.4.2 Results

Transmission electron microscopy (Figure 5.1) revealed differences in the internal structures of conidia obtained from the different conidium production treatments, and also in conidia stored for six months. Presentation of results of the TEM assessments focuses on the interactions of conidium production treatments (20°C *vs* 30°C and C:N ratios 5:1 *vs* 160:1) which were the production treatments used in the pot experiments described in Chapter 4, and also comparisons between conidia produced as 20°C with dextrose fresh (20°C-Dex) and after six months storage (20°C-Dex, 6 mo conidia) (Figure 5.1). Electron micrographs from the other conidium production treatments are presented in Appendix 5.10.

Fresh conidia produced at 20°C versus 30°C: Conidia produced at 20°C were mostly malformed (Figure 5.1). The cytoplasm of individual conidia was often retracted from the cell wall or largely filled with lipid droplets accumulated in the vicinity of the internal wall layers. Spaces were evident between the conidium cell walls and the plasma membranes. The nuclei were small or they were was replaced with

lipids. Plasma membranes were disrupted and there were many indentations in the membranes. When abundant lipid droplets were observed, mitochondria were disorganised or transformed from round to oval or oblong. Depending on how much of the inner conidium volume was occupied with lipids, the number of vacuoles decreased as lipid content increased. Conidia produced at 30°C had more complete integrity of nuclei, vacuoles and mitochondria, and had solid cell walls. The cell walls not separated from the plasma membranes as was observed in conidia produced at 20°C. The number of lipid droplets were similar to that seen in conidia produced at 20°C, and the lipid droplets in conidia with few droplets were dense with inclusion bodies.

Fresh conidia produced at C:N ratios of 5:1 versus 160:1: The external wall layers in conidia produced from growth medium containing a C:N ratio of 5:1 were thin compared with conidia produced at C:N ratio of 160:1, but had thicker internal wall layers. Conidia produced at C:N 160:1 had internal wall layers substituted with lipids. Plasma membranes in conidia produced at C:N 5:1 had a few indents but were not disrupted, while the plasma membranes of conidia from C:N 160:1 were disrupted with distinctive borders separated from wall layers and with accompanying lipid accumulation. The cytoplasm of conidia from the C:N 5:1 was more dense than that for conidia from the C:N 160:1 treatment. Nuclei in the conidia from C:N 5:1 were quite distinctive and large, but nuclei in conidia from the C:N 160:1 were much less distinct, and the nucleolus had moved to one side of the cytoplasm. The conidia produced from C:N 160:1 had many small lipid droplets, while those produced from C:N 5:1 usually contained one or two large lipid droplets.

Fresh conidia produced at 20°C-Dex versus after 6 month storage (20°C-Dex, 6 mo): Conidia produced from the 20°C-Dex growth treatment had thin external wall layers with the immediate internal wall layers occupied by lipids. For the conidia produced at 20°C-Dex after six months storage the external wall layers were also thin but the internal wall layers were stable and distinctive from the cytoplasm, with thin borders of lipid layers. Plasma membranes in conidia produced in the 20°C-Dex treatment were stable with a few indents. These conidia also had dense cytoplasm, numerous mitochondria, large nuclei and traces of lipid droplets, while the conidia after six months storage (20°C-Dex, 6 mo) had smaller mitochondria, smaller deformed nuclei, and greater numbers and larger lipid droplets.

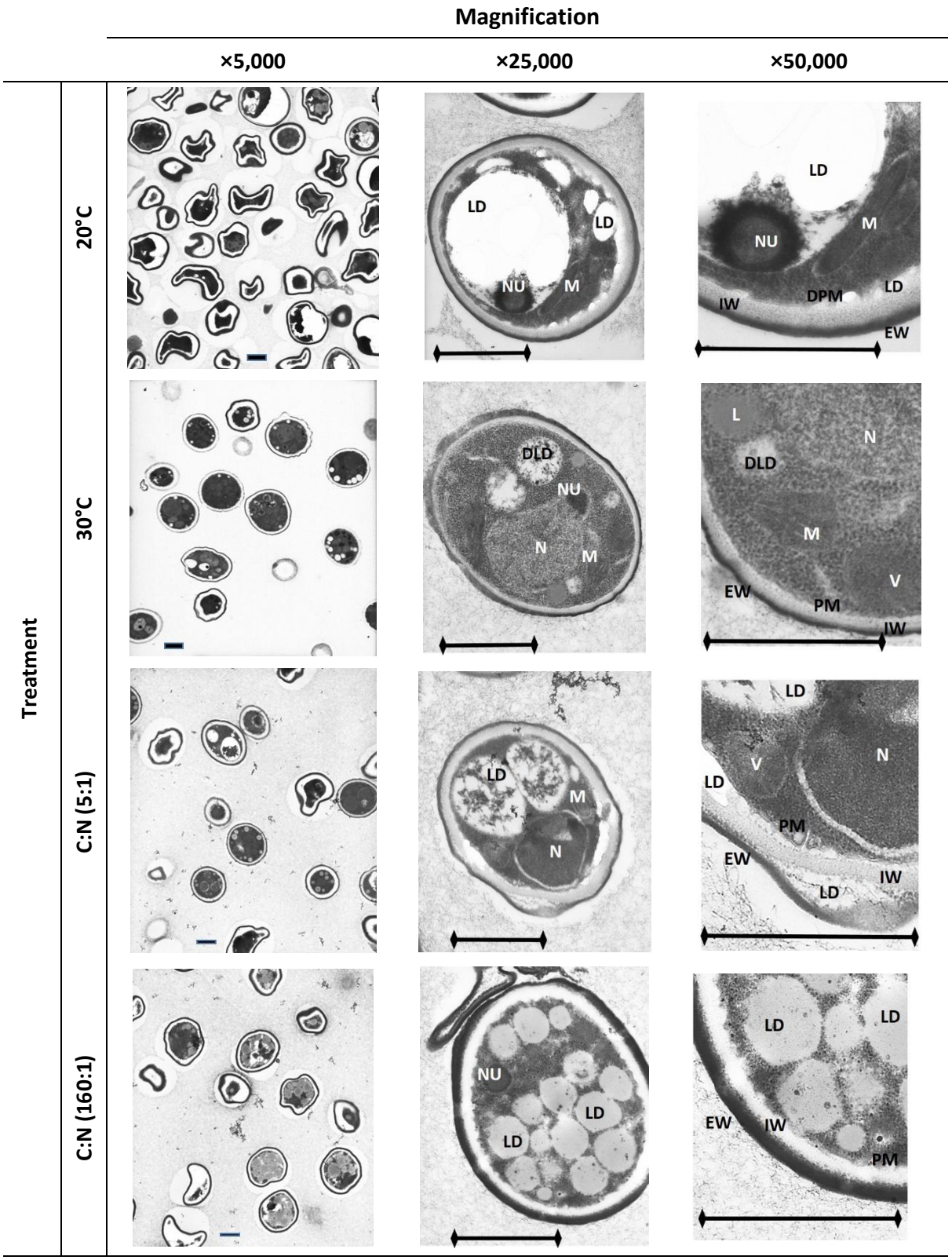
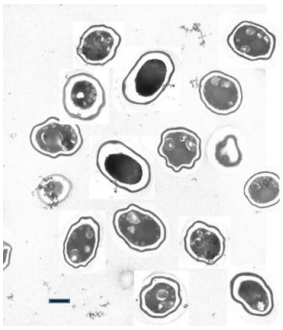
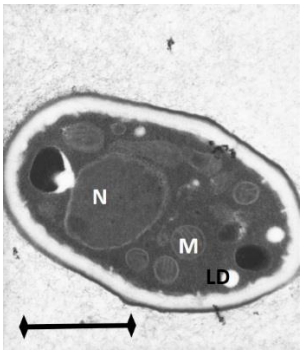
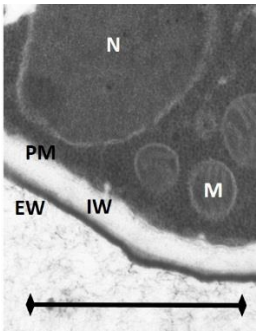
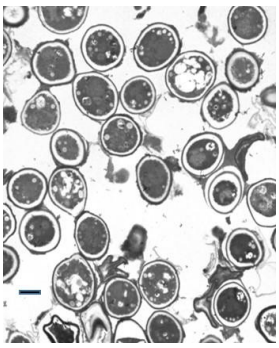
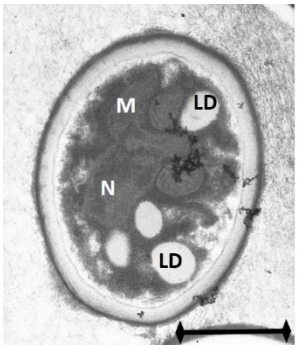
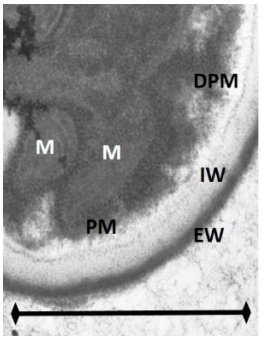


Figure 5.1 Electron micrographs of *Trichoderma atroviride* LU132 conidia produced from conidium production treatments of 20°C, 30°C, C:N ratio 5:1, C:N ratio 160:1 or at 20°C-Dex (produced at C:N ratio 5:1 at 20°C with dextrose) and also stored conidia of 20°C-Dex treatment after six months storage (20°C-Dex, 6 mo) at 30°C and 0% RH. Continued on the next page.

Continued from previous page

Treatment	Magnification ×5,000	×25,000	×50,000
20°C-Dex			
20°C-Dex, 6 mo			

DLD: dense lipid droplet, DPM: disrupted plasma membrane, EW: external wall layer, IW: internal wall layer, L: lomosome, LD: lipid droplet, M: mitochondrium, N: nucleus, NU: nucleolus, PM: plasma membrane, V: vesicle. Scale bars = 1 µm.

5.5 Discussion

***Sugar analyses*:** Analysis of the different sugar contents of *T. atroviride* LU132 conidia demonstrated that different culture conditions, including temperature, nutrients, water activity and pH, alone or in combinations (as seen in conidium production treatments in Chapter 4), significantly affected the concentrations of intracellular sugars in conidia, particularly their trehalose contents. Arabitol was the least abundant sugar detected in conidia. The levels of both glycerol and arabitol production are probably co-regulated in this fungus, as suggested by Luyten *et al*. (1995) and Hohmann *et al*. (2000). Glycerol was not detected in the present study. However, the amounts of this compound in the conidia could have been less than the limit of detection (>2 ppm) by HPLC used for the assays.

Of the sugars assayed, mannitol was produced by the conidia to the greatest amount compared with arabitol and trehalose. Accumulation of high molecular weight polyols such as mannitol have been proposed to suppress cellular enzyme activity (Chirife *et al*., 1984). However, low concentrations of mannitol have been suggested as being contributors to increased tolerance to water stress (Teixidó *et al*., 1998). In the present study, mannitol accumulation was generally greatest in conidia that gave the

least bioactivity after storage, except for the 30°C treatment. Much lower mannitol concentrations were detected for conidium production treatments such as C:N 5:1 and C:N 160:1, which gave optimum bioactivity after storage (Appendix 5.3). This indicates that lower concentration of mannitol could confer resistance in conidia to dry (0%) storage conditions during long periods after production. This result is consistent with a previous study by Teixidó *et al*. (1998). Furthermore, the role of mannitol in scavenging reactive oxygen species has been demonstrated, especially in plant pathogenic fungi (Smirnoff & Cumbes, 1989, Voegele *et al*., 2005). Mannitol in *Alternaria alternata* was shown to produce enzymes scavenging reactive oxygen species which protected the fungus from oxidative stress mediated by host plants. Jennings *et al*. (2002) showed that a mutant of tobacco, constitutively expressing manitol dehydrogenase, was able to catabolise mannitol of fungal origin resulting in no plant infection. In the present study, conidia produced at 30°C experienced several stresses including high temperature followed by water shortage and oxidative stress. These conditions gave conidia with high bioactivity and survival during storage. Thus, it is likely that high levels of accumulated mannitol in conidia produced at 30°C protected them from oxidation stress during the periods of storage.

High trehalose concentrations were detected in the conidia with the greatest bioactivity, suggesting that trehalose supported conidium fitness, although this was not the case for some conidia which contained high concentrations of trehalose (Table 5.2 and Appendices 5.1 and 5.2). For example, conidia from the 1.2 mM trehalose production treatment, containing high trehalose concentrations (53 mg/g of dry conidia), or similarly pH 7.5 conidia (55 mg/g of dry conidia), and these conidia did not show reasonable bioactivity after storage. From the results outlined in Chapter 3, it was suggested that excessive sucrose as a carbon source during conidium production inhibited absorption of trehalose into *T. atroviride* LU132 cells during growth and development. However, no expected beneficial effects of trehalose addition allowing them to withstand unfavourable environmental conditions during storage were observed for the conidia produced with 1.2 mM trehalose. This may have resulted from the conidia being in a state of deep dormancy following rapid drying before storage. The negative effects of rapid drying and subsequent rehydration of fungal conidia have been studied previously (Moore *et al*., 1997; Hong *et al*., 2000; Friesen *et al*., 2005). These studies have demonstrated that for formulation development of fungal BCAs, the conidia must be dried to induce dormancy which increases the shelf-life and also protects the resulting formulation from microbial contamination (Jin & Custis, 2011). However, dormancy results in physical separation of endogenous reserves (e.g. trehalose) from metabolic enzyme (trehalase) (e.g. Mandels and Maguire, 1972). Some of the conidia in the present study containing high concentrations of trehalose did not show expected bioactivity in relation to their trehalose content. Furthermore, high storage temperature (30°C) could

have elevated the levels of dormancy as an immediate or gradual response to the stresses of drying, at least for some of the test conidia.

Induction of trehalose was not solely correlated to heat shock (as has been demonstrated by Pedreschi *et al*., 1997) or external trehalose addition (as has been demonstrated by Kets & Bonts, 1994; Kets *et al*., 1996; Pedreschi *et al*., 1997; Bonaterra *et al*., 2005), as elevated levels were also recorded for some other conidia, such as those from the C:N 160:1 and pH 7.5 production treatments. Furthermore, increased trehalose accumulation was not observed for conidia produced under other cultural conditions when temperature was the only treatment difference applied, as for the treatments used in experiments described in Chapter 4 (20°C-Dex, 20°C-Suc, 30°C-Dex). An exception was the 30°C-Suc treatment. This indicates that while temperature is a key factor affecting trehalose content of conidia the high trehalose content could be supported in the presence of sucrose as a carbon source in the 30°C-Suc (50 mg/g of dry conidia) treatment compared with dextrose in 30°C-Dex (32 mg/g of dry conidia). However, for all of these four conidium production treatments, high levels of bioactivity after six months of storage were seen compared with other treatments (Appendix 5.3).

Fatty acid analyses: The results of the fatty acid analyses for conidia produced in different culture conditions showed the close relationship between culture conditions composition of fatty acids in conidia. Furthermore, the results showed qualitative and quantitative changes in fatty acid composition during culture production and storage. *Trichoderma atroviride* LU132 is categorised as a mesophilic fungus with optimum conidium production occurring at approximately 25°C. Mesophilic and psychrophilic fungi are similar in their proportions of unsaturation of cellular lipids, while thermophilic species contain less unsaturated lipids (Sumner *et al*., 1969). Saturated fatty acids are more stable than unsaturated fatty acids and may support greater longevity during storage, while greater proportions of unsaturated lipids (reflected as the number of double bonds in lipid molecules) rather than saturated lipids result in less stability and more susceptibility to oxidation (Rustan & Drevon, 2005). It has also been demonstrated that linoleic acid (18:2 *c*9, 12) is a major membrane component in conidia of *T. harzianum*, and decreased concentration of this fatty acid probably indicated conidium senescence or deterioration (Serrano-Carreon *et al*., 1992).

There are contradictory findings regarding effects of unsaturated fatty acids on microorganism shelf life. For example, high concentrations of linoleic acid (18:2 *c*9, 12) have been related to increased membrane sensitivity to peroxidation and cellular senescence (May & McCay, 1968; Mowri *et al*., 1984), while the percentage of unsaturated fatty acid content of *Trichoderma* conidia in the present study, and also in that of Agosin *et al*. (1997), were not found to be correlated with long term stability. In general, most of the tested conidia contained similar amounts of linoleic acid and also high levels

of unsaturation, at least for conidia produced from the growth treatments of 20°C-Dex (60% unsaturation) or 30°C (59% unsaturation).

High concentrations of total fatty acids are likely to reflect low bioactivity during storage for 20°C treatments (66 µg/g of dry conidia), resulting in deep dormancy or deterioration effects (Appendix 5.3). However, this result did not correlate with the amount of unsaturation. It has been demonstrated that low temperature (≤ 20°C) prompts accumulation of polyunsaturated fatty acids (Levin, 1972; Duan *et al*., 2011), which are susceptible to oxidation resulting in rapid conidium senescence (Rustan & Drevon, 2005). Furthermore, the large percentage of unsaturation for the 30°C conidium production treatment of was unexpected, as it was expected that saturated fatty acids would accumulate in these conidia.

When *T. atroviride* LU132 colonies were grown at a high carbon to nitrogen ratio (i.e. 160:1), which has been suggested to be detrimental to protein synthesis (Weete, 1980), the lipid content of the conidia was increased but a high sugar concentration in C:N 160:1 conidia resulted in protective effects during storage (Appendix 5.3). In fungi, it has been shown that high C:N ratio is associated with increased lipid accumulation (Weete, 1980). Conversion of excess carbon to lipids in conidia (Jackson, 1997) has been associated with excess glucose in culture media (Prill *et al*., 1935; Ward *et al*., 1935), and it has been suggested that high levels of carbon supply are needed for lipid synthesis (Woodbine, 1959). For example, increased carbohydrate concentration in the culture medium for *Aspergillus parasiticus* gave increased total lipid content (Shih & Marth, 1974).

High levels of unsaturated fatty acids were accumulated in 20°C-Dex conidia (60%) produced in culture medium with a C:N 5:1 (4.2 g/L carbon, dextrose), while conidia produced on media amended with sucrose had less unsaturated fatty acids. This agrees with the results of Ward *et al*. (1935), where simple sugars such as dextrose (glucose), rather than sucrose, were preferred for conversion to fatty acids.

***Ultrastructural analyses*:** The results of electron microscope examination revealed that *T. atroviride* LU132 conidia have different cell ultrastructure as they develop under different culture conditions, and also when stored for a long period. Ultrastructural changes could therefore be related to culture growth medium differences and also to variations in conidium fitness.

Conidia produced at 20°C showed the least viability and bioactivity after storage. Ultrastructural observations indicated that these characteristics could be related to disorganisation of conidium contents, malformation, and reduced amounts of less electron dense lipid droplets, while these conidia contained the greatest accumulation of fatty acids (66 µg/g of dry conidia) compared with other conidium production treatments assessed in this study. Conidia produced at 20°C showed the

least storage capability, indicating low heat tolerance to 30°C during storage following desiccation at 0% RH. It has been demonstrated that water shortage results in weak interactions between biological molecules in cells, since water is a key biological solvent (Szent-Gyorgyi, 1964). Desiccation will, in turn, cause accumulation of non-aqueous macromolecules such as lipids, resulting in frequent cellular vacuolisation (Buckley *et al*., 1966). Regarding the results of the storage experiments in the present study, production of the *T. atroviride* LU132 at 20°C did not support conidium viability and bioactivity after six months storage at 30°C and 0% RH. Conidia that can resist unfavourable conditions may have physical attributes (e.g. strengthened cell walls) or have accumulated beneficial compounds (e.g. trehalose), to allow them to resist drying and unfavourable storage conditions. Trehalose analysis for this treatment showed 13 mg/g of dry conidia, which was the least amount compared with other conidium production treatments.

Conidium production at 30°C gave conidia that were able to withstand stresses during storage while maintaining reasonable viability, apparently at least in part supported by structural properties. The culture conditioning at 30°C resulted in conidia that were possibly thermo-, osmo-, and oxidation-adapted. These conidia had electron dense cytoplasmic contents. Although the number of lipid droplets were less these were denser and retracted cytoplasm was not common, compared with that observed in conidia produced at 20°C. This suggests that dense lipid droplets are sources of energy supporting the viability of *Trichoderma* conidia produced at 30°C. Similar results have been reported in *Saccharomyces cerevisiae*. Athenstaedt *et al*. (1999) showed that lipid droplets in this yeast could store proteins, which could, under certain circumstances, become active. For instance, the Erg1p enzyme is a protein which has been shown to be stored in lipid droplets and is involved in steroid metabolism.

In the present study, accumulated lipid droplets could have been sources of energy for conidia produced at C:N 5:1 and 160:1, both of which were shown to contain highly electron dense lipids. Both of these conidium production treatments gave reasonable to optimum bioactivity after storage, but fresh conidia from the 160:1 C:N treatment resulted in low levels of productivity, germinability and bioactivity (indicated in Chapter 2). It has been demonstrated that a high C:N ratio is associated with low rates of protein synthesis and high lipid production, while a low C:N ratio is associated with protein metabolism (Weete, 1980). This is because at high C:N ratios, amino acid sources will be rapidly exhausted prior to carbohydrates, so that increased lipids is correlated with the presence of lipid droplets in conidia, apparently resulting from the conversion of excess carbon to lipids (Jackson, 1997). In the present study, from the large number of dense lipid droplets observed in *T. atroviride* LU132 conidia, it could be expected that these would support high levels of viability and bioactivity during storage, which were evident from the results from effects of long term storage. When the temperature

for production was 20°C and the C:N ratio was 5:1, the assessment of dextrose *versus* sucrose showed that dextrose in the culture medium was superior, giving greater viability and bioactivity compared with other combinations of temperature and sugars. The conidia produced from the 20°C-Dex treatment had structural characteristics of highly dense cytoplasm that was related to optimum production, shelf life and bioactivity.

This set of experiments has defined nutritional and physical growth conditions that affect conidium fitness in *T. atroviride* LU132 as a BCA, which were detected as physiological changes giving biochemical and ultrastructural differences. Moreover, physiological changes giving bioactivity variations resulted from manipulation of culture growth conditions for conidium production. These results are further confirmation that nutrient requirements of *T. atroviride* LU132 colonies are more important for production of high quality conidia than specific physical environmental conditions, such as temperature.

5.6 Summary of key results:

- Low trehalose content in conidia was associated with the least conidium fitness, although high trehalose content did not necessarily support conidium fitness.
- High proportions of total fatty acids in conidia were associated with the least conidium fitness. However, low proportion of fatty acids was not always associated with high levels of conidium fitness.
- Total fatty acids in conidia was increased when they were grown at high carbon to nitrogen ratio.
- Maximum unsaturation of fatty acids was related to greatest conidium fitness.
- Structurally sound conidia showed greatest fitness and survival during storage.
- Dense content of conidium cytoplasm was associated with the greatest conidium fitness (stability and bioactivity).
- Large numbers of lipid droplets in conidia were associated with less conidium fitness, unless the lipid droplets were dense.
- Optimised nutritional requirements are likely to be more important than manipulation of physical environmental conditions for production of stable and bioactive conidia of *T. atroviride* LU132.

5.7 References

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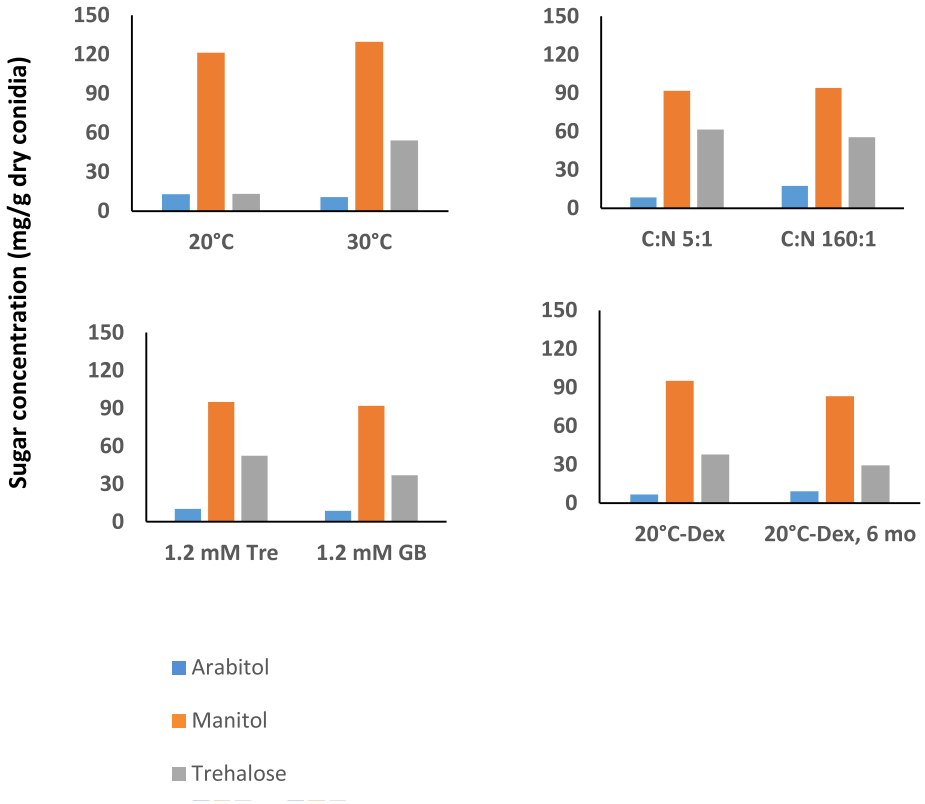
5.8 Appendices

Appendix 5.1 Mean concentrations of sugars (arabitol, mannitol and trehalose: mg/g of dry conidia) in *Trichoderma atroviride* LU132 conidia, either freshly harvested or after storage at 0% RH and 30°C for six months (6 mo) for valid treatment comparisons. These include: conidia produced at 20°C or 30°C on PDA, at C:N ratios 5:1 or C:N 160:1 amended with sucrose, at C:N 5:1 amended with sucrose and trehalose (1.2 mM Tre) or sucrose and glycine-betaine (1.2 mM GB), and at C:N 5:1 and 20°C and amended with dextrose (20°C-Dex) as fresh and after storage for six months (20°C-Dex, 6 mo).



Appendix 5.2 Mean amounts of sugars (mg/g of dry conidia) in *Trichoderma atroviride* LU132 conidia produced in different culture conditions (see Table 3.1), either freshly harvested or after storage at 0% RH and 30°C for six months (6 mo).

Treatment[1]	Sugar		
	Arabitol	Mannitol	Trehalose
20°C	13	121	13
30°C	11	130	54
a_w 0.985	6	144	24
a_w 0.961	7	104	32
C:N 5:1	8	92	62
C:N 160:1	17	94	55
1.2 mM Tre	10	95	53
1.2 mM GB	9	92	37
pH 5.5	21	144	37
pH 7.5	12	127	55
20°C-Dex	7	95	38
20°C-Suc	7	99	34
30°C-Dex	5	81	32
30°C-Suc	6	118	50
20°C-Dex, 6 mo	9	83	29
20°C-Suc, 6 mo	9	117	35
30°C-Dex, 6 mo	8	109	48
30°C-Suc, 6 mo	4	78	23
$LSD_{0.05}$	1.3	24.6	1.2
Overall mean	9	107	39
Significance of contrasts between valid comparisons[2]			
20°C *vs* 30°C	**	ns	***
C:N 5:1 *vs* C:N 160:1	***	ns	***
1.2 mM Tre *vs* 1.2 mM GB	ns	ns	***
20°C-Dex *vs* 20°C-Suc	ns	ns	***
30°C-Dex *vs* 30°C-Suc	ns	*	***
20°C-Dex *vs* 30°C-Dex	ns	ns	***
20°C-Suc *vs* 30°C-Suc	ns	ns	***
20°C-Dex *vs* 20°C-Dex, 6 mo	**	ns	***
20°C-Suc *vs* 20°C-Suc, 6 mo	**	ns	ns
30°C-Dex *vs* 30°C-Dex, 6 mo	ns	ns	***
30°C-Suc vs 30°C-Suc, 6 mo	**	**	***

[1] 20°C or 30°C conidia produced on PDA at 20°C or 30°C, Tre: trehalose, GB: glycine-betaine; 20°C-Dex or 30°C-Dex: conidia produced at 20°C or 30°C, amended with dextrose; 20°C-Suc or 30°C-Suc: conidia produced at 20°C or 30°C, amended with sucrose.

[2] *, **, ***: the effects of treatments are statistically significant at P = 0.05, 0.01, 0.001, respectively; ns: not significant (P > 0.05).

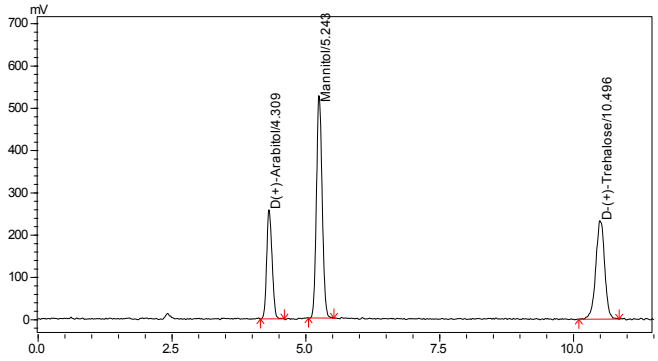
Appendix 5.3 Mean germination and bioactivity percentages of *Trichoderma atroviride* LU132 conidia produced in different culture conditions (see Table 3.1), using AUC (as described in Chapter 2, Section 2.2.1.7), either freshly harvested or after storage at 0% RH and 30°C for six months (6 mo). These data were obtained from culture conditions outlined in Chapter 3 and 4.

Treatment[1]	%Germination[2] (Fresh)	%Germination (6 mo)	%Bioactivity (Fresh)	%Bioactivity (6 mo)
20°C	55	9	74	28
30°C	74	44	77	66
a_w 0.985	21	17	68	56
a_w 0.961	78	31	74	54
C:N 5:1	79	48	77	65
C:N 160:1	49	47	61	63
1.2 mM Tre	78	36	75	55
1.2 mM GB	55	27	63	52
pH 5.5	63	18	69	46
pH 7.5	86	21	76	51
20°C-Dex	88	76	79	71
20°C-Suc	80	63	72	58
30°C-Dex	74	55	70	61
30°C-Suc	82	69	75	66

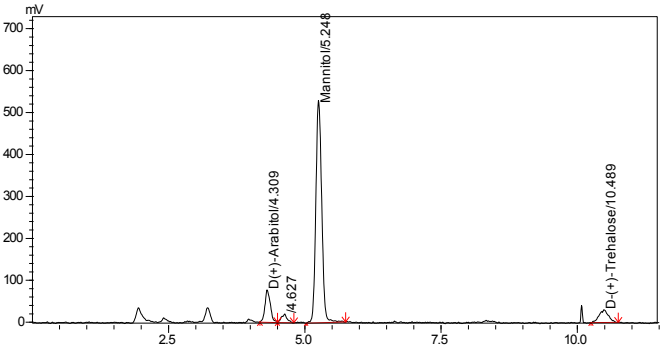
[1] 20°C or 30°C conidia produced on PDA at 20°C or 30°C, Tre: trehalose, GB: glycine-betaine; 20°C-Dex or 30°C-Dex: conidia produced at 20°C or 30°C, amended with dextrose; 20°C-Suc or 30°C-Suc: conidia produced at 20°C or 30°C, amended with sucrose.
[2] AUC: data is based on area under the curve.

Appendix 5.4 HPLC chromatographs of standard sugars: arabitol (20 ppm), mannitol (40 ppm) and trehalose (40 ppm), and sugars extracted from in *Trichoderma atroviride* LU132 conidia, either freshly harvested or after storage at 0% RH and 30°C for six months (6 mo) for valid treatment comparisons. These include: conidia produced at 20°C or 30°C on PDA, at C:N ratios 5:1 or C:N 160:1 ameded with sucrose, at C:N 5:1 amended with sucrose and trehalose (1.2 mM Tre) or sucrose and glycine-betaine (1.2 mM GB), and at C:N 5:1 and 20°C and amended with dextrose (20°C-Dex) as fresh and after storage for six months (20°C-Dex, 6 mo). Vertical axis is HPLC column absorbance (mV) and horizontal axis is retention time (min).

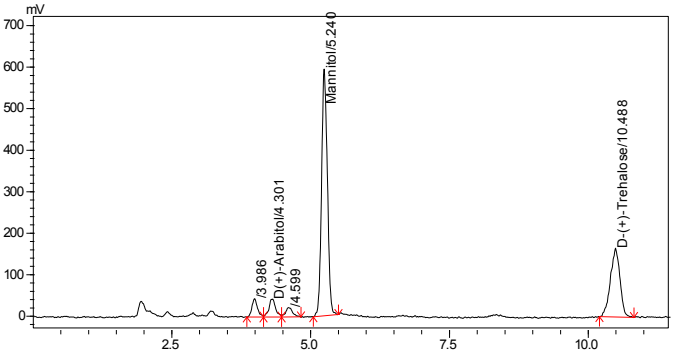
Standards



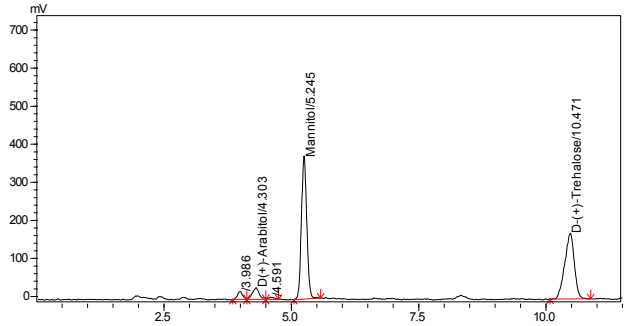
20°C



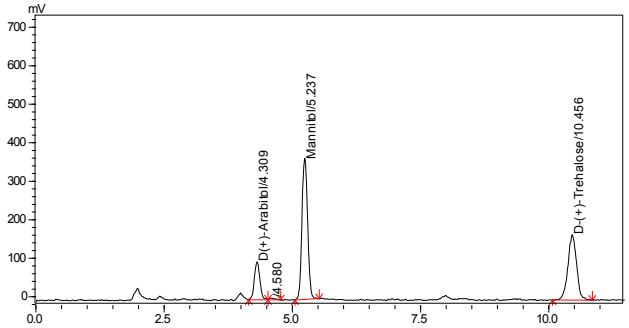
30°C



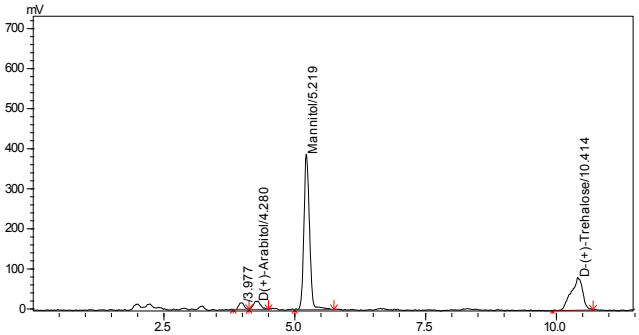
C:N ratio 5:1



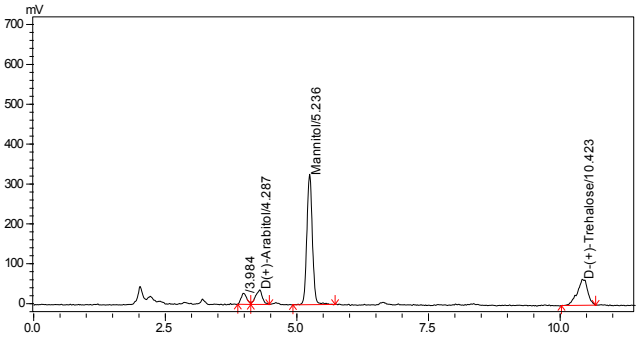
C:N ratio 160:1



20°C-Dex



20°C-Dex, 6 mo



Appendix 5.5 List of standards of fatty acids and proportions (%) of each fatty acid in each set of standards.

ME61 (1 mg/injection)	
Compound	**(%)**
C4:0	4
C6:0	2
C8:0	1
C10:0	3
C12:0	4
C14:0	10
C14:1 *c*9	2
C16:0	25
C16:1 *c*9	5
C18:0	10
C18:1 *c*9	25
C18:2 *c*9,12	3
C18:3 *c*9,12,15	4
C20:0	2

ME93 (1 mg/injection)	
Compound	**(%)**
C14:1 *t*9	5
C14:1 *c*9	10
C15:0 iso	3
C15:1	2
C16:1 *t*9	9.8
C17:0 iso	3
C17:1	5.2
C18:0 anteiso	2
C18:1 *t*11	10
C18:1 *c*6	4.9
C18:1 *c*11	5.1
C18:2 *t*9,12	5
C19:0	10
C18:3 *c*6,9,12	2
C20:1 *t*11	3
C22:1 *t*13	10
C24:0	5
C26:0	5

BR2 (0.25 mg/injection)	
Compound	**(%)**
C13:0 anteiso	12.5
C13:0	12.5
C14:0 iso	12.5
C14:0	12.5
C15:0 anteiso	12.5
C15:0	12.5
C16:0 iso	12.5
C16:0	12.5

BR3 (0.25 mg/injection)	
compound	**(%)**
C15:0 anteiso	12.5
C15:0	12.5
C16:0 iso	12.5
C16:0	12.5
C17:0 anteiso	12.5
C17:0	12.5
C18:0 iso	12.5
C18:0	12.5

GLC411 (1 mg/injection)	
Compound	**(%)**
C8:0	3.23
C10:0	3.23
C12:0	3.23
C12:1	3.23
C14:0	3.23
C14:1 *c*9	3.23
C16:0	3.23
C16:1 *c*9	3.23
C18:0	3.23
C18:1 *c*6	3.23
C18:1 *c*9	3.23
C18:1 *c*11	3.23
C18:2 *c*9,12	3.23
C18:3 *c*6,9,12	3.23
C18:3 *c*9,12,15	3.23
C20:0	3.23
C20:1 *c*5	3.23
C20:1 *c*8	3.23
C20:1 *c*11	3.23
C20:3 *c*8,11,14	3.23
C20:4 *c*5,8,11,14	3.23
C20:3 *c*11,14,17	3.23
C22:0	3.23
C22:1 *c*13	3.23
C22:2 *c*13,16	3.23
C22:3	3.23
C22:4 n-6	3.23
C24:0	3.23
C24:1 *c*15	3.23
C22:6 *c*4, 7, 10, 13, 16, 19	3.23

Continued from list of standards

GLC463 (1 mg/injection)				CLA *c*9 t11 (0.061 mg/injection)	
Compound	**(%)**	**Compound**	**(%)**	**Compound**	**(%)**
C4:0	1	C18:1 *t*11	1	CLA *c*9 t11	100
C5:0	1	C18:1 *c*6	1		
C6:0	1	C18:1 *c*9	4	**CLA C23:0 (0.126 mg/injection)**	
C7:0	1	C18:1 *c*11	1	**Compound**	**(%)**
C8:0	2	C18:2 *t*9,12	2	CLA C23:0	100
C9:0	1	C18:2 *c*9,12	4		
C10:0	2	C19:0	1		
C11:0	1	C19:1	1		
C11:1	1	C18:3 *c*6,9,12	1		
C12:0	4	C18:3 *c*9,12,15 (ALA)	4		
C12:1	2	C20:0	4		
C13:0	1	C20:1 *c*5	2		
C13:1	1	C20:1 *c*8	2		
C14:0	4	C20:1 *c*11	2		
C14:1 *c*9	2	C20:2 *c*11,14	2		
C15:0	1	C20:3 *c*8,11,14	1		
C15:1	1	C20:4 *c*5,8,11,14	1		
C16:0	4	C20:3 *c*11,14,17	2		
C16:1 *t*9	1	C22:0	2		
C16:1 *c*9	4	C22:1 *c*13	4		
C17:0	2	C20:5 *c*5,8,11,14,17 (EPA)	2		
C17:1	2	C22:2 *c*13,16	1		
C18:0	4	C22:3	2		
C18:1 *t*9	1	C22:4	1		
		C24:0	2		
		C24:1 *c*15	1		
		C22:5 *c*7,10,13,16,19 (DPA)	2		
		C22:6 *c*4,7,10,13,16,19 (DHA)	2		

Appendix 5.6 Mean total and main fatty acids contents (µg/g of dry conidia) and the mean percentage unsaturation in of *Trichoderma atroviride* LU132 conidia produced in different culture conditions (see Table 3.1), either freshly harvested or after storage at 0% RH and 30°C for six months (6 mo).

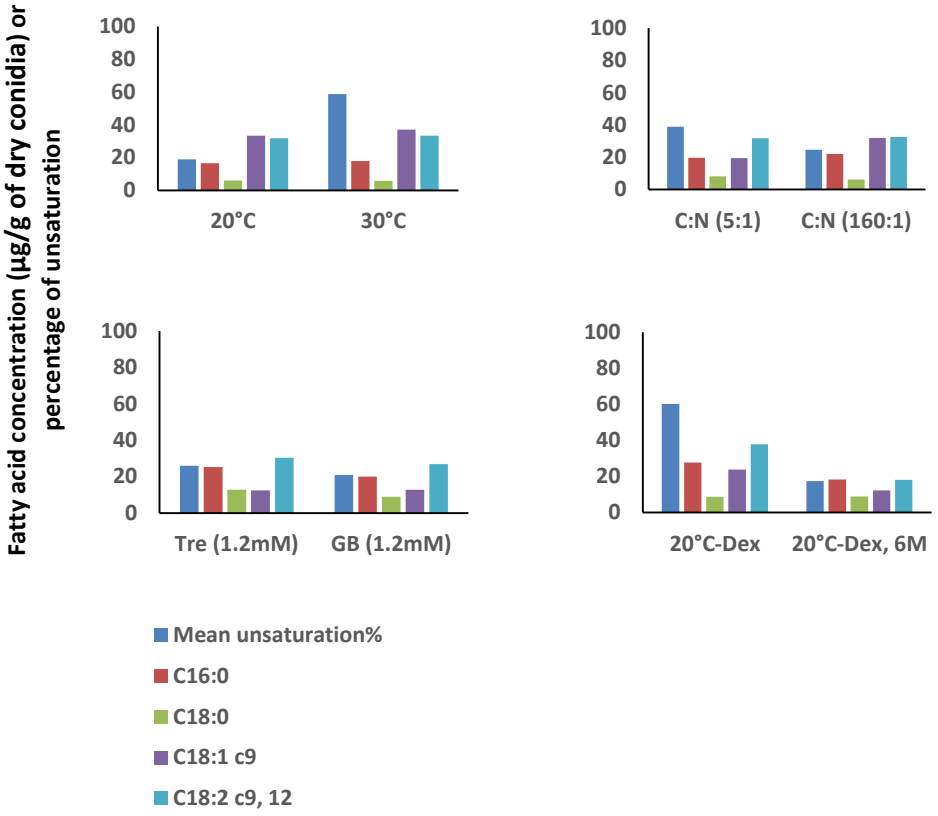
Treatment[1]	Total FA[2]	%Mean unsaturation	C16:0	C18:0	C18:1 *c*9	C18:2 *c*9, 12
20°C	66	19	17	6	33	32
30°C	35	59	18	6	37	33
a_w 0.985	32	33	19	7	34	27
a_w 0.961	31	27	20	6	34	30
C:N 5:1	12	39	20	8	19	32
C:N 160:1	48	25	22	6	32	33
1.2 mM Tre	15	26	25	13	12	30
1.2 mM GB	25	21	20	9	13	27
pH 5.5	32	21	20	9	26	26
pH 7.5	38	16	20	9	26	23
20°C-Dex	20	60	28	9	24	38
20°C-Suc	48	14	18	6	20	30
30°C-Dex	19	16	18	11	10	19
30°C-Suc	26	16	19	9	13	22
20°C-Dex, 6 mo	27	17	18	9	12	18
20°C-Suc, 6 mo	23	27	18	8	13	16
30°C-Dex, 6 mo	23	26	18	8	13	18
30°C-Suc, 6 mo	27	23	17	9	13	15
$LSD_{0.05}$	0.5	3	0.6	0.4	0.4	0.4
Overall mean	-	-	20	8	21	26
Significance of contrasts between valid comparisons[3]						
20°C *vs* 30°C	***	***	**	ns	***	**
C:N 5:1 *vs* C:N 160:1	***	***	***	***	***	**
1.2 mM Tre *vs* 1.2 mM GB	***	***	***	***	ns	***
20°C-Dex *vs* 20°C-Suc	***	***	***	***	***	***
30°C-Dex *vs* 30°C-Suc	***	***	***	***	***	***
20°C-Dex *vs* 30°C-Dex	***	ns	**	***	***	***
20°C-Suc *vs* 30°C-Suc	***	ns	**	***	***	***
20°C-Dex *vs* 20°C-Dex, 6 mo	***	***	***	ns	***	***
20°C-Suc *vs* 20°C-Suc, 6 mo	***	***	ns	***	***	***
30°C-Dex *vs* 30°C-Dex, 6 mo	***	***	ns	***	***	**
30°C-Suc vs 30°C-Suc, 6 mo	**	***	***	ns	ns	***

[1] 20°C or 30°C conidia produced on PDA at 20°C or 30°C, Tre: trehalose, GB: glycine-betaine; 20°C-Dex or 30°C-Dex: conidia produced at 20°C or 30°C, amended with dextrose; 20°C-Suc or 30°C-Suc: conidia produced at 20°C or 30°C, amended with sucrose.

[2] FA: fatty acid.

[3] *, **, ***: the effects of treatments are statistically significant at $P = 0.05, 0.01, 0.001$, respectively; ns: not significant ($P > 0.05$).

Appendix 5.7 Mean percentages of fatty acid unsaturation and concentration (µg/g of dry conidia) of main fatty acids (C16:0, C18:0, C18:1 *c*9, C18:2 *c*9 12) in *Trichoderma atroviride* LU132 conidia, either freshly harvested or after storage at 0% RH and 30°C for six months (6 mo) for valid treatment comparisons. These include: conidia produced at 20°C or 30°C on PDA, at C:N ratios 5:1 or C:N 160:1 amended with sucrose, at C:N 5:1 amended with sucrose and trehalose (1.2 mM Tre) or sucrose and glycine-betaine (1.2 mM GB), and at C:N 5:1 and 20°C and amended with dextrose (20°C-Dex) as fresh and after storage for six months (20°C-Dex, 6 mo).



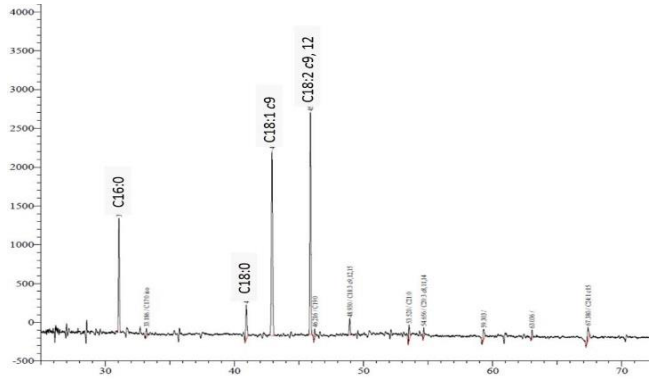
Appendix 5.8 Mean fatty acids (µg/g of dry conidia) in trace amounts in *Trichoderma atroviride* LU132 conidia produced in different culture conditions (see Table 3.1), either freshly harvested or after storage at 0% RH and 30°C for six months (6 mo).

Treatment[1]	C17:0 iso	C18:3 *c*9,12, 15	C19:0	C20:2 *c*11, 14	C20:5 *c*5, 8, 11, 14, 17	C21:0	C23:0
20°C[2]	0	2	2	0	0	3	0
30°C	0	0	0	0	0	0	0
aw(0.985)	0	0	0	0	5	0	0
aw(0.961)	0	0	2	0	3	2	2
C:N (5:1)	0	0	0	0	6	0	3
C:N (160:1)	0	0	1	0	1	0	1
Tre (1.2 mM)	2	0	3	0	3	0	4
GB (1.2 mM)	0	0	2	2	3	0	2
pH (5.5)	1	0	2	2	2	0	2
pH (7.5)	0	0	2	2	2	0	1
20°C-Dex	1	0	0	0	0	0	0
20°C-Suc	3	3	2	2	2	0	2
30°C-Dex	3	0	5	4	3	0	4
30°C-Suc	0	0	3	3	2	0	2
20°C-Dex, 6 mo	0	1	2	0	0	2	0
20°C-Suc, 6 mo	3	0	2	0	0	2	0
30°C-Dex, 6 mo	0	0	6	0	0	6	3
30°C-Suc, 6 mo		0	3	0	0	3	2
$LSD_{0.05}$	0.1	0.1	0.3	0.2	0.4	0.2	0.3
Overall mean	1	2	2	1	2	1	1

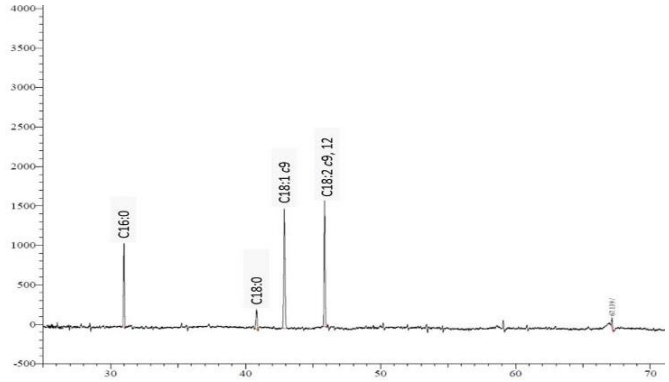
[1] Tre: trehalose, GB: glycine-betaine; 20°C-Dex or 30°C-Dex: conidia produced at C:N (5:1) at 20°C or 30°C, amended with dextrose (Dex) or sucrose (Suc).
[2]20°C or 30°C conidia produced on PDA at 20°C or 30°C

Appendix 5.9 Gas chromatographs of fatty acids analysis for *Trichoderma atroviride* conidia, either freshly harvested or after storage at 0% RH and 30°C for six months (6 mo) for valid treatment comparisons. These include: conidia produced at 20°C or 30°C on PDA, at C:N ratios 5:1 or C:N 160:1 amended with sucrose, at C:N 5:1 amended with sucrose and trehalose (1.2 mM Tre) or sucrose and glycine-betaine (1.2 mM GB), and at C:N 5:1 and 20°C and amended with dextrose (20°C-Dex) as fresh and after storage for six months (20°C-Dex, 6 mo). Vertical axis is GC column absorbance (mV) and horizontal axis is retention time (min).

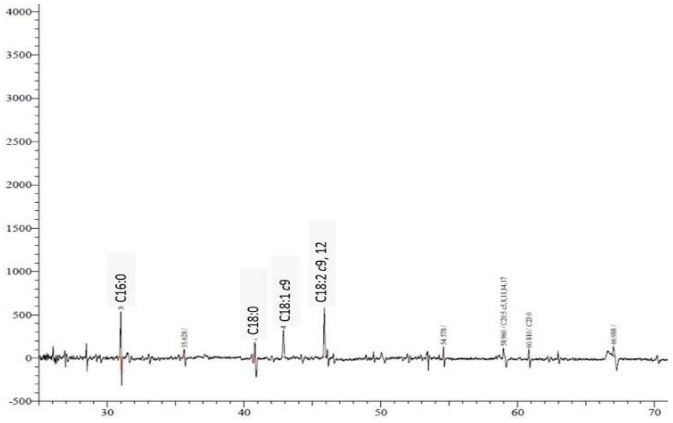
20°C



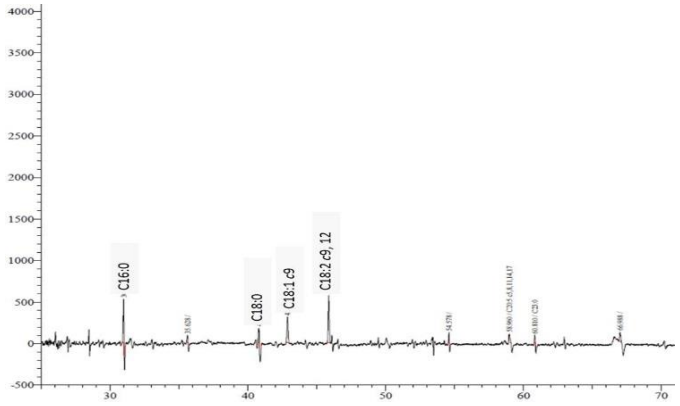
30°C



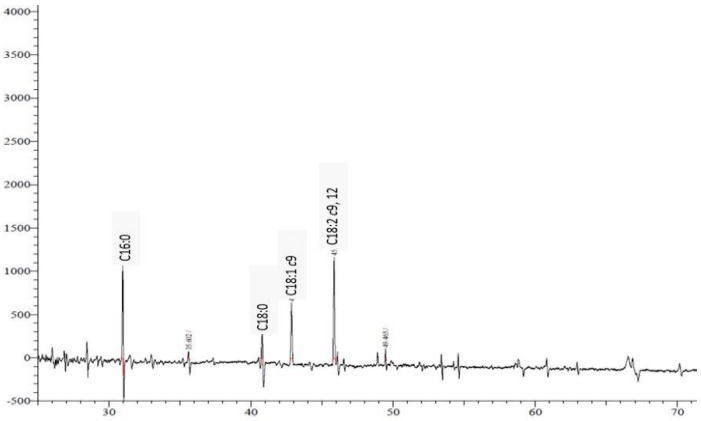
C:N ratio5:1



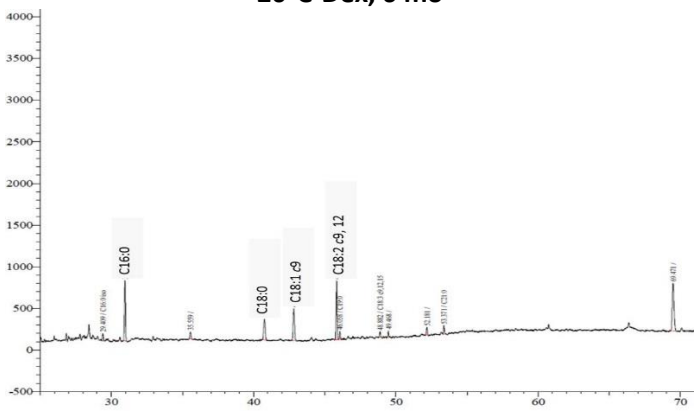
C:N ratio160:1



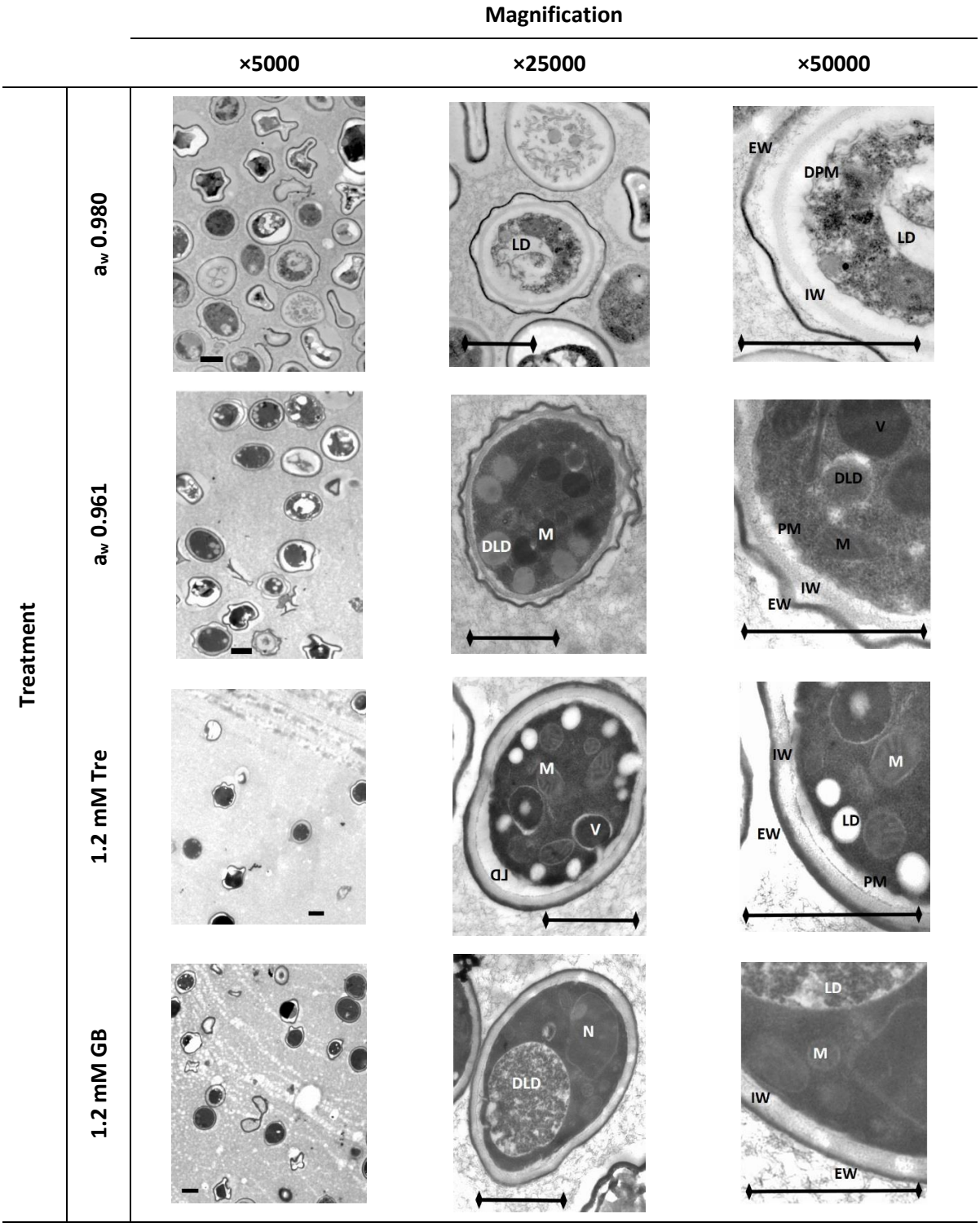
20°C-Dex



20°C-Dex, 6 mo



Appendix 5.10 Electron micrographs of *Trichoderma atroviride* LU132 conidia produced at conidium production treatments of a_w 0.985, a_w 0.961, 1.2 mM Tre amended with sucrose and trehalose, 1.2 mM GB amended with sucrose and glycine-betaine, pH 5.5, pH 7.5, 20°C-Suc, 30°C-Dex, 30°C-Suc (produced at C:N 5:1 at 20°C or 30°C with dextrose or sucrose) and also stored conidia for six months (20°C-Suc, 6 mo; 30°C-Dex, 6 mo; 30°C-Suc,6 mo) at 30°C and 0% RH.



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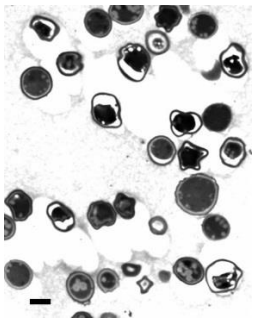
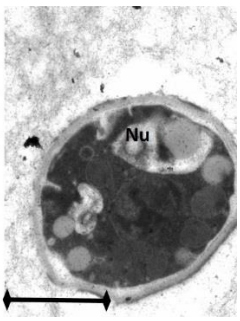
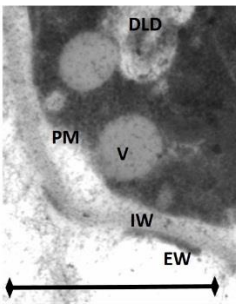
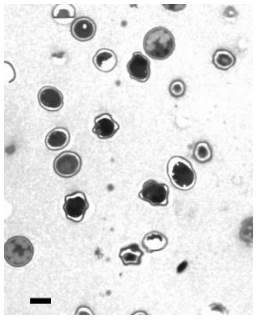
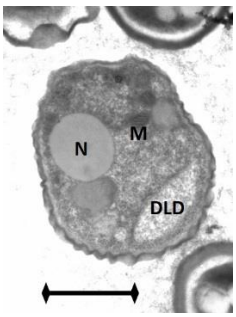
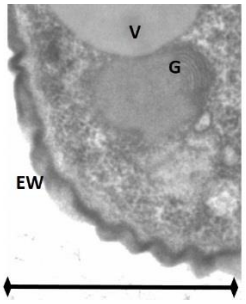
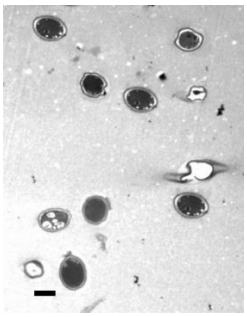
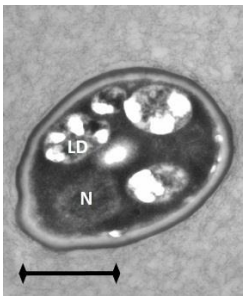
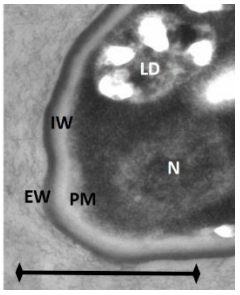
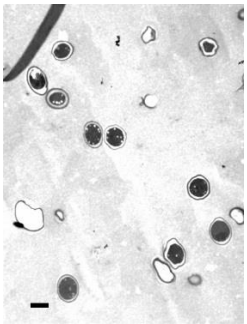
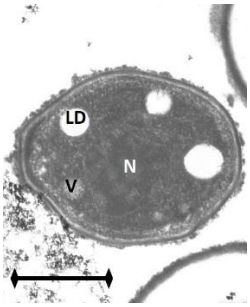
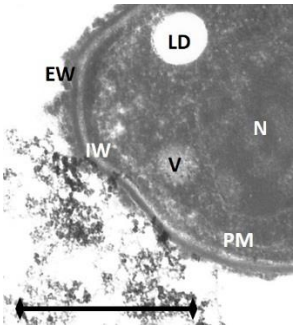
Treatment	Magnification		
	×5000	×25000	×50000
pH 5.5			
pH 7.5			
20°C-Suc			
20°C-Suc, 6 mo			

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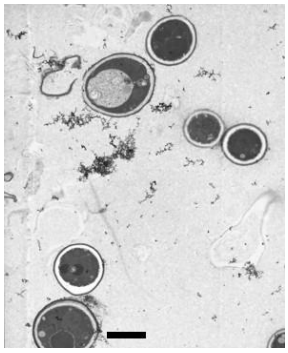
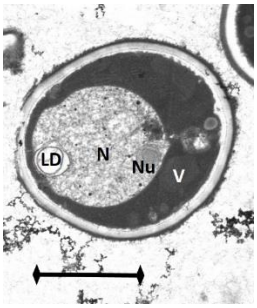
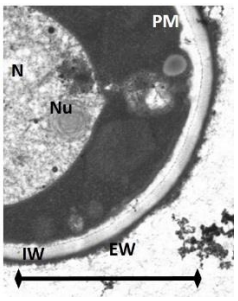
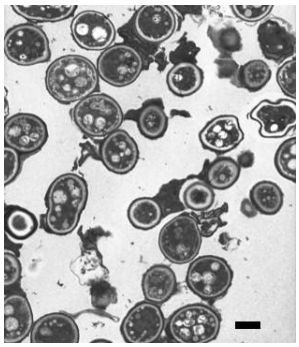
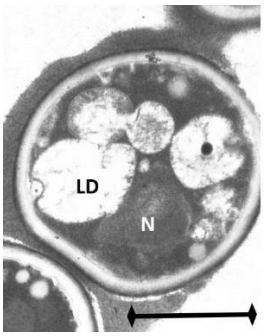
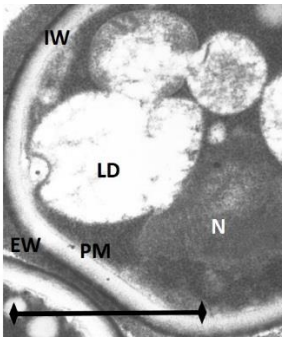
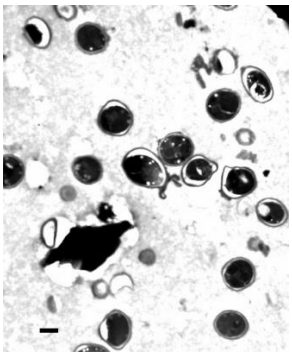
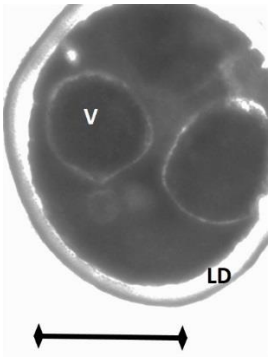
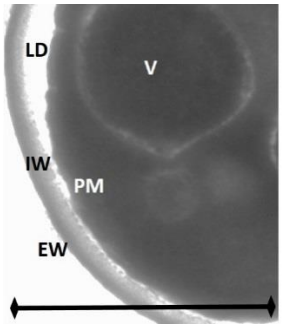
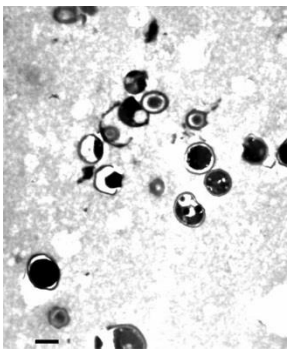
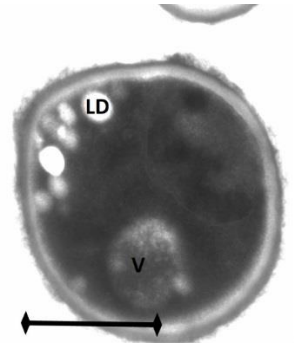
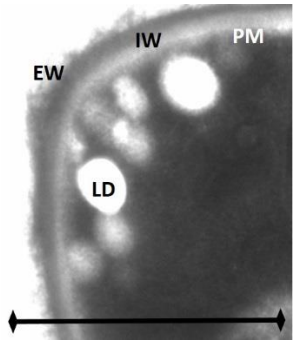
Treatment	Magnification ×5000	×25000	×50000
30°C-Dex			
30°C-Dex, 6 mo			
30°C-Suc			
30°C-Suc, 6 mo			

DLD: dense lipid droplet, DPM: disrupted plasma membrane, EW: external wall layer, IW: internal wall layer, L: lomosome, LD: lipid droplet, G: golgi apparatus, M: mitochondria, N: nucleus, NU: nucleolus, PM: plasma membrane, V: vesicle. Scale bars = 1 µm.

Chapter 6

Outcomes, general discussion and future research

The main constraint for use of biological control agents (BCAs) is inconsistent performance in the field under changeable environmental conditions. The present study aimed to develop new knowledge of effects of growth conditions, to increase persistence and efficiency of the key agent *Trichoderma atroviride* LU132, and to understand the factors which may influence conidium fitness for biological control formulations of conidia that are robust (long-surviving) and active against target plant pathogens. The effects of culture conditions on *T. atroviride* LU132 conidia were examined for productivity, germinability and bioactivity, and for shelf life. These were studied in laboratory and glasshouse pot experiments, including studies in association with the soil-borne plant pathogen, *Rhizoctonia solani*. The study has been presented as integrated sections, where the results of each set of experiments have been evaluated and discussed. The present chapter presents a summary of the outcomes from the study, a general discussion, and suggestions for further research.

6.1 Summary of outcomes

This is the first report indicating that the temperature at which conidia of *T. atroviride* are produced affects germination and bioactivity. Temperatures near 25°C and incubation periods of 15 d were shown to be optimum for production of *T. atroviride* LU132. Conidium production of this biological control strain was shown to be a continuous process, and a scheduled dark/light regime increased conidium production. Conidium production is likely to be on 20 d base cycle, which is probably dependent on colony age rather than abiotic factors. This finding is also the first report of bimodal conidium production in a *Trichoderma* BCA. Exposing *T. atroviride* LU132 colonies to specific culturing conditions (i.e. ecophysiological manipulation), improved conidium fitness in terms of increased stress tolerance, biological control performance and shelf life. Physiological changes in the conidia produced were attributed to bioactivity variations which resulted from manipulation of culture conditions during conidium production, and these changes were evident from biochemical analyses and ultrastructural observation using transmission electron microscopy.

Carbon to nitrogen ratio of 5:1 was determined as an appropriate nutritional balance for *T. atroviride* LU132 for optimum conidium fitness. A C:N ratio of 5:1 with carbon concentration of 4.2 g/L was optimal for conidium production, as assessed for numbers of produced and for biological control efficacy against *R. solani*. This medium composition, along with temperatures close to 20°C and incubation period of 15 d gave high levels of conidium fitness, as indicated by optimised numbers of

conidia produced and longevity of conidium viability. Furthermore, the conidia produced under these conditions gave the greatest bioactivity against *R. solani* in a ryegrass host plant bioassay, where rhizosphere and bulk soil *Trichoderma* colonisation assessments were carried out. *Trichoderma* population levels in the host rhizosphere soil and bulk potting mix changed during the duration of the pot experiment, with two distinct peaks observed similar to the bimodal conidium production cycle seen on agar plates (Chapter 2), but in an approx. 15 d cycle. This may indicate that simulated natural conditions shortened the life cycle of *T. atroviride* LU132, and this may be linked to greater metabolic activity due to environmental induction compared with conditions applying in agar culture.

6.2 General discussion

Many BCAs show inconsistent disease control efficacy after applications in field environments (Berger *et al*., 1996; Stewart, 2001; Alabouvette *et al*., 2006; Gerbore *et al*., 2013). Considerable research effort has been made to address these inconsistencies by improving the quality and vigour of BCA inocula (bacteria and fungi), through development of optimised culture conditions to increase capabilities of the agents during storage and to withstand environmental variations (Desai *et al.,* 2002; Verma, 2007; Edel-Hermann *et al*., 2009). The present research is a novel case study for increasing conidium fitness in *Trichoderma*. Manipulation of culture conditions will have eco-physiological effects on stability, stress tolerance and performance, and has the potential to improve the efficiency of biological control activity. Medium composition and physical growth conditions, including temperature, water activity, light and pH, are likely to be key factors, associated with appropriate nutrient amendments and colony age, influencing conidium quantity and quality of biological control fungi (Darby & Mandels, 1955; Hjeljord *et al*., 2000; Schubert *et al*., 2009; Dagno *et al*., 2010; Xue *et al*., 2013). These abiotic factors separately and in combinations will influence the fitness of *T. atroviride* LU132 as a BCA (Hallsworth & Magan, 1996).

The first section of the present study examined effects of temperature on conidium production, as a principal abiotic factor affecting numbers of conidia produced and their germination and bioactivity. Effects of colony age were also assessed to determine the optimum incubation periods to provide the greatest bioactivity. Temperature as an ecophysiological requirement was studied for *T. atroviride* isolate SC1 demonstrating that this strain grew best at 25°C (Longa *et al*., 2008). Similarly, in the present study, the incubation temperature of 25°C was found to be optimum for conidium production. For many fungi, the optimum temperatures for production have not been optimal for conidium germination or bioactivity (Campbell *et al*., 1996; Thomas & Jenkins, 1997; Zhao & Shamoun, 2006). Culture age also affects BCA metabolite production and bioactivity, and inoculum quality (Hallsworth & Magan, 1996). In the present study, *T atroviride* LU132 bioactivity differed with the duration of

incubation, so that temperatures near 25°C and incubation periods of 15 d were shown as likely to be optimum for conidium production of *T. atroviride* LU132 for biocontrol applications.

The bimodal conidium production observed during extended incubation periods of up to 50 d suggested that the first cycle occurred at 5 to 25 d and the second cycle between 25 to 50 d. Considering these cycles of conidium production and colony age, a 15 d incubation period was shown to be optimum, since the conidia from this stage of culturing were neither too young, as seen for 10-d-old conidia, nor too old and mixed with the first cycle of conidia at 20 d. Bimodal conidium production has been reported previously for pathogenic fungi in respect to the seasonal dynamics of conidium production (e.g. Copes & Hendrix, 2004; Scherm *et al*., 2008). In order to investigate the factors involved in bimodal conidium production, manipulation of light regimes for growing cultures of *T. atroviride* LU132 suggested that a scheduled dark/light regime gave increased conidium production compared with continuous light or dark regimes. This indicates that *Trichoderma* colonies require a period of darkness to initiate more conidiation, probably mediated by gene regulation. This has been demonstrated previously for *Exserohilum turcicum,* the pathogen causing Northern leaf blight in maize (Flaherty & Dunkle, 2005). From the results of experiments performed in this part of the study, including effects of temperature, extended incubation period and light/dark regimes, *T. atroviride* LU132 conidium production is likely to follow a 20 d primary cycle. A second cycle of conidium production follows that is probably dependent on colony age under gene regulation rather than abiotic factors during culturing. The results indicated that culture conditions only accelerate or delay the *Trichoderma* cycles of conidium production.

For the culture medium conditions, including nutrients, pH and water activity (at constant temperature of 25°C for 15 d), which resulted in low and high levels of *T. atroviride* LU132 conidium fitness, their effects on viability and bioactivity after long term storage for six months at 30°C and at 0 or 50% RH were assessed. Based on valid treatment comparisons, conidia produced at 30°C apparently survived unfavourable storage conditions by means of physical attributes (e.g. strengthened cell walls) or *via* accumulation of beneficial compounds (e.g. trehalose). In contrast, conidia produced at 20°C were not able to withstand unfavourable storage conditions. Levels of intracellular accumulation of trehalose, lipid profiles and cell wall proteins have been shown to affect the survival and stress resistance in fungal spores (Hottiger *et al*., 1987; Hallsworth & Magan, 1996; Agosin *et al*., 1997; Agosin & Acuilera, 1998; Hounsa *et al*., 1998). Conidia produced at 30°C probably experienced several stresses including high temperature followed by water shortage and oxidative stress. Temperature and water activity interdependently affect fungal growth (Brownell & Schneider, 1985). These stressful culture conditions probably resulted in the strengthening of conidium cell walls and elevated content of polyol compounds, resulting in thermo-, osmo-, and oxidation-adapted conidia with

reasonable bioactivity and viability. The numbers of conidia produced at the higher temperature were low, however. For example, high levels of accumulated mannitol in conidia produced at 30°C probably protected *T. atroviride* LU132 colonies from oxidation stress during the periods of culturing. The role of mannitol to scavenge reactive oxygen species has been previously demonstrated (Smirnoff & Cumbes, 1989). Conidium production treatments assessed in Chapter 4 (20°C-Dex, 20°C-Suc, 30°C-Dex, except 30°C-Suc) did not increase trehalose content of conidia. Optimum bioactivity indicated that temperature was likely to be a key factor affecting trehalose content (50 mg/g of dry conidia), as seen in conidia produced at 30°C on media containing sucrose as a carbon source rather than dextrose (32 mg trehalose /g of dry conidia). Since the trehalose content of the conidia did not directly reflect conidium fitness, other sugars may contribute to the viability and bioactivity of *T. atroviride* LU132 conidia obtained from different culturing conditions.

In this study, conidia produced at 20°C with the least bioactivity during storage contained large amounts of fatty acids (66 µg/g dry conidia). It was expected that the greatest fatty acid accumulation would be in conidia grown with a C:N ratio of 160:1, as it has been suggested that high concentrations of carbon will be converted to fatty acids due to the presence of excess carbon in the culture media leading to lipid synthesis (Woodbine, 1959). It has also been demonstrated that high levels of hydrocarbon source can increase the viability of conidia during storage, but also result in weak conidium fitness in fresh conidia (Nicholson & Moraes, 1980; McRae & Stevens, 1990). This supports the results obtained for conidia produced from the C:N 160:1 treatment in the present study. The culturing conditions in the C:N 160:1 treatment produced conidia which were probably protected from desiccation during storage. Excess hydrocarbon in the culture medium probably provided a physical barrier to protect conidia in storage, rather than protecting them through accumulation of lipids.

Changes in fatty acid structure were not influenced by culture conditions. Culturing at 30°C gave high proportions of unsaturation of fatty acids, while it was expected that there would be accumulation of saturated fatty acids, as suggested by Levin (1972) and Duan *et al*. (2011). Low temperatures in the culture media can cause increases in concentrations of polyunsaturated fatty acids, resulting in increased susceptibility to oxidation and rapid conidium senescence (Rustan & Drevon, 2005). However, in the present study, the least accumulation of unsaturated fatty acids (19 µg/g of dry conidia) was recorded in conidia produced at 20°C. The qualitative effect of fatty acid composition in *T. atroviride* LU132 is therefore likely to be an isolate-specific characteristic, or colony age may be a factor affecting the fatty acid profile.

Ultrastructural examination of conidia produced from different culture conditions showed how the integrity of conidium structures may be involved in conidium stability and viability. Ultrastructural differences of conidia (e.g. in external cell walls) have been linked to differences in conidium survival

and successful biological control establishment (Munoz *et al*., 1995). Conidia produced at 20°C showed significant disorganisation of cellular structures. Low electron density of conidium contents and accumulation of lipid droplets were associated with less integrity and viability. Lipid droplets filled with electron dense bodies possibly support conidium fitness, as it has been demonstrated that lipid droplets could store proteins such as the Erg1p enzyme in *Saccharomyces cerevisiae.* This enzyme becomes active for steroid metabolism under certain circumstances (Athenstaedt *et al*., 1999).

In this study, some conidia showed high levels of vacuolisation. This could be related to lack of water availability resulting in accumulation of non-aqueous macromolecules such as lipids (Buckley *et al*., 1966). The negative effect of desiccation was seen in conidia produced at 20°C, where they showed the least viability and bioactivity in storage. These conidia contained the least amounts of trehalose, which was measured by biochemical analyses. Although, the biochemical contents detected in conidia produced on dextrose and at 20°C (20°C-Dex treatment) did not give them optimum stability or bioactivity, these conidia showed undisrupted ultrastructure, they maintained viability during storage and also retained significant bioactivity in agar plate and pot experiments.

Study on the interactions between hydrocarbon source (dextrose or sucrose at a C:N 5:1), and temperature (20°C *vs* 30°C) established optimum conditions for producing large numbers of high quality conidia. Those produced on dextrose and at 20°C (20°C-Dex conidia) had the greatest ability to colonise the rhizospheres of host ryegrass plants and bulk potting mix in the absence of the plant pathogen *R. solani*. Hydrocarbon metabolism could be increased at high temperatures resulting in conversion of nutrient resources to complex compounds, which may not be as easily available as energy sources (Bossert & Bartha, 1984; Leahy & Colwell, 1990). Furthermore, simple carbohydrates such as glucose and organic nitrogen are preferred nutrient sources for microorganisms, so that metabolism of non-preferred nutrients (complex carbohydrates and organic nitrogen) are repressed (New *et al*., 2014). The present study has shown that dextrose is therefore likely to be a preferable carbon source to provide adequate physiological metabolism for *T. atroviride* LU132 at an incubation temperature of 20°C and C:N ratio of 5:1. Although, disaccharides (e.g. sucrose in the present study) have been recognised as the best carbon sources protecting microorganisms from environmental stresses and giving high survival rates (Redway & Lapage, 1974; Van Laere, 1989), in the present study, the monosaccharide dextrose gave superior colonisation potential in pot experiments. Sucrose was metabolically optimised in *T. atroviride* LU132 conidia at incubation temperatures of 30°C (30°C-Suc) and gave greater populations for 14 d after sowing in both rhizosphere and bulk potting mix. Sucrose was probably then rapidly exhausted as an energy source and the population did not recover during the remaining duration of the experiment. These findings suggest that dextrose would be optimal as a sugar at low temperatures close to 20°C, while sucrose could be optimal at temperatures from 25°C

(suggested from previous experiments in this study) to close to 30°C. The results of the pot experiment, where conidia produced at 20°C-Dex gave greatest control of the effects of *R. solani* on ryegrass plants, did not reflect results from dual culture assays carried out in Chapter 2, where 30°C conidia gave greatest inhibition of *R. solani*. This indicates a well-known requirement for confirming laboratory bioassay activity with host/pathogen/BCA assessments in pot or field trials (Berger *et al*., 1996; Stewart, 2001; Alabouvette *et al*., 2006; Gerbore *et al*., 2013).

6.3 Future directions

A primary step towards provision of new knowledge useful for increasing the efficiency of *T. atroviride* LU132 as a BCA was achieved in the present study, using manipulation of medium composition along with defined culture temperature and length of incubation. However, a BCA is likely to be commercially successful, with regard to cost-effective production, only where large numbers of conidia can be produced. The results of this study showed that a period of darkness during growth will increase conidium production. Furthermore, conidium production in changing light regimes revealed the inhibitory effect of constant light on conidium production compared with light/dark regimes. The inhibitory effects of light on conidium germination of *Aspergillus nidulans* are likely to be under phytochrome genes regulation, which changed conidium polar growth to isotropic (Röhrig *et al*., 2013). Study of genes involved in initiation of conidiation under light/dark regimes will help to optimise the duration of light required for maximum conidium production. This could be achieved *via* the quantification of light-receptor genes which encode RNA transcripts for each duration of light or dark during colony incubation. Quantitative Polymerase Chain Reaction (qPCR) experiments could be employed during periods of incubation (e.g. 15 d), as indicated in the present study. Duration of light or dark treatments could be from a few hours to days, to establish the optimum light requirements for *Trichoderma* colony growth and conidium production. Further, study of gene expression throughout the *Trichoderma* life cycle would assist identification of genes acting at different stages. Linking genetic information from gene expression in *Trichoderma* life cycles would provide basic knowledge to assist the development of more effective and economic biological control formulations.

Despite the determination of incubation time for *T. atroviride* LU132 colony growth in this study, the physiological maturation of the conidia was not determined. It has been suggested that conidium bioactivity results from the relationship between morphological and physiological maturity (Hodges, 1972). Understanding the time required for physiological and morphological maturation will therefore assist with the choice of appropriate times for harvesting *T. atroviride* LU132 conidia for different purposes, such as for storage or for immediate application against target pathogens, to provide optimum biological control.

In the storage experiment described in Chapter 3, the impact of drying and hydration after storage was not studied, while these factors are likely to affect the quality of conidia for use as BCAs. Drying and hydration of *T. atroviride* LU132 conidia should be optimised to reduce conidium mortality during preparation and storage. Although desiccation will put conidia into dormancy, different rates of drying could render the conidia into a deep dormancy accompanied with physiological separation between cellular contents. In this situation, the enzymes involved in activation of dormant conidia would be unavailable.

Quantitative PCR techniques could be usefully applied to studies where enumeration of conidia are required. The present study used labour-intensive plate assays counting colony forming units for *Trichoderma* population quantification, in host rhizospheres and in bulk soil. Quantitative PCR would require specific primers for *T. atroviride* LU132. This technology could also be employed for rapid detection of *T. atroviride* LU132 as a potential endophyte in ryegrass plants. Endophytic colonisation of *T. atroviride* LU132 was not studied in depth, as this was beyond the scope of the present study. Strain-specific qPCR could be used for comprehensive assessment in field situations to determine if this fungus internally colonises host plants. However, early results of an endophytic study carried out in a field experiment carried out at the Bio-Protection Research Centre (Lincoln University, New Zealand) have shown that several *T. atroviride* strains, including *T. atroviride* LU132, in competition with indigenous endophytic fungi, did not successfully establish endophytic relationships with ryegrass plants (W. Kandula, personal communication, 2014).

A study of complex interactions between *T. atroviride* LU132, plants and pathogens using proteomics and metabolomics would provide valuable information regarding the complex tripartite interactions (*Trichoderma*-host plant-pathogen) involved, and this would assist optimal application of this fungus for biological control. The pot experiments in the present study were carried out in a pasteurised potting mix. Application of *T. atroviride* LU132 in soil under field conditions and using proteomics and metabolomics technologies may provide more information for effective formulation development. *Trichoderma* isolates have the ability to produce a wide range of metabolites, including volatile and non-volatile compounds, in response to stimuli from other organisms or in different environments, while metabolite production is reduced in other situations (Reino *et al*., 2008; Stoppacher *et al*., 2010). However, only a few studies have fully addressed this property of *Trichoderma* species (Wheatley *et al*., 1997; Nemčovič *et al*., 2008; Vinale *et al*., 2008). *Trichoderma* metabolites would have significant attributes in biological control activity against target pathogens, and also affect the success of colonisation of plants roots in soil. Production of these metabolites may protect a variety of plants *via* antibiotic effects and/or induction of host defence systems, and identification of these metabolites and their activity could provide new strategies for management of plant diseases.

Biochemical analyses, particularly fatty acid profiles, revealed a wide range of entities on chromatograms, indicating that *T. atroviride* LU132 conidia sampled in the present study could be discriminated differentially based on fatty acid content. Sugar analysis was performed based on three standard sugars including arabitol, mannitol and trehalose. The HPLC column used in these analyses was not sensitive enough to detect a wide range of sugars. Besides more sensitive fatty acid and sugar analyses, protein analyses could also provide better understanding of the relationships between the biochemical content of conidia and their bioactivity and viability during storage.

Improvement in shelf life and efficacy of BCAs is the first priority to attain market acceptance of any biological control product. The next step for *T. atroviride* LU132 should be focused on increasing the stability of BCA formulation, along with consideration of the costs of production before application. Some advanced formulation technologies, such as encapsulation by biopolymeric materials (e.g. xanthan, gum arabic, alginate, chitosan) may provide increased protection of *Trichoderma* conidia, and evaluation of these should also be part of future research. Furthermore, *T. atroviride* LU132 metabolites with optimum bioactivity could be encapsulated for application in the vicinity of plant parts to prime resistance and growth promotion.

6.4 Conclusions

Results obtained from this research provide additional insights which can be utilised for improving efficiency of production of the key biological control agent, *Trichoderma atroviride* LU132. These results show that ecophysiological manipulation of culture conditions for conidium production is closely related to fungal physiology and physiological changes, which are likely to result in the variations seen in bioactivity. This study has shown that exposing *T. atroviride* colonies to different culturing conditions using appropriate ecophysiological manipulation may improve conidium fitness, including improved shelf life, increased stress tolerance and enhanced biological control performance. Carbon to nitrogen ratio of 5:1 was determined as the most optimum for production of high quality conidia. Furthermore, media with a C:N ratio of 5:1 with carbon concentrations of 4.2 g/L is likely to provide conidia with optimal biological control activity, as was demonstrated in assays against the pathogen, *R. solani*. Although the temperature used for conidium production has been recognised previously as a principal factor among abiotic factors influencing conidium quality in BCAs , the results of this study indicate that the nutrient composition of culture media is likely to have a greater influence on conidium fitness than temperature, since high temperature gave decreased conidium production. Nevertheless, conidia produced at high temperatures retained high levels of bioactivity and longevity during storage.

6.5 References

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General Appendix A

Culture media and procedures for TEM in this study

Potato Dextrose Agar (PDA): 39 g of PDA powder (Difco™) was suspended in 1 L of distilled water in a 1 L capacity Schott bottle. Medium was sterilised by autoclaving at 121°C for 15 min (10.34 Kpa). After cooling down in water bath at 50°C, the medium was poured into Petri plates in a laminar air flow cabinet, and the plates were kept until complete solidification of medium. Plates were then sealed with parafilm tape and stored at 4°C.

Potato Dextrose Broth (PDB): Preparation of PDB medium was performed as described for PDA preparation but at concentration of 24 g/L.

***Trichoderma* Selective Medium (TSM)**: Following materials were suspended in distilled water and autoclaved as decribed for preparation of PDA. After cooling down in water bath at 50°C, chloramphenicol was added as antibiotic.

Bacteriological agar (Scharlau®)	20.0 g/L
Glocose	3.00 g/L
Ammonium nitrate (NH_4NO_3)	1.00 g/L
Dipotassium hydrogen orthophosphate trihydrate ($K_2HPO_4 \bullet 7H_2O$)	0.90 g/L
Magnesium sulphate 7 hydrate ($MgSO_4 \bullet 7H_2O$)	0.20 g/L
Potassium chloride (KCL)	0.15 g/L
Terrachlor® 75 WP (quintozene 750 g/kg a.i.)	0.20 g/L
Rose Bengal	0.15 g/L
Iron sulphate (Ferrous sulphate) 7 hydrate ($FeSO_4 \bullet 7H_2O$)	1.00 g/L
Manganous sulphate tetrahydrate ($MnSO_4 \bullet 4H_2O$)	0.65 g/L
Zinc sulphate ($ZnSO_4 \bullet 7H_2O$)	0.90 g/L
Chloramphenicol (after atoclave)	2.50 mg/L

The above recipe is a modified version of the medium initially developed by Elad *et al*. (1981), modified by Elad & Chet (1983) and modified again by Askew & Laing (1993).

Fixation and processing for resin embedding for transmission electron microscopy

Fixation: Normal fixative is a solution of 2.5% glutaraldehyde, 2% formaldehyde in a 0.1M phosphate buffer solution (pH 7.2). Example: 10 ml stock (25%) glutaraldehyde, 20 ml 10% fresh formaldehyde

solution, 50 ml 0.2 M phosphate buffer, made up to 100ml with distilled water. Different proportions of phosphate buffers (1 M KH_2PO_4 or K_2HPO_4) were mixed and final pH was adjusted at 7.2.

Higher concentrations of fixative (up to 5% glutaraldehyde and 4% formaldehyde may be required for some specimens). Glass pipettes not plastic will be used to transfer fixatives (bulb not mouth).

Samples were each agitated with a pipette tip to break up the conidium mass, and were then left under vacuum for 10 min to remove air bubbles. After standing for 24 h at 4°C, samples were washed three times with phosphate buffer (pH 7.2), and post-fixation was performed in 1% (w/v) aqueous osmium tetroxide.

Dehydration: Ethanol dilution series of 10%, 20%, 30%, 50%, 70%, 95% and 100% were made and the samples were dehydrated for 15 min at each step.

Resin embedding: Spurr's resin was used in this stage. This is a 4 component low viscosity epoxy resin that is mixed prior to use. The firmness and viscosity of the resin depend on the ratio of the components used. The general method is as follows:

ERL-4221 (vinylcyclohexene dioxide)	5 g
DER-736 (diglycidyl ether of a propylene glycol)	3 g
NSA (nonenylsuccinic anhydride)	13 g
DMAE (dimethylaminoethanol)	0.18g

The first three components in the order given will be added into a 100 ml tripour beaker with a dry magnetic stirrer bar. The mixture will be covered on magnetic stirrer for three minutes. DMAE will be added to the mixture and the recent mixture will be covered for at least 30 minutes before use. Mixture remains usable for the rest of the day and can be stored (sealed with parafilm) for two days stored at 4°C.

After last rehydration, 100% ethanol will be replaced with 50:50 Spurr's resin (making up resin at least 30 minutes before use). Samples in resin will be agitated on a rotator overnight. The embedding procedure will be repeated twice with two changes of pure resin the next day. The embedded sample will be left in an oven at 70°C overnight (8 h maximum).

Sectioning: Embedded samples were sectioned using a Leica UCT ultramicrotome (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland) at a thickness of 110 nm. Sections were placed on grids and stained with 1% (w/v) aqueous uranyl acetate followed by aqueous lead citrate.

General Appendix B

Outputs from this research

- Daryaei, A., McLean, K. L., Glare, T. R. and Stewart, A. (2012). *Effect of temperature and length of incubation period on Trichoderma atroviride LU132 conidial production, germination and bioactivity.* Programme and Abstracts, the 12th International *Trichoderma* and *Gliocladium* Workshop, Lincoln University, New Zealand, 27-30 August 2012: 97.
- Daryaei, A., Falloon, R.E., McLean, K.L., Glare, T.R. and Stewart, A. (2013). *Temperature and incubation period affect Trichoderma atroviride conidium production, germination and bioactivity.* Abstracts, the 10th International Congress of Plant Pathology, Beijing, China, 25-30 August 2013. *Acta Phytopathologica Sinica* **43** (Supplement): 32.
- Daryaei, A., Falloon, R.E., Glare, T.R., Jones, Alizadeh, H. and Stewart, A. (2013). *Understanding the survival, persistence, and bioactivity of the biocontrol agent Trichoderma atroviride LU132.* Abstracts, the Microbial Products Symposium, Lincoln, New Zealand, 14 September 2013.
- Daryaei, A., Falloon, R.E., Glare, T.R. Jones, E.E., Alizadeh, H. and Stewart, A. (2013). *Germination and bioactivity of Trichoderma atroviride affected by culturing and storage conditions.* Abstract and Programme Book, the 19th Australasian Plant Pathology Conference, Auckland, New Zealand, 25-28 November 2013: 49.
- Daryaei, A., Falloon, R.E., Glare, T.R., Jones, E.E., Alizadeh, H. and Hallett, I.C. (2014). *Bioactivity, biochemical and ultrastructural characteristics of Trichoderma atroviride conidia influenced by different culturing conditions: factors affecting biocontrol potential.* Abstracts, the XIVth International Congress of Mycology and Eukaryotic Microbiology, Montreal, Canada, 27 July – 1 August 2014: 700.

Effect of temperature and length of incubation period on *Trichoderma atroviride* LU132 conidial production, germination and bioactivity

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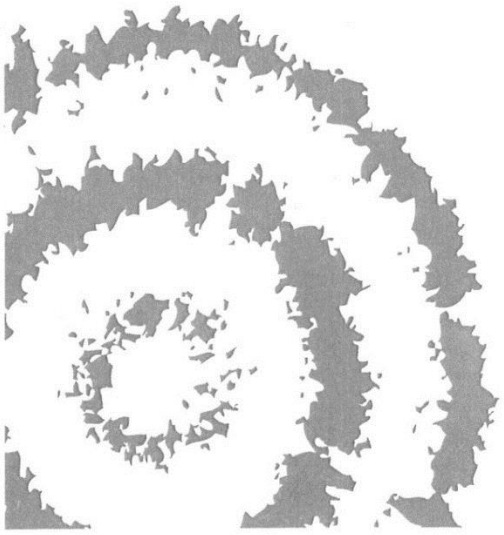
Trichoderma atroviride LU132 has been commercialised in New Zealand as Tenet® and Sentinel® for the control of *Sclerotium cepivorum* and *Botrytis cinerea*, respectively. To determine the effect of culturing conditions on efficacy of *T. atroviride* LU132, the present research examined the influence of incubation temperature (20, 25, and 30°C) on the production of conidia under constant light over a 25 day period. Two measures of quality of the conidia produced were also assessed - germination and bioactivity of the subsequent colony against *Rhizoctonia solani*. Maximum conidial production occurred at 25°C and after 20 days of growth. A decline in conidial production at 25 days was observed. The conidia produced at 30°C, germinated significantly faster than those produced at 25 and 20°C, respectively. Incubation temperature did not have a significant effect on subsequent colony bioactivity against *R. solani*. However, a significant increase in bioactivity was seen with colonies arising from spores produced after 25 days incubation compared with the shorter incubation periods. To determine if the decline in conidial production continued after 25 days, the experiment was repeated at 25°C for 50 days. Extending the incubation period resulted in a second peak in conidial production after 45-50 days. The produced conidia in the extended experiment had an optimum germination after 20 days incubation and the optimum bioactivity was achieved with conidia harvested after 15 days. This study suggests that *T. atroviride* formulations based on conidia produced to optimise quantity may not be the most bioactive.



27 - 30 August 2012
Commerce Building
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O03.012 *Pichia anomala* and *Candida oleophila* in biocontrol of post-harvest diseases of fruits: 20 years of fundamental and practical research's

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Fungal pathogens such as *Botrytis cinerea*, *Penicillium expansum* and *Gloeosporides* group are mainly responsible of important economical losses of post-harvest apples and pears. Application of biological control agents (BCA's) is an emerging alternative to synthetic fungicides. However, before becoming an economically feasible alternative to chemical control, BCA's have to satisfy different requirements related to biological, technological and toxicological properties. The different steps for a successful strategy of disease control (selection, production and formulation, study of mechanisms of action, ecological characterization, molecular monitoring, pilot efficacy trials, registration) will be reviewed considering the 2 antagonistic yeasts: (1) *Pichia anomala* strain K as scientific model and (2) *Candida oleophila* strain O. Two decades ago, both strains were selected for their high and reliable antagonistic activity against *B. cinerea* and *P. expansum* on apples and pears. The lack of efficient and reliable BCA's constitutes until now the major drawback to commercialise biopesticides based on these BAC's. In that context, the studies of mode of action and ecological fitness are important because they can lead to a better efficacy of antagonistic yeast strains. Recent advanced molecular techniques have contributed to improve knowledge on the modes of action. Thanks to the identification of genes involved in biocontrol properties, the genetic basis of action mechanisms can be understood. That approach was adopted for *P. anomala* (strain K) and lead to the identification of genes coding for exo-β-1,3-glucanases implicated in the efficacy. Based on that identification, a formulation involving β-1,3-glucans was developed and applied with higher efficacy in controlled conditions. The importance of ecological characterisation is also crucial in the context of pre-harvest application for both antagonistic strains of yeast. UV light, temperature and humidity were identified as major factors influencing strain K and strain O populations. Models taking into consideration temperature and humidity were developed and could be useful in deciding whether pre-harvest treatment is sufficient to allow fast colonization of wounds prior to the arrival of wound pathogens, or whether it is wise to apply further post-harvest treatment to increase the yeast population density.

Furthermore, the scientific background obtained for *Candida oleophila* strain O lead to the development of a formulated biopesticide called Nexy® and registrated in US and some European countries. Practical applications of Nexy® was carried out against postharvest diseases of apples, pears, citrus and bananas. The presentation will concentrate also on these practical applications taking into account the necessity to integrate Nexy® with other methods of post-harvest disease control. Such integration must lead to decrease the global level of chemical residues on fruit surface while keeping a sufficient level of efficacy. Finally, the presentation of 20 years of work will be also the opportunity to highlight the difficulties and challenges to transfer results from scientific laboratory to industrial level, keeping in mind the satisfaction of fruit growers and consumers.

O03.013 Temperature and incubation period affect *Trichoderma atroviride* conidium production, germination and bioactivity

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Trichoderma atroviride LU132 has been commercialised in New Zealand as Tenet® and Sentinel® for the control, respectively, of *Sclerotium cepivorum* and *Botrytis cinerea*. This study examined the influence of incubation temperature (20, 25, or 30°C) on the production of conidia under constant light over a 25 d period. Two measures of quality of the resulting conidia were also assessed-germination and subsequent bioactivity against *Rhizoctonia solani*. Maximum conidium production occurred at 25°C after 20 d. Production of conidia declined at 25 d. Conidia produced at 30°C germinated more rapidly than those produced at 20 or 25°C. Incubation at 30°C gave greatest bioactivity against *R. solani* in comparison with incubation at 20 or 25°C. An incubation period of 25 d increased bioactivity compared with shorter incubation periods. The experiment was repeated at 25°C for 50 d. Extending the incubation period resulted in a second peak of conidial production at 45-50 d. These conidia had an optimum germination after 20 and 25 d incubation, and the optimum bioactivity for the colonies was achieved with conidia harvested after 15 d. This study suggests that *T. atroviride* formulations based on optimised production of conidia may not result in optimal bioactivity.

O03.014 Control of *Botrytis cinerea* secondary inoculum within grape bunches by applications of biological control agents and natural products

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Infected necrotic tissues inside the grape bunch represent an important secondary inoculum source for *Botrytis*

Understanding the survival, persistence, and bioactivity of the biocontrol agent *Trichoderma atroviride* LU132

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Abstract Identification of the production and storage factors that affect conidial germination and bioactivity (fitness) will assist the success of biological control agents. Abiotic factors (temperature, nutrients, water activity, pH) during production were studied and then the effects of culturing conditions on conidial fitness of *Trichoderma atroviride* LU132 were examined in different storage conditions over time. Conidia from the culturing regimes which resulted in greatest and least bioactivity against *Rhizoctonia solani* in dual culture were selected to assess effects of storage condition on conidial fitness. Fitness of the test conidia was examined after storage at 30˚C and at 0 or 50% relative humidity (RH) over 6 months. Fitness declined over time, and the decline was greater for 50% RH than 0% RH. The greatest number of conidia and germination percentage resulted from conidia produced at 25°C, but greatest bioactivity resulted from those produced at 30˚C. Different C to N ratios (5:1 or 160:1) did not affect these parameters. However, fewer conidia were produced at 30˚C, and the least germination and bioactivity resulted from conidia produced at 20°C. Conidia can be divided into two groups: those adapted to extreme culturing conditions (e.g. high temperature), and those protected by nutrients during storage. However, environmental factors are not independent. For example, conidial production at 30°C is probably accompanied by water stress, oxidation, and rapid pH change which may also affect fitness.

Keywords *Trichoderma,* survival, fitness, abiotic factors, bioactivity

Germination and bioactivity of *Trichoderma atroviride* affected by culturing and storage conditions

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Identification of the production and storage factors that affect conidial germination and bioactivity (fitness) will assist the success of biological control agents. Effects of culturing conditions on conidial fitness of *Trichoderma atroviride* LU132 were examined in different storage conditions over time. Abiotic factors (temperature, nutrients, water activity, pH) during production were studied. Conidia from the culturing regimes which resulted in greatest and least bioactivity against *Rhizoctonia solani* in dual culture were selected to assess effects of storage condition on conidial fitness. Fitness of the test conidia was examined after storage at 30˚C and at 0 or 50% relative humidity (RH) over 6 months. Fitness declined over time, and the decline was greater for 50% RH than 0% RH. The greatest number of conidia and germination percentage resulted from conidia produced at 25˚C, but greatest bioactivity resulted from those produced at 30˚C. Different C to N ratios (5:1 or 160:1) did not affect these parameters. However, fewer conidia were produced at 30˚C, and the least germination and bioactivity resulted from conidia produced at 20˚C. Conidia can be divided into two groups: those adapted to extreme culturing conditions (e.g. high temperature), and those protected by nutrients during storage. However, environmental factors are not independent. For example, conidial production at 30˚C is probably accompanied by water stress, oxidation, and rapid pH change which may also affect fitness.

Effect of *Aureobasidium* isolates on mycelium growth of three major bunch rot pathogens of grapes

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Currently, fungicide treatments represent the primary method for the control of bunch rot disease of grapes. But chemical control methods have consequences with social and environmental perspectives. Public concerns about fungicidal residues in grapes and development of fungicidal resistant strains of the pathogens have promoted the search for alternative means, less harmful to environment and human health. Recently, considerable success has been achieved by utilizing the microbial antagonists to control post and pre-harvest diseases of fruits. *Aureobasidium pullulans*, an important cosmopolitan yeast-like fungus, colonize on the surfaces of many fruits and vegetable is a potential biocontrol agent for plant pathogens. In this experiment, different *Aureobasidium* isolates were isolated from surfaces of Chardonnay grapes collected from four different berry development stages during 2012 vintage. Altogether 27 *Aureobasidium* isolates were screened against the three major bunch rot pathogens of grapes such as *Botrytis cinerea*, *Colletotrichum acutatum* and *Greeneria uvicola* under *in-vitro* condition. According to the results, *Aureobasidium* isolates showed high level of suppression on mycelium growth of the fungus *Greeneria uvicola* when compared to the other tested two pathogens *Colletotrichum acutatum* and *B.cinerea*.

Workshop Sessions

MEM-WK305.02 - Bioactivity, biochemical and ultrastructural characteristics of Trichoderma atroviride conidia influenced by different culturing conditions: factors affecting biocontrol potential

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Identification of production and storage factors that affect conidial germination and bioactivity (fitness) will assist the success of biological control agents. Effects of abiotic factors (temperature, nutrients, water activity, pH) on conidial fitness of Trichoderma atroviride LU132 during production were studied. Conidia from the culturing regimes which resulted in greatest and least bioactivity against Rhizoctonia solani in dual culture were selected to assess effects of storage conditions on conidial fitness over time. Fitness of the test conidia was examined after storage at 30˚C and at 0 or 50% relative humidity (RH) over 6 months. Fitness declined over time, and the decline was greater for 50% RH than 0% RH. The greatest number of conidia and greatest germination resulted from growth at 25°C, but greatest bioactivity resulted from conidia produced at 30˚C. Different C to N ratios (5:1 or 160:1) did not affect these parameters. However, fewer conidia were produced at 30˚C, and the least germination and bioactivity resulted from conidia produced at 20°C. The different environmental factors were not independent. For example, conidial production at 30°C is probably accompanied by water stress, oxidation, and rapid pH change which may also affected fitness. Further study was performed based on temperature and hydrocarbon type. Biochemical and ultra-structural studies were carried out to determine relationships between quality variations and cellular characteristics for conidia produced in different culturing conditions. Results of fatty acid, and sugar (trehalose and polyols) analyses, and ultrastructural observations are presented in this paper.