Defence of *Agaricus bisporus* against

toxic secondary metabolites from *Trichoderma aggressivum.*

by

Calvin P. Sjaarda, B.Sc.

A Thesis

submitted to the Department of Biological Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

September, 2013

Brock University

St. Catharines, Ontario

© Calvin P. Sjaarda, 2013

Abstract

Trichoderma spp are effective competitors against other fungi because they are mycoparasitic and produce hydrolytic enzymes and secondary metabolites that inhibit the growth of their competitors. Inhibitory compounds produced by *Trichoderma aggressivum*, the causative agent of green mold disease, are more toxic to the hybrid off-white strains of *Agaricus bisporus* than the commercial brown strains, consistent with the commercial brown strain's increased resistance to the disease. This project looked at the response of hybrid off-white and commercial brown strains of *A. bisporus* to the presence of *T. aggressivum* metabolites with regard to three *A. bisporus* genes: laccase 1, laccase 2, and manganese peroxidase.

The addition of *T. aggressivum* toxic metabolites had no significant effect on *MnP* or *lcc*1 transcript abundance. Alternatively, laccase 2 appears to be involved in resistance to *T. aggressivum* because the presence of *T. aggressivum* metabolites results in higher *lcc2* transcript abundance and laccase activity, especially in the commercial brown strain. The difference in laccase expression and activity between *A. bisporus* strains was not a result of regulatory or coding sequence differences. Alteration of laccase transcription by RNAi resulted in transformants with variable levels of laccase transcript abundance. Transformants with a low number of *lcc* transcripts were very sensitive to *T. aggressivum* toxins, while those with a high number of *lcc* transcripts had increased resistance. These results indicated that laccase activity, in particular that encoded by *lcc*2, serves as a defense response of *A. bisporus* to *T. aggressivum* toxins and contributes to green mold disease resistance in commercial brown strains.

Acknowledgements

First, I thank Dr. Alan Castle for continual encouragement and support throughout my doctorate studies. I thank him for the opportunity to work in his lab, and for his coaching through the research, writing and review process. Dr. Castle's door is always open to listen to good or bad results, and he is always very able to provide solutions, clarifications, ideas, and insight. I would also like to thank Dr. Castle for his attention towards furthering my career, for the occasion of attending conferences and making contacts.

I would like to thank the member of my advisory committee, Dr. Yousef Haj-Ahmad and Dr. Charles Deprés, for the encouragement, suggestions and ideas through several progress seminars. I am indebted for the time they have spent reading and reviewing this manuscript.

Thank you to the internal external examiner, Dr. Ana Sanchez for her time and comments on this thesis and her questions during the defence. Thank you to my external examiner, Dr. Richard Kerrigan for reviewing this manuscript as well as attending my defence. I would like to thank Dr. Kerrigan for his time, interest and insight in my research, especially for the comments and perception into the heteroallelism of *A. bisporus* and for his feedback and questions during the defence.

There are many people I have made contact with during my time here at Brock University that have helped me in one capacity or another. I would like to thank many colleagues that I had in Dr. Castle`s lab including Dr. Kamal Abubaker for teaching me everything the first couple years of my research; Dr. Katrina Brudzynski for reviewing manuscripts, providing feedback and encouragement; several students in the lab including Jonathan Gaiero, Nancy Gad, Tony Wang and Patrick Viel. Thank you to Dr. Liette Vasseur for her help and guidance with the statistical analysis of my results.

Table of Contents

List of Tables

List of Figures

General Introduction

Interactions between fungal species are extremely common in natural ecosystems where species compete for limited resources and space for reproduction. Competition and antagonism are frequently manifested by mycoparasitism (Chet, Harman & Baker, 1981), the production of toxins (Reino et al. 2008) and/or cell wall degrading enzymes (Yang et al. 2009) that impair growth of a neighbouring species. Production of both toxin and cell wall degrading enzymes are observed during confrontation between *Trichoderma aggressivum* f. *aggressivum* and *Agaricus bisporus*, the button mushroom (Krupke, Castle & Rinker, 2003; Guthrie & Castle, 2006; Abubaker, Sjaarda & Castle, 2013).

In the mid-1980s extensive crop losses attributed to *T. aggressivum* occurred on mushroom farms in the British Isles (Seaby, 1987). Over the past 25 years the disease has spread across Europe and into Western Asia (Geels, 1997; Mamoun et al. 2000a; Sobieralski et al. 2009b). Similar problems developed in Ontario and British Columbia in the early 1990s, where *T. aggressivum* has spread and caused severe problems in many areas across North America (Rinker, 1993; Rinker et al. 1997; Anderson, Beyer, & Wuest, 2001). This problem is known world-wide as "green mold disease". The very common off-white strain U1, its derivatives, and white strains are very sensitive to *T. aggressivum*. Commercial brown strains are much more resistant and crop loss is much less severe than that of the U1 strain (Rinker & Alm, 1997; Anderson et al. 2001).

T. aggressivum produces 3,4-dihydro-8-hydroxy-3-methylisocoumarin, a toxin that inhibits the growth of *A. bisporus* (Krupke et al. 2003). The inhibition of the brown strain SB65 was significantly less than that observed with the white strain S130, an observation that is consistent with brown strain resistance to green mold disease. The mechanism of resistance is unknown but a plausible explanation is that commercial brown strains are able to degrade the

toxin more rapidly than white or off-white strains. If so, possible candidate enzymes would include laccases and peroxidases.

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are extracellular, multi-copper enzymes that use molecular oxygen to degrade numerous phenolic substrates. The main role of these enzymes in basidiomycetes is lignin utilization but they may also have roles in defense. Several fungal antagonists, including *T. harzianum*, have been shown to increase laccase activity in the basidiomycetes *Trametes versicolor* and *Pleurotus ostreatus* during co-cultivation (Baldrian, 2004). In addition, *Lentinula edodes* produced increased laccase activities when challenged with spent medium from *T. harzianum* cultures (Savoie, Mata and Billette, 1998).

In this thesis project, cultures of *A. bisporus* were treated with extracellular extracts of *T. aggressivum* medium and measured transcript abundances of three genes, laccase 1, laccase 2 and manganese peroxidase; as well as enzymatic activity of laccase. The objectives were to determine if *T. aggressivum* extracellular secondary metabolites induced expression and activity of hydrolytic enzymes in *A. bisporus*, and if induction was more pronounced in a green mold resistant brown strain than in a sensitive off-white strain. *Lcc1* and *lcc2*, including upstream regulatory elements, were sequenced from both brown and off-white strains to see if any differences in transcriptional control sequences or in predicted protein structure could account for any potential activity differences between the strains. Finally, we generated transgenic off-white and brown strains expressing an hpRNA cassette to determine whether altering laccase expression impacts susceptibility to *T. aggressivum* toxins.

Chapter 1: Literature Review

1.1. *Agaricus bisporus*

1.1.1. Economic importance

In the last 50 years, commercial production of mushrooms and truffles worldwide has increased from under 1 billion dollars annually in 1961 to over 13.3 billion dollars in 2010. Currently, the largest producer is the People's Republic of China, producing over 65% of the
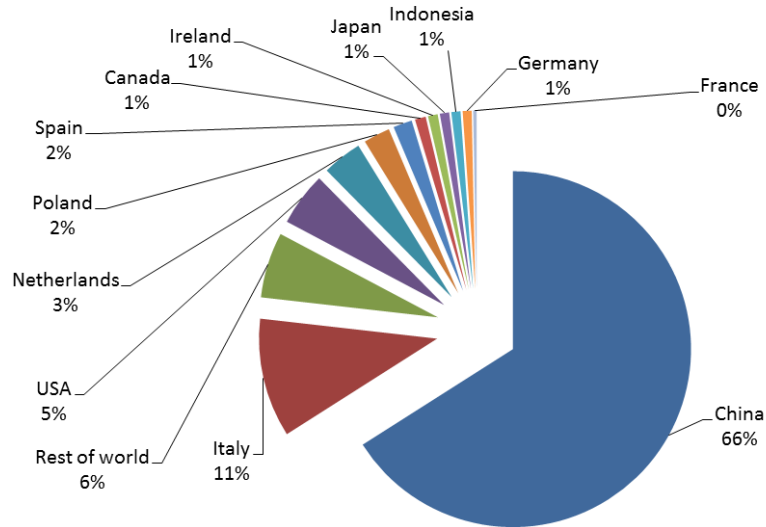
Figure 1: Mushroom and truffle gross production value generated by the top twelve countries in the world (FAOSTAT 2012).

world's mushrooms and truffles (FAOSTAT 2012) (Figure 1). Canada is the seventh largest producer, producing over 86 thousand tons of mushrooms valued over 250 million dollars annually (Figure 2). The average Canadian ate 1.25 kg of mushrooms in 2010, and the rest was exported predominantly to the United States (Statistics Canada).

The majority of mushrooms produced and consumed in Canada are the white button, crimini, and portabella mushrooms (all *Agaricus bisporus*). Specialty mushrooms include shiitake (*Letinula edodes*), oyster (*Pleurotus ostreatus)* and enoki mushrooms *(Flammulina velutipes*).
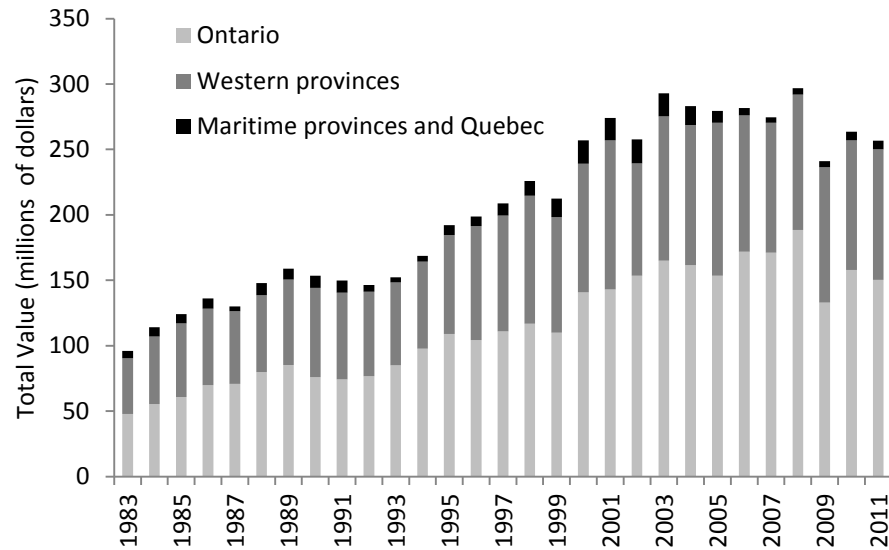
Figure 2: Total value of the fresh and processed mushroom generated in Canada from 1983 until 2011 (Statistics Canada).

A. bisporus mushrooms are an attractive food source because they have low protein content, high fiber, all the essential amino acids, and all the essential vitamins except iron (Chakravartey, 2011; Sadler, 2003). Mushrooms also have significant antimicrobial and antioxidant potential (Aban & Aban, 2010).

1.1.2. Life cycle

The lifecycle of a basidiomycete fungus is shown in Figure 3A. Two haploid mycelia with compatible mating type ❶ can undergo plasmogamy (cytoplasm fusion) to form a dikaryotic mycelium. ❷ Under the right conditions the dikaryotic mycelium will form a fruiting body containing gills lined with the basidia. ❸ Within the basidium, the nuclei will fuse (called karyogamy) to form a diploid nucleus. ❹ Following karyogamy, the cells will go through meiosis to form a basidium containing four haploid nuclei. ❺ Each nucleus will be incorporated into its own basidiospore on the end of the basidium. The basidiospores get dispersed ❻ and germinate to form haploid mycelia starting the life cycle over. ❼

16
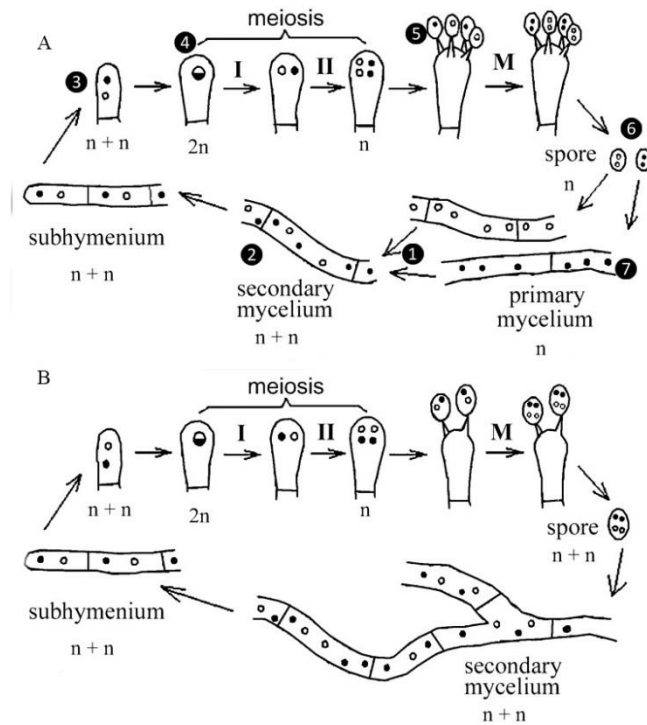
Figure 3: A: Heterothallic life cycle typical of most Basidiomycetous fungi. B: Secondary homothallic life cycle of *A. bisporus* v. *bisporus* (Figure taken with modification from Kamzolkina et al. [2006]).

    A. bisporus v. *bisporus* deviates from the typical life cycle of Basidiomycetes because it is secondary homothallic (Figure 3B). Secondary homothallic refers to a fungus derived from a heterothallic (outbreeding) form that is self-fertile. In *A. bisporus,* rather than four haploid nuclei splitting into four separate spores, only two basidiospores will develop on each basidium. Each of these basidiospores receive non-sister nuclei with compatible mating type determinants, and when they germinate they form hyphae that are multinucleate and self-fertile (Castle, Horgen, & Anderson, 1987; Castle, Horgen, & Anderson, 1988). There are no haploid, monokaryotic cells within the life cycle of *A. bisporus*. In addition, as the hypha grows, the mitotic divisions that occur generate cells that each contains 2 to 8 nuclei. Kamzolkina, et al. (2006) showed that over 90% of *A. bisporus* cells have 3 to 5 nuclei per cell. This life style has made genetic studies and manipulation of *A. bisporus* very difficult and labour intensive.

17

1.1.3. Cultivation

1.1.3.1. Substrate

Mushrooms are grown on compost consisting of plant fibre (wheat straw), nitrogen (animal manure – usually horse and chicken), gypsum (calcium sulphate) for balancing pH, and lots of water. Before these raw materials can be inoculated by *A. bisporus* mycelium, it must go through two stages of composting.

1.1.3.2. Phase I – Composting

The raw materials are mixed together and placed into concrete bunkers, or piles 1.5m high and square on top. The bunkers and piles need to be watered and mixed every three days, and continually aerated to support aerobic microbial growth. Bacteria and fungi grow in the pile and compost the raw ingredients by breaking down the simple carbohydrates (Ross & Harris, 1983). A compost pile with enough microbial growth should maintain an internal temperature of 55 – 60°C. At the end of 12 – 14 days, the compost should be pliable and dark brown in colour.

1.1.3.3. Phase II – Pasteurization and conditioning

The composted substrate is moved into trays or bed where it is pasteurized to remove unwanted microorganisms, and conditioned to reduce ammonia content. The growing rooms are heated, typically with steam, to 60°C for 8 hours to kill unwanted organisms including fly larvae, bacteria and molds. Over several days, the compost cools to 30°C allowing ammonia to escape from the compost. Compost that has successfully gone through both phases should have around 65% moisture, a carbon to nitrogen ratio of 19:1, 2.2% total nitrogen content, pH = 7.4, and free of competitive molds (Zeid et al. 2011).

1.1.3.4. Spawning

Spawn is the mushroom "seed" that is used to inoculate the bed of compost. Most mushroom growers buy spawn from a supplier to ensure a good mushroom crop. A central mushroom supplier is more able to maintain healthy productive strains and produce spawn under aseptic conditions than each individual farmer. The substrate for spawn production includes rye

grain, calcium carbonate (lime), calcium sulfate (gypsum), and water. After sterilization, mushroom mycelium is inoculated aseptically into the substrate. After the fungus has completed colonized the substrate, the spawn is sent to the mushroom farm, where the farmer will mix it into the compost (0.5% w/w). The compost temperature is maintained at 27°C and 95-99% relative humidity for the 14 days it takes for the mushroom mycelia to colonize the compost.

1.1.3.5. Casing and pinning

The mycelium growing within the compost will not produce a good mushroom harvest without a casing layer. The casing consists of 80% peat moss, 20% limestone and saturated to 80% moisture and is spread over the compost about 3-4 cm thick. The compost is maintained at 24°C and 99% humidity. The fungus from the compost will colonize the casing layer; after six days the casing is raked to break up mycelium to facilitate complete colonization. After 11 days the temperature is cooled to 20°C which stimulates the fungus to switch from mycelial growth to fruiting body formation. The mycelium starts to clump together in structures called rhizomorphs. The rhizomorphs develop small outgrowths, calls pins, and the pins grow into mushrooms. The pins are very sensitive to desiccation, so lots of water and high humidity are very important. Typically the first mushrooms can be harvested 21 days after the casing layer was applied.

1.1.3.6. Harvest

Mushrooms are produced in a series of "breaks" or "flushes" at 7 day intervals. Mushrooms are picked by hand to minimize damage to the mushroom and mycelium. After the second break, mushroom production declines very rapidly, and most mushroom producers will terminate the crop after the second or third flush. A mushroom harvest will yield between 0 to 39 kg of mushrooms per square meter of compost (Royse & Beelman, 2007).

When the mushroom harvest is complete, steaming the growing rooms at 65°C kills all bacterial and fungal organisms. The spent compost gets disposed. It is estimated that 36 million m^3 of compost gets disposed in the Unites States annually (Royse, 2010). Double cropping

involves using compost for more than one crop of mushroom and provides growers with the opportunity to increase production efficiency. By placing landscape fabric between the compost and casing layer, Royse (2010) describes removing the casing after mushroom harvest is complete. After the spent casing is removed the compost can be fragmented (mixed), supplemented and recased. Double cropping mushroom compost enables growers to increase mushroom production up to 40% (Royse et al. 2008).

1.1.4. *A. bisporus* strains

In 1975, the Horst research station was asked to combine the properties of white and off-white strains of *A. bisporus* (Fritsche, 1986). The white mushrooms had nice shape and white colour, while the off-white was hardier and larger size. Fritsche collected monospore isolates of white and off-white strains, and tested them for compatibility. Fertile combinations were tested for desirable quantity and quality of mushroom production. Of the four successful hybrids, U1 and U3 were selected for commercial marketing. The U1 strain possessed the round, white and smooth cap from the white parent and the bigger size from the off-white parent. The U3 strain also had the white smooth cap from the white parent and the size and thick stipe from the off-white parent. The U3 mushroom quality was found to be dependent on the casing water levels, dry casing resulted in small mushrooms and wet casing produced large mushrooms (Fritsch, 1986). Due to its desirable properties, over 90% of commercial white button mushrooms in North America and Europe today are the Horst U1 hybrid off-white strain (Chen et al. 2003).

Commercial brown strains commonly referred to as Portabella or Crimini mushrooms, were not part of a hybrid selection process and have been shown to be more resistant to mushroom diseases (Anderson et al. 2001; Krupke et al. 2003; Moquet et al. 1998).

1.2. Diseases of commercial mushrooms

1.2.1. Viral diseases

La France disease also known as brown disease, X-disease, and dieback is one of the most serious diseases of the commercial mushroom. Studies show that La France disease is caused by

nine dsRNAs encapsidated in a 36-nm isometric virus-like particle (Borodynko et al. 2010; Goodin, Schlagnhaufer & Romaine, 1992; Romaine, Schlagnhaufer & Goodin, 1994; Romaine & Schlagnhaufer, 1995; Wach, Sriskantha, & Romaine, 1987). The primary source of infection of the virus is within the basidiospores. Infection causes decay of the mycelia resulting in slow and aberrant growth, reduced mushroom yield and malformation of the fruiting body (Borodynko et al. 2010; Romaine & Schlagnhaufer, 1995). *A. bisporus* has no resistance to the virus, so management is limited to good hygiene practices and elimination of infected fungal tissue (Romaine & Schlaghaufer, 1995).

1.2.2. Bacterial diseases

Bacterial blotch is the most common bacterial disease of the commercial mushroom. Bacterial blotch is caused by *Pseudomonas tolaasii* and affects button, oyster, and shiitake mushrooms (Lee, Jeong & Cha, 2002). *P. tolaasii* produces an extracellular toxin called tolaasin, which forms ion channels in the fungal membrane leading to disruption of plasma and vacuole membranes (Brodey et al. 1991; Largeteau & Savoie, 2010; Lee et al. 2002). *P. tolaasii* is endemic in compost and casing soil, and can switch between pathogenic and non-pathogenic form in response to metabolites produced by the host (Largeteau & Savoie, 2010). Damp and wet casing and mushrooms accelerate proliferation of the bacteria. Mushrooms infected with *P. tolaasii* are of reduced quality due to pitting and the development of brown spots on the pileus (Oh et al. 2000). Although several treatments including using chlorinated water (Oh et al. 2000), bacteriophage TO1 (Munsch & Olivier, 1995), tolaasin detoxifying bacteria (Tsukamoto, Murata & Shirata, 2002) and others have been shown to reduce disease severity, none are completely successful.

1.2.3. Fungal diseases

1.2.3.1. Dry bubble

Dry bubble is caused by the fungal pathogen *Lecanicillium fungicola* (previously known as *Verticillium fungicola*) and plagues mushroom growers worldwide (Collopy et al. 2001; Mills et

al. 2000). Infection of *L. fungicola* on a mushroom farm usually results from contaminated

casing ingredients (Berendsen et al. 2010). *A. bisporus* vegetative hyphae are resistant to

infection by *L. fungicola* so infection only occurs in the fruiting bodies (Berendsen et al. 2010).

If the infection develops late in mushroom development, it only results necrotic lesions,

characterized by brown and grey discolouration in the cap and stipe of the mushroom. However,

if the infection occurs early in the mushroom development, major symptoms including stipe

blowout (stipe splits and peals) and dry bubble (mushroom tissue grows into an undifferentiated

amorphous mass) develop (Berendsen et al. 2010; Del Carmen et al. 2002; Mills et al. 2000).

Control of *L. fungicola* depends on strict hygiene and application of fungicides. Most effective

chemical treatments of the disease also affect the host resulting in a very limited number of

fungicides that can be used, and *L. fungicola* has developed resistance to most of them

(Berendsen et al. 2010; Del Carmen et al. 2002).

1.2.3.2.   Wet bubble

Wet bubble or La Molé is a common disease of the commercial mushroom caused by

Mycogone pernicosa. Wet bubble has been reported everywhere that *A. bisporus* is grown

commercially (Fletcher et al. 1995). It only causes major crop damage when infection is early

and is left untreated (Fletcher & Gaze 2008). *M. pernicosa* causes *A. bisporus* mushrooms to

become large and irregular with tumorous, undifferentiated lumps of tissue called sclerodermoid

mushrooms (Fletcher et al. 1995; Tanovic et al. 2009). Initially the lumps of tissue are white and

spongy, but they turn brown and often exude amber coloured droplets (Gea, Tello & Navarro,

2010). A well-managed farm with high level of sanitation will have a very low or null incidence

of wet bubble disease (Gea et al. 2010).

1.2.3.3.   Cobweb

Cladobotryum dendroides is the causal agent of cobweb of the commercial mushroom. *C.

dendroides* can be identified by coarse mycelium that grows over the affected mushrooms

(Tanovic, et al. 2009). The infecting fungus grows over *A. bisporus* mushroom in a manner that

the takes the shape of a spider web giving rise to the name Cobweb disease.   The overgrowth causes spots to form on the mushroom cap, rendering them unsellable (Chang & Miles, 2004). *C. dendroides* infection usually results from contaminated casing material, and its growth is promoted by excessive moisture on the casing layer and on the mushroom. The infection of *C. dendroides* can be managed if prompt action is taken as soon as the fungus' presence is perceived. Covering the affected area with a damp paper towel stops the movement of spores and treatment with salt effectively manages its growth (Chang & Miles, 2004).

1.2.3.4.   Green mold

Green mold disease (caused by *Trichoderma aggressivum*) was first described in Northern Ireland in 1985 by Seaby (1987) and in North America in 1992 (Rinker et al. 1997). *T. aggressivum* rapidly overgrows compost and *A. bisporus* mycelium, and produces large numbers of green spores. Infection with green mold inhibits the formation of *A. bisporus* fruiting bodies, and loss in mushroom yield can be up to 100% (Anderson et al. 2001). Several chemical compounds have been used for treatment of green mold disease. Benylate, based on the fungicide benomyl, can be applied to the spawn as a preventative control of *T. aggressivum,* but is ineffective against established green mold infections (Chang & Miles, 2004). However commercial benylate production ceased at the end of 2003. Carbendazim, a benzimidazole metabolite of benomyl, has been shown to be effective for treatment of green mold disease but many strains of *Trichoderma* have already developed resistance (Chang & Miles, 2004; Romaine et al. 2006). Currently, the primary approach to managing *T. aggressivum* infection is sanitation and hygiene; especially post crop steam off and early spawn run (Rinker et al., 1997). This project deals with green mold disease of the commercial mushroom, so a review of the *Trichoderma* genus is provided.

1.3. *Trichoderma*

1.3.1. **Ecology and phylogenetics**

Trichoderma species are free living fungi that are distributed all over the world. They are common in nearly all temperate and tropical soil and root ecosystems at concentrations of 10^1 to 10^3 propagules per gram of soil (Harman et al. 2004). Part of *Trichoderma*'s success comes from its ability to satisfy its energy requirements from a wide range of organic compounds including mono and disaccharides, complex polysaccharides, purines, pyranidines, amino acids, tannins and catechins, aldehydes and organic acids (Papavizas, 1985).

The first *Trichoderma* fungus was described in 1794 by Persoon (Samuels, 2006). In 1865 the link between *Trichoderma*`s anamorph (asexual state) and teleomorph (sexual state) was described by the Tulasne brothers (Samuels, 2006). The teleomorphs of several species have been identified and classified in the genus *Hypocrea*; however, most species of *Trichoderma* have only been found in the asexual state. While *Trichoderma* is often found in soil and root ecosystems, *Hypocrea* often colonizes woody and herbaceous plant material (Harman et al. 2004).

Prior to the 1990s, phylogeny in *Trichoderma* was determined based on phenotype, cytology, physiology and ultrastructure (Samuels, 2006). Different *Trichoderma* species have very similar morphological character and these techniques have identified only 40 species (Druzhinina et al. 2010). The advent of routine DNA sequencing in the mid-1990s allowed phylogeny analysis based on different nucleotide sequences. Most phylogenic taxa of *Trichoderma* and *Hypocrea* species today are based on sequence differences between transcription elongation factor 1α (EF-1α) and the rDNA internal transcribed spacer (ITS) (Samuels, 2006). Phylogenic speciation based on DNA loci has allowed the identification of over 100 different species of *Trichoderma* and *Hypocrea* (Druzhinina et al. 2010).

1.3.2. Biological Control Agents

Successful pathogen control has been accomplished predominately through the use of agricultural chemicals. However, environmental contamination and pest resistance have driven the development of biological control agents (BCAs) (Lewis & Papavizas, 1991). *Trichoderma* spp. have several features that enable it to be successfully used as a BCA. Different *Trichoderma* species are antagonistic to many different soil phytopathogens including fungi, invertebrates (insects and nematodes), bacteria, and plants (weeds) (Verma et al. 2007). *Trichoderma* spp. reportedly are able to reduce the pathogenicity or disease severity of several fungal plant pathogens including *Aspergillus flavus, Botrytis cinerea, Fusarium* spp.*, Rhizoctonia solan*i, *Sclerotinia sclerotiorum, Sclerotium rolfsii,* and *Sclerotium trifoliorum* (Benhamou & Chet, 1993; Calistru, McLean & Berjak, 1997; Celar, 2003; Chet & Baker, 1981; Elad, Chet & Katan, 1980; Elad, 1996; Sivan & Chet, 1986; Wells, Bell & Jaworski, 1992).

The success of *Trichoderma* as an organism and as a BCA results from its ability to out-compete other organisms, mycoparasitism, and production of enzymes and antibiotic compounds. Other commercial applications of *Trichoderma* beyond the scope of this report include induction of systemic resistance in plants, rendering them more resistant to pathogens (Howell et al. 2000; Viterbo et al. 2002; Yedidia, Benhamou & Chet, 1999), and the use of enzymes produced by *Trichoderma* in bioremediation (Ezzi & Lynch, 2002; Karam & Nicell, 1997).

1.3.3. Competition

There is severe competition between microorganisms for the few nutrients contained in soil, typically only a few μg of simple sugars and amino acids per mL of soil water (Blackman, 1978). As a result, starvation is the most common cause of death for microorganisms (Benitez et al. 2004). *Trichoderma* spp. are well equipped for this competitive environment because they grow very rapidly in soil, can sense and change extracellular pH, are proficient at obtaining

nutrients, and can overcome toxicity in the soil (Benitez et al. 2004). A few relevant examples illustrate the genetic and biochemical attributes of *Trichoderma* that make it an efficient competitor in nutrient limiting conditions.

The ability to thrive over a wide range of external pH conditions gives *Trichoderma* an advantage over other competing organisms. Coping with variable pH conditions requires efficient intracellular pH homeostasis, as well as the ability to regulate the synthesis of extracellular enzymes and metabolites only at the pH when they will be active (Arst & Penalva 2003). Many fungi that are able to sense environmental pH, including *Trichoderma*, possess the Pal signalling pathway that regulates the activity of the PacC transcription factor. PacC is a wide domain zinc finger transcription factor that recognizes the 5` GCCARG 3` DNA sequence (Eisendel et al. 2004). Figure 4 shows the model of pH regulations starting with PacC being translated in the cytosol as 72 kDa protein ($PacC^{72}$); $PacC^{72}$ is inactive under acidic conditions (Arst & Penalva, 2003; Prusk & Yakoby, 2003). Alkaline conditions are sensed by cell membrane receptors, PalI or PalH, which starts a signal cascade resulting in PalB cleaving $PacC^{72}$ to $PacC^{53}$ (53 kDa). Independent of pH, $PacC^{53}$ is cut to the active $PacC^{27}$ which is translocated into the nucleus (Prusk & Yakoby, 2003). The active $PacC^{27}$ will bind its target sequence and is responsible for repression of acid pH genes and expression of alkaline pH genes (Eisendel et al. 2004; Prusk & Yakoby, 2003). PacC mutants of *T. harzianum* are unable to detect alkaline pH and under alkaline conditions will have a similar response as the wild type under acidic conditions.

PacC regulation has been shown to control many different pathways within the cell. Steyaert, Weld & Steward (2010) describes a role in *Trichoderma* conidiation. Benitez's group show PacC regulation involved in production of extracellular enzymes including β-glucanase and aspartyl protease (Delgado-Jarano, Pintor-Toro & Benitez, 2000; Delgado-Jarano, Rincon & Benitez, 2002). Some species of *Trichoderma* are better adapted to low pH conditions, and able to control the pH of their environment (Benitez, et al. 2004).
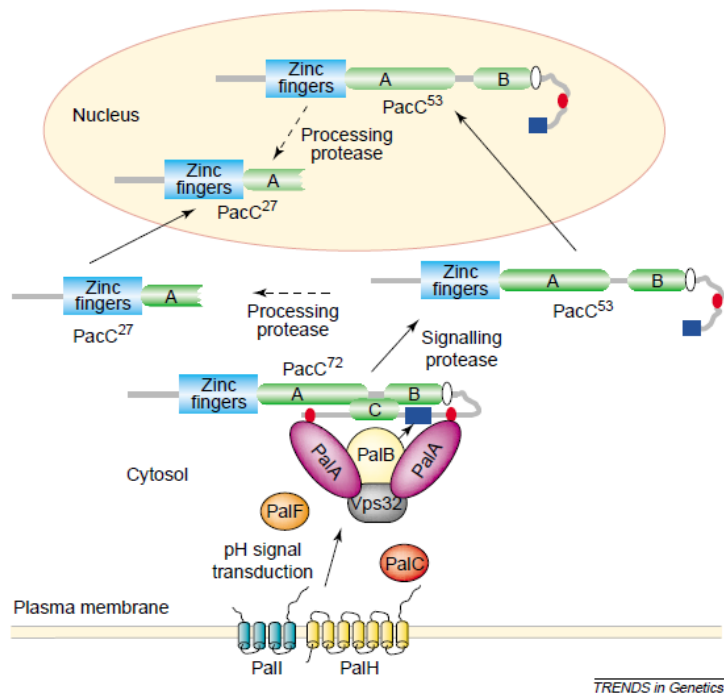
Figure 4: Model of pH regulation in *Aspergillus nidulans*. The PalI and/or PalH membrane proteins will transduce an extracellular alkaline pH signal to the signalling protease PalB. PalB will cleave PacC^{72} to PacC^{53}, which in turn is cleaved, independent of pH, into PacC^{27}. PacCc^{27} is active and will bind regulatory elements within the genome (Figure taken from Arst & Penalva, 2003).

Trichoderma spp. possess a glucose transport protein (coded for by the *gtt*1 gene) which allows *Trichoderma* to utilize nutrients from their environment more efficiently than competing organisms (Delgado-Jarana, Moreno-Mateos & Benítez, 2003). Logically, high glucose conditions have been shown to induce expression of the glucose transporter, *gtt*1 while carbon starvation results in repression of the gene (Delgado-Jarana et al. 2003). Variable pH has been demonstrated to also influence *Trichoderma* expression of *gtt*1. Acidic pH results in a 40% decrease in glucose transport, so to counteract the lower levels of transport *Trichoderma* is able to increase expression of *gtt1*. Similarly, under alkaline pH conditions, *gtt*1 expression is repressed (Delgado-Jarana et al. 2003). The presence of a competitive fungus, *Rhizoctonia solani,* stimulates *Trichoderma* to maximize its carbon uptake thereby assisting *Trichoderma* to outcompete the competitor. The presence of a glucose transporter with a high affinity for glucose enables *Trichoderma* to obtain energy from hydrolyzed polymers and rapidly take up

sugars molecules, which is very useful when competing for a limited number of nutrients (Delgado-Jarana, et al. 2003).

Filamentous fungi also require and compete for the utilization of iron (Benitez et al. 2004). Under iron starvation conditions, *Trichoderma* (and many other fungi) can produce siderophores, low molecular weight ferric iron-specific chelators. The SreA (iron regulator) and PacC site (pH regulator) regulatory elements control expression of three genes involved in siderophore biosynthesis and uptake include (Eisendel et al. 2004). Similar to glucose transport, environmental pH has several major effects on iron uptake (Eisendel et al. 2004). Acidic conditions increase siderophore uptake because siderophores are co-transported with protons. In addition, acidic conditions increase iron solubility making it more readily available for uptake. On the other hand, low pH reduces siderophore affinity for iron because protons compete for the binding sites. The ability to regulate siderophore synthesis with variable pH conditions makes *Trichoderma* an effective competitor at obtaining iron from the environment.

Trichoderma is able to utilize nitrogen faster than competing fungi including *Fusarium* spp., *R. solani, S. sclerotionium* (Celar, 2003) and *B. cinerea* (Elad, 1996). By outcompeting *B. cinerea* for nutrients (including nitrogen) *T. harzianum* reduced *B. cinerea* germination and reduce disease severity of rot symptoms (Elad, 1996).

1.3.4. Mycoparasitism

The first description of the mycoparasitic ability of *Trichoderma* was made by Weindling in 1932. He described *Trichoderma* hyphae coiling around the hyphae of *R. solani*, followed by penetration and dissolution of the host hyphae. Mechanisms underlying the ability of *Trichoderma* to parasitize other fungi have been well described by a number of researchers.

The process of seeking out a host is not a coincidence or collision occurrence. *Trichoderma* grows directly toward its host with a swollen region at the tip of its hyphae, characteristic of a chemotactic response to a chemical cue (Chet et al. 1981). This chemical cue

is probably chitin oligomers, cut from the host cell walls by *Trichoderma* chitinases secreted constitutively at low levels (Benetez et al. 2004; Lora et al., 1994).

Trichoderma follows the chemical cue and when it reaches the host produces hook shaped contact branches, and either grows parallel to or coils around the host hyphae (Benhamou & Chet, 1993; Chet et al. 1981). To determine whether the coiling was a mechanical response to the physical structure of the host hyphae, or a chemical response to a molecule on the host hyphae, Chet et al. (1981) added plastic threads the same diameter as the host hyphae and reported that *Trichoderma* would not coil around the threads. In 1992, Inbar and Chet showed the carbohydrate lectin, a sugar binding protein in cell membranes, is a recognition signal which caused *Trichoderma* to coil. They showed that the lectin caused *Trichoderma* to produce a mucilaginous material which aids in adhesion (Inbar & Chet, 1994) and triggers a general parasitic response in *Trichoderma* (Inbar & Chet, 1995). Since the lectin has an obvious role in the mycoparasitism mechanism, Benhamou et al. (1999) covered fibers with lectins and found that *Trichoderma* would coil around the fibers demonstrating that the coiling and subsequent mycoparasitic responses is a response to the chemical stimulus.

The signal cascade resulting from interactions between the host and antagonistic fungi has been described by Omero et al. (1999) and Zeilinger & Omann (2007). Mastoparan and fluoroaluminate are G-protein activators, and the presence of either of these compounds results in an increase in intracellular cAMP, which causes *Trichoderma* hyphae to coil around bare fibers similarly to mycoparasitism (Omero et al. 1999).   Heteromeric G-protein signalling is composed of a G-protein couple receptor and a heteromeric G-protein consisting of α, β, and γ subunits (Neer, 1995). When a ligand binds the receptor, there is a conformation change which causes GDP to be exchanged for GTP on the G_α subunit. The phosphorylated G_α subunit dissociates from the $G_{\beta\gamma}$ subunit and both subunits can regulate the activities of downstream effectors (Birnbaumer, 1992; Gutkind, 1998; Kaziro et al. 1991; Neer, 1995). *Trichoderma* G_α subunits are divided into three subgroups; subgroup I ($G_{\alpha i}$), coded for by the *tga*1 gene inhibits

adenylate cyclase and has been shown to be involved in coiling and conidiation (Rocha-Ramirez, et al. 2002), $G_{\alpha ii}$ (coded by *tga2*) is not well described, and $G_{\alpha iii}$ (coded by *tga3*) functions by increasing cytosolic cAMP (Bölker, 1998; Turner and Borkovich, 1993). *Trichoderma* mutants have been generated for all three G_α subunits; tga1 mutants have lower chitinase activity and are unable to overgrow and lyse host fungi (Mukherjee et al. 2004; Reithner et al. 2005), tga2 mutants do not have any major phenotypic defects (Mukherjee et al. 2004), and *tga*3 mutants have decreased cAMP levels, lower chitinase activity, are unable to produce mycoparasitic related structure and are avirulent (Zeilinger et al. 2005).
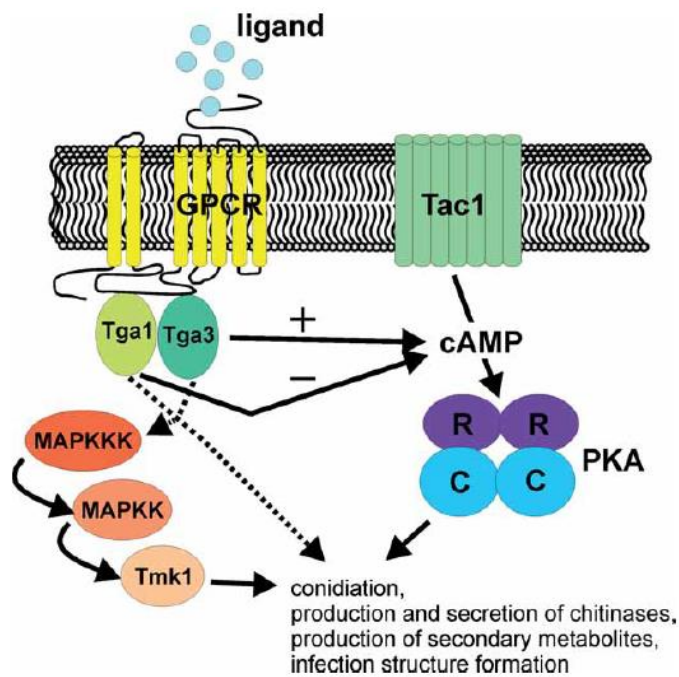
Figure 5: Model of G-protein signalling in *Trichoderma*. The G-protein coupled receptor senses the presence of a ligand and activates the Gα subunit (Tga1 or Tga2). The subunits modulate cAMP levels which regulate conidiation, production of chitinases, secondary metabolites and mycoparasitic structures (Zeilinger & Omann, 2007).

Figure 6 shows a series of pictures showing the mycoparasitic events that take place during interaction between *Trichoderma* and a host fungus, *R. solani*. After coiling around host hyphae, *Trichoderma* produces appressorium-like structures and in conjunction with the production and secretion of active lytic enzymes, will penetrate the hyphal wall (Chet et al. 1981; Lo et al.

1998). *Trichoderma* hyphae will grow inside the host hyphae utilizing nutrients from the host (Chet et al. 1981).
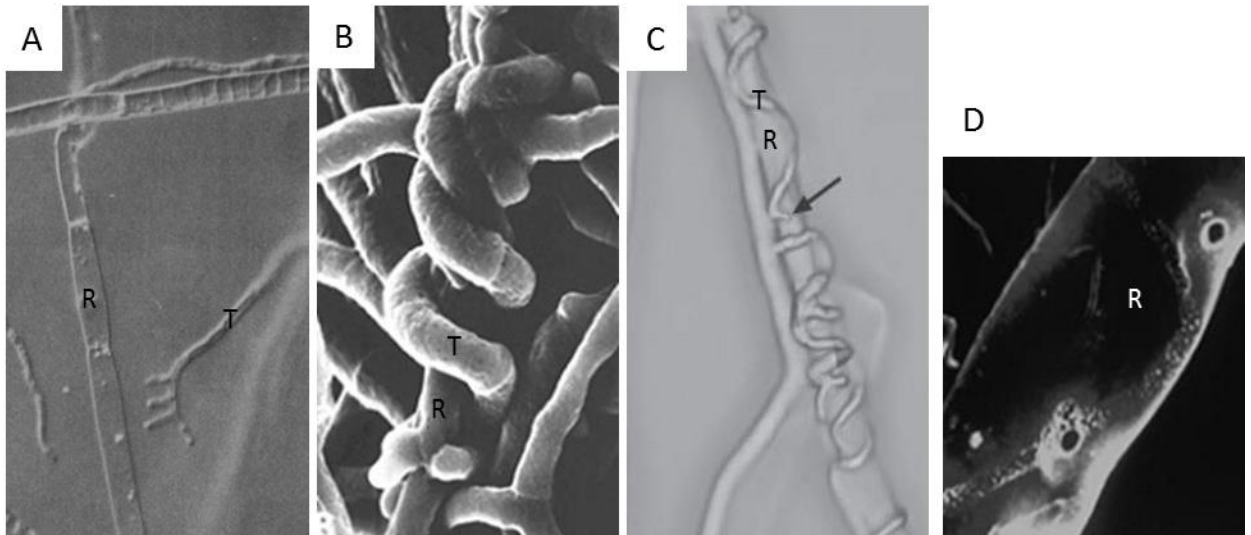
Figure 6: Mycoparasitism of *R. solani* by *Trichoderma* starts with directed growth towards host hyphae (A: Chet et al. 1981), followed by coiling (B: Benhamou & Chet, 1993) and penetration – shown by arrow (C: Steyaert et al. 2003). *R. solani* hyphae displaying penetration site after *Trichoderma* has been removed (D: Harman et al. 2004). *Trichoderma* hyphae are labelled T and *R. solani* hyphae are labelled R.

In 2011, Reithner et al. performed a high throughput transcriptome-wide sequencing to identify genes that are related to sensing and parasitizing other fungi. When *Trichoderma* was confronted with a host, they found 74 genes upregulated, 98 down regulated, and 3 with variable expression. The majority of the up-regulated gene were metabolic, suggesting the lysis of host improves the availability of nutrients.

1.3.5. Hydrolytic enzyme production

Fungal cell walls are composed of 80% polysaccharides, up to 20% proteins, and minor components include lipids, pigments and inorganic salts. The polysaccharides and related structures of the fungi include β-glucans, chitin and glycoproteins (Steyaert et al. 2003). To attack and penetrate the hyphae of phytopathogens, *Trichoderma* produces a battery of hydrolytic enzymes (Table 1) under regulatory control that enables a coordinated attack (López-Mondéjar, Ros & Pascual, 2011). Regulation of hydrolytic enzymes is accomplished by

repression under nutrient rich conditions, and induction during mycoparasitism. The

mycoparasitism-related genes are repressed by the CRE1 protein under high glucose conditions

Table 1: Cell wall degrading enzymes isolated from *Trichoderma* spp.
Chitinase names are based on apparent size from SDS-PAGE; therefore,
cloning and sequencing may reveal that some genes are represented by
more than one enzyme (Table modified from Steyaert et al. 2003).

Group	Enzyme	Gene (accession number)
Chitinases	Chitobiosidase, CHIT40	
	Endochitinase, CHIT52	
	Endochitinase, CHIT42	*ech42* (*T. atroviride* X79381)
		chit42 (*T. hamatum* AY258898)
	Endochitinase, CHIT37	*chit36* (*T. harzianum* AY028421)
	Endochitinase, CHIT33	*chit33* (*T. harzianum* X80006)
	Endochitinase, CHIT31	
	β-N-acetylhexosaminidase, CHIT102	
	β-N-acetylhexosaminidase, CHIT72 CHIT73)	*nag1* (*T. atroviride* S83231)
		exc1 (*T. harzianum* AJ314642)
	β-N-acetylhexosaminidase, CHIT64	
	β-N-acetylhexosaminidase, CHIT28	
β-glucanases	Exo-β-1,3-glucanase (110 kDa)	*lam1.3* (*T. harzianum* L48994)
	Exo-β-1,3-glucanase (40 kDa)	
	Exo-β-1,3-glucanase (31 kDa)	
	Exo-β-1,3-glucanase (70 kDa)	
	Endo-β-1,3-glucanase (78 kDa)	*bgn13.1* (*T. harzianum* X84085)
	Endo-β-1,3-glucanase (70 kDa)	
	Endo-β-1,3-glucanase (36 kDa)	
	Endo-β-1,6-glucanase (51 kDa)	
	Endo-β-1,6-glucanase (43 kDa)	*bgn16.1* (*T. harzianum* X79197)
	β-1,3-glucosidase (78 kDa)	*gluc78* (*T. atroviride* AF256421)
Cellulases	Cellobiohydrolase I	*cbh1* (*T. harzianum* AF315381)
	Cellobiohydrolase II	*cbh2* (*T. reesei* M55080)
	Endo-β-1,4-glucanase I	*egl1* (E00390*T. reesei* M15665))
	Endo-β-1,4-glucanase II	*egl2* (*T. viride* AB021657)
	Endo-β-1,4-glucanase III	*egl3* (*T. reesei* M19373)
	Endo-β-1,4glucanase IV	*egl4* (*T. reesei* Y11113)
	Endo-β-1,4-glucanase V	*egl5* (*T. reesei* Z33381)
	β-glucosidase I	*bgl1* (*T. reesei* U09580)
Proteinases	Serine protease	*prb1* (*T. atroviride* M87518)

and by the GATA motifs during nitrogen rich conditions (Steyaert et al. 2003). *Trichoderma*

also possesses mycoparasitism response elements (MYRE) that are able to induce genes in

response to the presence of a host fungus (Pozo et al. 2003).

1.3.5.1. Chitinases

Chitin is a long chain β1-4 polymer of N-acetyl-β-D-glucosamine (GlcNAc), and the major cell wall component most filamentous fungi (Farkas, 1979). Chitinases are enzymes that degrade chitin, and are the focus of considerable study as enzymes likely to be involved in biocontrol. Chitinases catalyze the cleavage of chitin between the C1 and C4 bonds of two consecutive GlcNAc units and are divided into endochitinases, exochitinases and 1,4-β-N-acetylglucosaminidases (Sahai & Manocha, 1993).

Endochitinases cleave β linkages randomly within the chitin chain and in *Trichoderma* spp. include CHIT52, CHIT42, CHIT37, CHIT33 and CHIT31. CHIT42 has been shown to be an important enzyme in the mycoparasitic interaction. Omero et al. (1999) and Zeilinger et al. (1999) showed that the gene *ech*42, which encodes CHIT42, is induced by a diffusible stimulus (presumably chitin oligomers) even in the absence of contact with the host fungus. CHIT52 and CHIT33 are induced by CHIT102 and products of chitin degradation. They are involved in catabolizing the fungal cell wall just before penetration of the host fungus, and are antagonistic to the growth of the phytopathogens that are targeted by the *Trichoderma* BCAs (Dana et al. 2001; Haran et al. 1996; Limón, Pintor-Toro & Benítez, 1999).

Exochitinases (aka chitobiosidases) cleave β-linkages from the non-reducing end of chitin and its oligomers. Only one exochitinase, CHIT40, has been described and is able to improve the biological activity of *Trichoderma* (Harman et al. 1993; Lorito et al. 1993).

The third class of chitinases called 1,4-β-N-acetylglucosaminidases or hexosaminidases cleave chitin, its oligomers and chitobiose from the non-reducing end (Steyaert et al. 2003). CHIT102 is unique in that it triggers the expression of other chitinases including CHIT73 (*nag*1), CHIT52 and CHIT33 (Haran et al. 1996; Mach et al. 1999).

1.3.5.2. β-glucanases

β-glucans are composed of chains of D-glucose linked by β1-3, β1-4, or β1-6 glycosidic bonds. After chitin, the second most abundant polysaccharide in the fungal cell wall is β1-3-

glucan chains, called laminarins, which are branched via β1-6 glycosidic linkages (Gruber & Seiboth, 2012). β1-4 glucan chains (aka cellulose) are the major component in plant cell walls, but they can also be found in fungal cell walls (Farkas, 1979). β-1,3- and β-1,6-glucanases are enzymes that catalyze the breakdown of the bonds between C1 and C3 or C6, and β-1,4-glucanases, break down cellulose – the bond between C1 and C4.

Both β-glucanases (β-1,3- and β-1,6-) and cellulases (β-1,4-) contain three subclasses of enzymes that breakdown different parts of the β-glucan. Exo-β-1,3-gluconases cut at the non-reducing end of the chain, Endo-β-1,3-glucanases cleave randomly with the β-glucan chain and oligomers, while β-glucosidases will cleave oligo- and disaccharides (Steyaert et al. 2003). Glucanases are important for *Trichoderma* antagonism of oomycetes (for example, *Pythium*) because their cell walls contain β-(1-3) and (1-6) glucans rather than chitin (Viterbo et al. 2002). The best characterised *Trichoderma* β-glucanase is a 78 kDa endo-β-1,3-glucanase which can inhibit *B. cinerea* spore germination (Lorito et al. 1994), inhibit the growth of *S. rolfsii* (El-Katatny et al. 2001) and hydrolyze yeast and fungal cells walls. The 43 kDa endo-β-1,3-glucanase was shown to inhibit the growth of several pathogens (Lora et al. 1995). Likewise, cellulases are divided into three sub-classes: cellobiohydrolases cleave cellobiose (disaccharide) units from the end of cellulose chains; endo-β1,4-glucanses cut randomly with the cellulose chain; and β-glucosidase cleaves cellobiose into glucose (Steyaert et al. 2003). Most research involving cellulases has been with *Trichoderma reesei* composting cellulose from plant waste. However, overexpression of the *egl*1 gene in transformed *T. longibrachiatum* resulted in greater biocontrol activity against *P. ultimum* than the wild type, indicating a role of cellulases in mycoparasitism (Migheli et al. 1998).

1.3.5.3. Proteases

Proteases can be divided into alkaline, neutral and acidic proteases. Acidic proteases are pH regulated, and their function is to degrade overexpressed extracellular proteins including chitinases, glucanases and cellulases. Most alkaline and neutral proteases are induced under

carbon starvation conditions (Viterbo et al. 2002). One protease known to contribute in mycoparasitism of other fungi is encoded by *Trichoderma*'s *prb*1 gene. The *prb*1 gene is induced by fungal cell wall of *R. solani*, and overexpression in *T. harzianum* improved its biological control over soil borne pathogens (Flores, Chet & Herrera-Estrella, 1997). A *T. virens* homologue to *prb*1, called *tvsp*1, was identified by Pozo et al. (2003). This gene coded for an extracellular serine protease whose over expression increased *Trichoderma*'s ability to protect plants against *R. solani*. A third serine protease with trypsin activity was identified as *Pra*1 (Suarez et al. 2004). *Pra*1 is induced by the same conditions that stimulate antagonism, indicating that it may have a role in antagonism.

1.3.6. Antibiosis

Trichoderma species are able to synthesize and secrete antimicrobial compounds that inhibit and/ or kill microorganisms in their vicinity (Verma, 2007). The different metabolites can be classified as low molecular weight and volatile metabolites which would have effects in the space around the fungus or high molecular weight, polar metabolites which exert their effects during direct interactions between fungi (Benitez et al. 2004; Reino et al. 2008). Over 185 antibiotic compounds within 24 families produced by different species of *Trichoderma* were reviewed by Reino et al. (2008). To provide an overview of the diversity of metabolites produced by *Trichoderma,* this paper provides basic information and a representational structure for important and common families as described in Reino et al. (2008).

1.3.6.1. Anthraquinones

Anthraquinones were first isolated from *T. viride* in 1967 (Slater, et al.). The biological activity of this family of molecules is typically related to pigmentation, but they also have bacteriostatic, antimicrobial and antifungal properties.

1 $R_1 = R_2 = H$
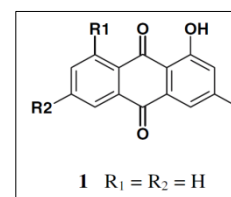
1.3.6.2. Pyrones

The pyrone, 6-pentyl-2H-pyran-2-one (shown to the right), was identified by Collins and Halim in 1972. This compound is common through most of the *Trichoderma* genus and is responsible for the coconut aroma associated with several species. Metabolites in this group have been shown to reduce the grown of *R. solani* by 70%, *F. oxysporum* by 32%, *B. cinerea,* and *S. rolfsii* by over 90%.
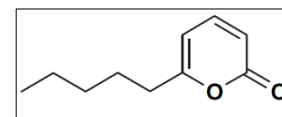
1.3.6.3. Viridins

Viridins have a structure that resembles steroidal antibiotics (with an extra furan ring). Viridin was described in 1945, and was shown to prevent germination of spores from *Botrytis allii, Fusarium caeruleum, Penicillium expansum, Aspergillus niger,* and others.
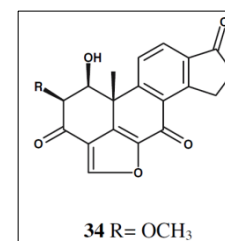
1.3.6.4. Nitrogen heterocyclic compounds

The pyridine ring appears in several fungal metabolites, and metabolites in this class have significant antifungal activity against *B. cinerea, R. solani, P. ultimum,* and *Gaeumannomyces graminis.* They have also been shown to inhibit protein phosphatases and mushroom tyrosinases.
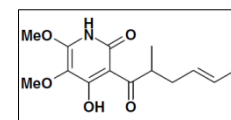
1.3.6.5. Butenolids

All the butenolids have shown significant antagonism towards the take-all fungus *Gaeumannomyces graminis* (wheat and grain pathogen). They are also inhibitory towards the growth of *R. solani* and *P. ultimum.*
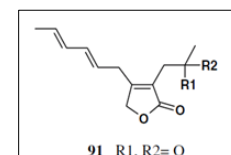
1.3.6.6. Isocyano metabolites

Metabolites in this class have a characteristic five membered ring with various levels of oxidization. Isocyano cyclopentenes were first reported by Pyke and Dietz (1966) and Meyer (1966), and several had their antibiotic activity patented (Coats et al. 1971; Yamano et al. 1970). Their activity has been shown in three areas:
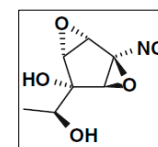
induction of bacteriostatsis of rumen bacteria; effects on fungal morphology; and inhibition of the enzyme tyrosinase.

1.3.6.7.   Diketopiperazines

The production of a 'lethal principle' was first described by Weindling in 1934 and was identified as gliotoxin in 1941 (Weindling). Gliotoxin displays a wide range of biological effects including antiviral, antibacterial, antifungal, and immunosuppressive properties.
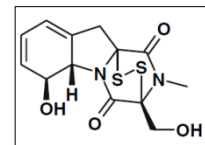
1.3.6.8. Peptaibols

Trichoderma species are the main producers of peptaibols, first described in *T. viride* by Meyer and Reusser (1967). Peptaibols are linear peptides containing 7-20 amino acids, with an acylated N-terminus, and C-terminal amino alcohol.

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phe-OH
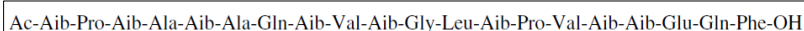
Peptaibols have strong antimicrobial activity against gram positive bacteria.

1.3.6.9. Coumarins

A methylisocoumarin was isolated and identified from *Trichoderma aggressivum* f. *aggressivum* (Krupke et al. 2003). The compound was found to be inhibitory against the growth of the commercial mushroom, *A. bisporus,* and other fungal species. This metabolite is the focus of the research presented in this project.
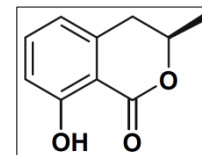
1.3.7.   Synergism

Trichoderma's ability to inhibit the growth of many other fungi is a result of the combined action of hydrolytic enzymes and antibiotic secondary metabolites (Benitez et al. 2004; Reino et al. 2008). Lo et al. (1998) suggests that mycoparasitism occurs after *Trichoderma* weakens the host cell wall by secreting enzymes and toxic metabolites. The effectiveness of *Trichoderma* species as BCAs and fitness in the environment results from a synergistic effect of all properties already described.

One concern with any BCA including *Trichoderma* spp. is how it will affect the rest of the ecosystem. Six species of *Trichoderma* (*T. citrinoviride, T. harzianum, T. koningii, T. longibracheatum, T. pseudokoningii,* and *T. viride*) have been identified as etiologic agents of infection in immune-compromised patients including dialysis patients, transplant recipients, and bone marrow transplant recipients (Kredics et al. 2003). *T. aggressivum* causes green mold disease of the commercial mushroom in North America and Europe (Samuels et al. 2002).

1.4. Green mold disease

1.4.1. History

Prior to 1985, green mold caused by *Trichoderma* spp. had been considered of minor importance. However, in the spring of 1985, farmers in Northern Ireland reported that bags of compost being colonized by *A. bisporus* would turn green with *Trichoderma* spores (Seaby, 1987). Compost or casing infected with green mold produced very little to no mushrooms (Castle et al. 1998). In addition, red pepper mites (*Pymephorus mesembrinae*) would swarm the bags to feed on the *Trichoderma* spores, rendering any mushrooms that managed to develop unpickable (Krupke et al. 2003). Seaby (1987) collected samples of green mold from infected mushroom farms and found that *T. viride, T. aureoviride, T. pseudokoningii,* and *T. hamatum* did not cause significant problems in mushroom production while three strains of *T. harzianium* were found to be virulent and he called them *Th*1*, Th*2*,* and *Th*3.

A similar problem developed on Canadian mushroom farms in 1992 and the contaminant was called *Th*4 (Rinker et al. 1997; Seaby, 1996a). The term "aggressive" became associated with the *Th*2 and *Th*4 strains not due to their pathogenicity, but due to their ability to reduce mushroom quality and yield (Williams et al. 2003). In fact, *Th*1 produced enzymes and metabolites that were more pathogenic to *A. bisporus* than the aggressive strains, resulting in the two fungi colonizing their own distinct areas of the compost (Krupke et al. 2003; Mumpuni, Sharma & Brown, 1998; Williams et al. 2003). Using a different strategy, *Th*2 and *Th*4 have reduced toxicity and greater tolerance towards *A. bisporus* allowing the *Trichoderma* strains to

colonize the entire compost. The presence of *Trichoderma* strains *Th*2 and *Th*4 drastically reduced the number of mushrooms produced by *A. bisporus*. Since the aggressivum strains were able to colonize the entire mushroom bed, they could obliterate mushroom production.

During the green mold epidemic in Europe, the vast majority of infected farms harboured the aggressivum strain *Th*2, but it was rarely detected in compost uncolonized by *A. bisporus* (Seaby, 1987; Seaby 1989). The *Th*2 strain is inhibited by compost bacteria and is only able to colonize spawned compost or compost that had been sterilized (Seaby, 1987; Seaby, 1996b). Initially it was considered that the lower level of toxicity of the aggressive strains was advantageous because they are dependent on *A. bisporus* to lyse compost bacteria to enable their survival in the compost (Seaby, 1996b). In contrast to Seaby's finding, several studies found that *Th*2 is able to infect uncolonized compost, but is hard to visualize because it has very thin hyphae (Largeteau-Mamoun, Mata & Savoie, 2002; Mamoun et al. 2000b; Mumpuni et al. 1998; Savoie et al. 2001a). One factor common in all studies is that heavy sporulation of *Trichoderma* only occurs on spawned compost, and that sporulation coincides with pathogenicity of *Trichoderma* (Mamoun, et al. 2000b). In addition, aggressive forms of *Trichoderma* are adapted to growing on compost colonized with *Agaricus*.

Rinker et al. (1997) and Seaby (1996) describe how *Trichoderma* is easily spread by dust, machinery, workers, and insect vectors. These strains of *Trichoderma* are able to survive the post-crop steam off, so spent compost was a huge source of infection in the new crop. The most effective means to control the spread of *Trichoderma* is though sanitation and hygiene (Rinker et al. 1997).

1.4.2. Genetic diversity

The different strains of *Trichoderma* have similar morphology. In order to effectively manage the newly emerged green mold disease, it was important to have an accurate and sensitive method for identification of the pathogenic strains (Castle et al. 1998). Using restriction fragment length polymorphisms (RFLPs) and randomly amplified polymorphic DNAs

(RAPDs) with ribosomal and mitochondrial DNAs, Muthumeenakshi et al. (1994) showed that the aggressive colonizers (*Th*2) could be differentiated from the non-aggressive *Th*1 and *Th*3. *Th*1 and *Th*3 were identical to isolates contained in the international culture collection while *Th*2 was not, suggesting that *Th*2 is not the selective product of one of the other strains (Muthumeenakshi et al. 1994; Hermosa et al. 2000). Similar tests were performed using the *Th*4 strain in North America, and was determined that it was also a genetically distinct form of aggressive *T. harzianum* (Castle et al. 1998; Chen et al. 1999; Dodd et al. 2000; Muthumeenakshi, Brown & Mills, 1998). *Th*1 was identified through RFLP and RAPD as being typical *T. harzianum* and *Th*3 was identified as *T. atroviride* (Castle et al. 1998). Based on differences in the ITS1 region of nuclear ribosomal DNA and *EF1α*, Samuels et al. (2002) determined that *Th*2 and *Th*4 were taxonomically different than *Th*1 and renamed them as *T. aggressivum*. Based on the similarities between the aggressive European and North American strains, Samuels et al. (2002) determined they were sister groups and different biotypes. Therefore, *Th*2 (European strain) was named *T. aggressivum* forme *europaeum* and *Th*4 (North American strain) was named *T. aggressivum* f. *aggressivum* (Samuels et al. 2002).

1.4.3. Distribution

The new aggressive strains of *Trichoderma* have spread to most areas where mushrooms are cultivated, causing severe losses in mushroom production. *T. aggressivum* f. *europaeum* was first described as causing severe green mold disease in the spring of 1985 in Northern Ireland (Seaby, 1987). The disease spread through Ireland in 1986, and into England and Scotland by 1989 (Seaby, 1989). It was reported in the Netherlands in 1994 (Geels, 1997), France and Spain by 1997 (Mamoun, et al. 2000a; Mamoun et al. 2000b), and through Eastern Europe including Hungary, Poland and Croatia by 2002 (Hatvani et al. 2007; Szczech et al. 2008; Sobieralski, Siwulski, & Frużyńska-Jóźwiak, 2009a; Sobieralski et al. 2009b).

T. aggressivum f. *aggressivum* was first found in Ontario and British Colombia in 1992 (Rinker, 1997) and devastated the mushroom industry in Chester County, Pennsylvania in 1995

(Anderson et al. 2001). Romero-Arenas (2009) reported the presence of green mold in Mexico in 2004. Considerable success in reducing green mold disease has been achieved by increased hygiene and sanitation; however, recent outbreaks around the world demonstrate that it is still a serious problem for mushroom growers (Largeteau & Savoie, 2010; Rinker & Alm, 2002).

1.4.4. *Trichoderma* interactions with *A. bisporus*

Different methods used by *Trichoderma* spp. to succeed against competing fungi include competition for nutrients, mycoparasitism, and the production of hydrolytic enzymes and toxic metabolites.

Competition between fungi is often a battle to utilize the few nutrients present in the medium. This is not the case with green mold disease. Seaby (1996) reports that after *Th*2 had sporulated, *A. bisporus* was able to grow over the infected compost and produce a crop. In addition, on a culture containing *A. bisporus* and *T. aggressivum*, Guthrie & Castle (2006) killed the *Trichoderma* with benomyl, and *A. bisporus* was able to resume its growth. Compost is a rich and complex medium, so the competition that exists between *A. bisporus* and *T. aggressivum* is not to utilize the abundance of carbohydrates and amino acids in the compost.

Some species of *Trichoderma* are able to show directed growth towards a host fungus, followed by coiling and penetration of the host. These interactions have not been seen between *T. aggressivum* and *A. bisporus* (Abubaker et al. 2013; Krupke et al. 2003; Williams et al. 2003) and are not involved in this competition.

Many strains of *Trichoderma* produce a battery of hydrolytic enzymes consisting of chitinases, β-glucanases and proteases to dissolve hyphal walls of a competitor. Williams et al. (2003) looked at depolymerases that could attack *A. bisporus* cell walls, and found rapid production of chymoelastase and trypsin-like proteases produced by *T. aggressivum* but not by *T. harzianum.* In another study looking at chitinase production, *T. aggressivum* was shown to produce five chitinases in response to the presence of *A. bisporus* (Guthrie, Khalif & Castle, 2005; Guthrie & Castle, 2006). One of these chitinases, the 122 kDa N-acetylgluosaminidase

41

showed the greatest activity and may be an indicator of antifungal activity. Abubaker et al. (2013) found three genes, a chitinase (*ech42*), a β-1,3 glucanase and a protease (*prb1*) were all expressed at higher levels when co-cultivated with *A. bisporus*. The authors also report that *prb1* and *ech42* knockdown mutants caused less disease than wild-type *T. aggressivum*, indicating a role of these genes in green mold disease.

Lastly, several groups have discussed the involvement of volatile and diffusible *T. aggressivum* metabolites that are toxic to the growth of *A. bisporus* (Krupke et al. 2003; Mumpuni et al. 1998; Savoie & Mata, 2003). One toxic secondary metabolite was identified as 3,4-dihydro-8-hydroxy-3-methylisocoumarin (Krupke et al. 2003). The focus of this research project was to investigate ways in which *A. bisporus* responds to this metabolite.

1.4.5. Disease sensitivity of different *A. bisporus* strains

A study by Anderson et al. (2001) examined and quantified the yield losses associated with different strains of *A. bisporus* resulting from exposure to *T. aggressivum*. The white strain suffered a loss in mushroom yield of 96%, 3 off-white strains experience yield loss ranging from 56 – 73%, and 3 brown strains suffered losses of only 8 – 14%. Initially Anderson et al. (2001) suggested that the resistance of the different strains correlated with the colour of the mushroom cap, namely that dark capped mushrooms were more resistance than white capped mushrooms. However, studies using wild-collected isolate of *Agaricus* with mushrooms of differing cap colours showed that there is no correlation between cap colour and resistance to green mold (Foulongne-Oriol, Minvielle & Savoie, 2011; Mamoun et al., 2000a).

Even though a brown cap does not correlate with *A. bisporus* resistant to green mold disease, the commercial brown strains have been shown to be naturally more resistant to green mold disease. When white, off-white and commercial brown strains of *A. bisporus* were inoculated with *T. aggressivum,* it only took 12 days for green mold to appear on the white strains, 13 – 24 days on the off-white strains, and 30 to 42 day on the brown strains (Anderson et al. 2001). Even though the brown strain is more resistant, *Trichoderma* is always able to

produce symptoms of green mold. The ability of the commercial brown strains to slow down the progression of the disease is extremely important in terms of mushroom production. By the time green mold appears on the brown strains at day 30, the first flush of mushrooms has already been produced. Since the first flush of mushrooms is the largest, developing green mold after the first flush the causes the impact on mushroom production to be less severe.

Few studies have tried to explain the difference in susceptibility of the different strains of mushroom to green mold. Krupke et al. (2003) found that the commercial brown strains were more resistant to *T. aggressivum* toxic secondary metabolites that the off-white strains. Guthrie & Castle (2006) hypothesized that *T. aggressivum* would produce different chitinases, and in different quantities in the presence of different strains of *Agaricus*. However, they found no difference in *Trichoderma* chitinase expression, and concluded that commercial brown strain resistance is not a result of differential chitinase expression by *T. aggressivum*. Guthrie and Castle (2006) did report an *A. bisporus* 96 kDa *N*-acetylglucosaminidase produced earlier and in greater quantities by the commercial brown strains when co-cultivated with *T. aggressivum*. How this enzyme contributes to resistance to green mold disease is not known. Abubaker et al. (2013) reports that *T. aggressivum ech42,* β-1,3 glucanase, and *prb1* were induced to greater levels in the commercial off-white strains of *A. bisporus* than in the commercial brown strains. The increased levels of these hydrolytic enzymes may enable *T. aggressivum* to overcome the white and off-white strains of *A. bisporus* more easily than the commercial brown strains.

To date there are insufficient reports describing the difference between the commercial brown strains and the hybrid off-white strains of *A. bisporus* accounting for the difference in sensitivity to green mold. It is believed that increased resistance to *Trichoderma* is an attribute found in select brown strains and is not related to cap colour. The purpose of this thesis project is to examine the response of *A. bisporus* to the presence of *T. aggressivum* toxic secondary metabolites, and if that response illuminates the mode of increased resistance of the commercial brown strains.

1.5. Specific aim of this study

The aim of this work is to assess the response of *A. bisporus* to *T. aggressivum* secondary metabolites; in particular to evaluate a role of laccases in the increased resistance of the commercial brown strains to green mold disease. A role for laccase in *A. bisporus* resistance to *T. aggressivum* metabolites was considered under the following hypothesises.

- Commercial brown strains of *A. bisporus* increase transcription of laccases (*lcc*1, *lcc*2) when exposed to toxic *T. aggressivum* secondary metabolites; indicating a role for these enzymes in commercial brown strains' increased resistance to green mold disease (Chapter 2).

- Likewise, the presence of toxic *T. aggressivum* secondary metabolites increases laccase activity in commercial brown strains of *A. bisporus*. On the other hand, using glutathione to reverse the reaction catalysed by laccase will increase sensitivity of *A. bisporus,* indicating a direct role for laccases in metabolizing toxic phenolic compounds (Chapter 3).

- Differences in upstream regulatory elements account for the differences in laccase transcriptional activity and differences in predicted amino acid sequence account for difference in laccase enzymatic activity between the strains of *A. bisporus* (Chapter 4).

- Knockdown of laccase transcriptional activity using RNAi will increase sensitivity of *A. bisporus* strains to *T. aggressivum* secondary metabolites (Chapter 5).

Chapter 2: Response of *A. bisporus* to toxins from *T. aggressivum* – transcript abundance

2.1. Introduction

Trichoderma species use a variety of methods to inhibit the growth of competitive organisms including the production of toxic secondary metabolites. In 2003, Krupke et al. isolated two compounds from *T. aggressivum* that are toxic to the growth of *A. bisporus*. One of these toxins was identified through GC/MS and ^{1}H NMR as 3,4-dihydro-8-hydroxy-3-methylisocoumarin, while the second toxin remains unknown. The ability to divest the secondary metabolites of their toxicity would increase the competitiveness of *A. bisporus* against green mold disease. Based upon the structure of the methylisocoumarin, likely candidate enzymes to detoxify the secondary metabolites include laccases and peroxidases.

Laccase (EC 1.10.3.2) was first described in the Japanese lacquer tree (*Rhus vernicifera*) by Hikorokuro Yoshida in 1883, and the first fungal laccase was reported by Bertrand in 1896 (as cited in Baldrian, 2006). Laccases have diverse functionality and have been shown to be involved in the synthesis of melanin, conidiation, formation of fruiting bodies, stress defense, lignin degradation and catalyzing the oxidization of a wide range of phenolic compounds (Baldrian, 2006; Lundell, Mäkelä & Hildén, 2010; Mayer & Staples, 2002; Thurston, 1994). Baldrian (2006) suggested that laccases may degrade phenolic antibiotics that inhibit the growth of other fungi.

Laccases have at least one type I (T1) copper center which removes one electron from an aromatic carbon to generate a free radical (Figure 7). The electron in T1 site is transferred through a conserved histidine -cysteine- histidine motif to the T2/T3 site, consisting of one T2 copper center and two T3 copper centers arranged in a cluster. After accepting electrons from the T1 site, the T2/T3 site transfers them to molecular oxygen, ultimately transforming it to water (Baldrian, 2006; Larrondo et al. 2003; Thurston, 1994). The free radical generated from the one electron oxidation is unstable and can be degraded by enzymatic or non-enzymatic

reactions. Laccases can enzymatically remove a second electron to generate a quinone, or the radicals can undergo non-enzymatic hydration or polymerization (Thurston, 1994).
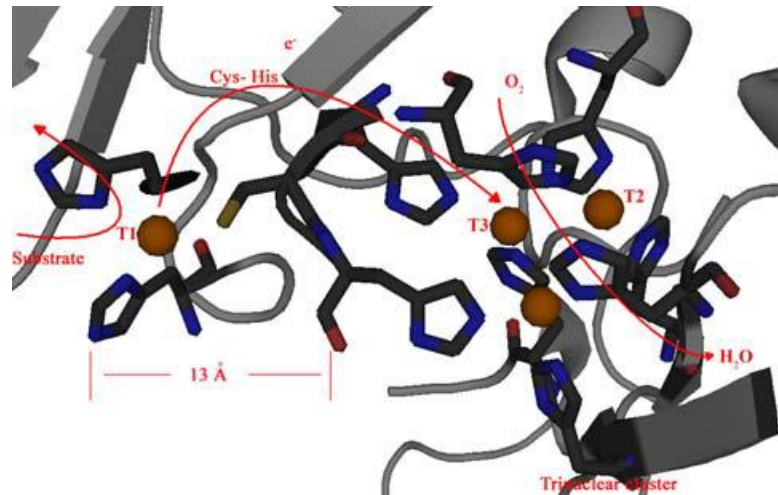
Figure 7: Catalytic cycle of laccase (Giardina, *et al*., 2010).

When *A. bisporus* colonizes compost, laccase is produced abundantly, comprising 2% of mycelial protein (Wood, 1980a). Initial experiments indicated that laccase was constitutively expressed, and the potential inducers had no effect on laccase expression (Wood, 1980a). However, laccase production is extremely variable during *A. bisporus* life cycle, increasing during mycelia grown, followed by a rapid decrease shortly before fruiting body formation (Ohga et al. 1999; Smith et al. 1989). The controlled expression of laccase at particular phases of the life cycle indicates that the genes are regulated. In addition, many laccase inducers including heat shock, anilines, methoxyphenolic acids, lignin derivatives, tannic acid, xylidine, and other phenolic compounds have been described in different fungal species (Carbajo et al. 2002; Kaluskar et al. 1999; Leonowitcz et al. 2001). Baldrian (2004) reports laccase induction in two white rot fungi as a general response to interactions with other fungi and bacteria. Therefore, it is likely that laccase in *A. bisporus* is also inducible, although the conditions have not yet been described.

Increased laccase expression has been demonstrated as a host defence response in several fungal/pathogen interactions. The basidiomycetes *Trametes versicolor* and *Pleurotus ostreatus* increase laccase activity during co-cultivation with several fungal antagonists, including

46

Trichoderma harzianum, (Baldrian, 2004). The shitake mushroom (*Letinula edodes*), like *A. bisporus,* suffers from green mold disease though not as severely. When challenged with *T. harzianum* mycelium or just the spent medium from *T. harzianum* culture, *L. edodes* responded with an increase in laccase activity (Savioe et al. 1998; 2001b). The resistance of *L. edodes* to green mold can be improved when environmental conditions stimulate laccase production by the addition of lignin and phenols to the substrate (Savoie & Mata, 2003). These reports indicate that *L. edodes* increase laccase activity in response to the presence of *Trichoderma*, and that the increase in laccase confers greater resistance to green mold disease. These studies form the premise validating the study of laccase involvement in the commercial brown strain of *A. bisporus* resistance to green mold disease caused by *T. aggressivum.*

Manganese peroxidase (EC1.11.17) was first described in the white rot fungus *Phanerochaete chrysoporium* in 1985 by William Gold's (Glenn & Gold, 1985; Kuwahara et al. 1986) and Ronald Crawford's groups (Paszczyński, Huynh & Crawford, 1985; 1986), and has been described in all white rot fungi to date, and in only a limited number of other basidiomycetes (Hofrichter, 2002; Whitwam et al. 1997). MnP is produced extracellularly by basidiomycetes and has a crucial role in lignin biodegradation. The enzyme is glycosylated and contains a heme group, which is essential for its catalytic cycle (Hatakka, 1994; Whitwam et al. 1997). MnP activity follows a classical peroxidase reaction cycle (Hofrichter, 2002; Whitwam et al. 1997). The reaction begins with the enzyme binding hydrogen peroxide to form an iron-peroxide complex. The heme group gives two electrons to reduce peroxide resulting in the loss of water molecule. This generates a Fe^{4+}-oxo-porphyrin radical complex called MnP-compound 1. Compound 1 can be reduced by a variety of electron donors, including Mn^{2+} ions and phenolic compounds forming an oxidized enzyme intermediate called MnP-compound 2. Compound 2 is reduced back to the ferric MnP enzyme only by Mn^{2+} complexed to a chelator. The Mn^{2+} ions are oxidized to Mn^{3+}, which gets stabilized by organic acids like oxalate. The

Mn^{3+}/organic acid complex are diffusible and will non-specifically attack organic compounds

abstracting protons and electrons generating radicals (Figure 8). The phenolic radicals are the
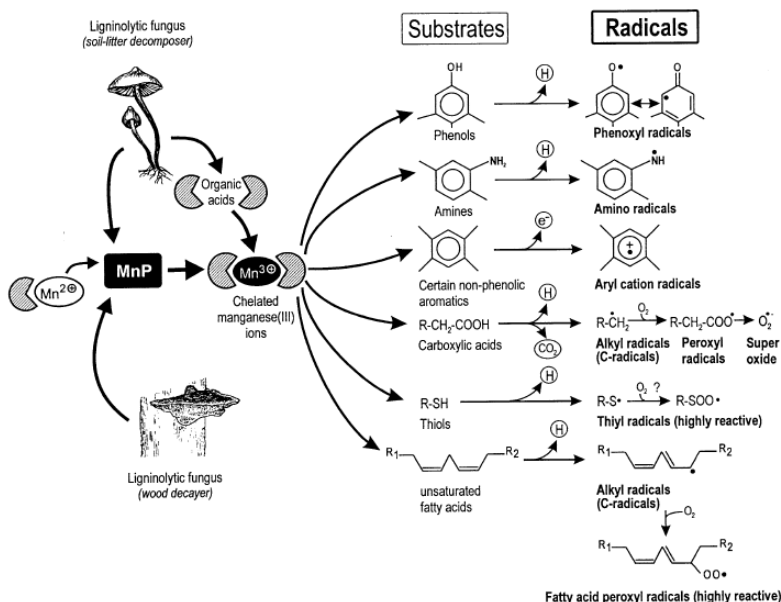
Figure 8: Manganese peroxidase oxidizes Mn^{2+} to Mn^{3+}, which non-specifically oxidizes many organic substrates forming radicals. (Picture taken from Hofrichter, 2002).

starting point of further spontaneous reactions leading to breakdown of compounds (Hatakka,

1994). This sequence of chemical reactions are important for white rot fungi because the Mn^{3+}

ions will generate phenolic radicals within the lignin polymer resulting in oxidative

depolymerisation of lignin (Whitwam et al. 1997).

The presence of MnP was first described in *A. bisporus* compost by Bonnen, Anton &

Orth (1994). Sequencing and characterization of the MnP gene was described in 2005 (Lankinen

et al.). MnP activity correlates with the loss of lignin from compost, indicating that it has a role

in lignin degradation by *A. bisporus* (Bonnen et al. 1994).

T. aggressivum represses the growth of *A. bisporus* through the production of toxic

compounds containing phenolic functional groups. The commercial brown strains of *A. bisporus*

are more resistant to these toxins that the white and off-white strains (Krupke et al. 2003), for

reasons that have not been described. *A. bisporus* produces laccases and MnP, which are able to

modify or break down phenolic compounds, potentially including toxic phenolic metabolites

48

produced by *T. aggressivum*. We hypothesized that the resistant commercial brown strains of *A. bisporus* may be able to degrade *Trichoderma* metabolites more efficiently than the off-white or white strains through differential expression of laccases and MnP. This chapter looks at expression of laccase and MnP in *A. bisporus* off-white and brown strains to determine if *T. aggressivum* secondary metabolites can affect transcript abundance of these two genes.

2.2. Methodology

2.2.1. Fungal and bacterial cultures

2.2.1.1. *Agaricus bisporus*

A. bisporus commercial strains Horst U1 off-white, and large brown Sylvan SB65 (Sylvan America Inc.) were cultured on complete yeast medium (CYM: 2 % agar, 2 % dextrose, 0.2 % yeast extract, 0.2 % peptone, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.046 % KH_2PO_4) (Rapier et al. 1972). Sub-cultures were made monthly, using a sterile scalpel to transfer a 5mm x 5mm plug to a fresh CYM plate. The cultures were maintained in a 28 °C incubator with minimal light exposure.

Liquid cultures of *Agaricus* were grown in CYM broth (2 % dextrose, 0.2 % yeast extract, 0.2 % peptone, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.046 % KH_2PO_4). The outer 2 cm of a mature plate culture of *Agaricus* was removed by scalpel (the center portion of the plate is older culture and often consists of empty hyphae) and added to 50 mL of CYM broth in a sterile Sorvall homogenizing tube. The mycelium was homogenized using a Sorvall omni-mixer for 30 seconds. The contents were added to 0.5 L of CYM broth and aliquot to empty sterile Petri dishes or Erlenmeyer flasks and incubated at room temperature until needed.

The slurry of homogenized *Agaricus* mycelium was also used to grow a mycelial lawn. The end of a 1 mL pipette tip was cut to accommodate larger mycelial fragments, and 200 μL was spread on a fresh CYM plate to yield a uniform lawn.

2.2.1.2. *Trichoderma aggressivum*

Trichoderma aggressivum f. *aggressivum* # 586 (Krupke et al. 2003) was grown on malt extract agar (MEA: 2 % malt extract, 2 % agar). The fungus was sub-cultured biweekly by spreading a loop of conidia onto a new MEA plate. These cultures were incubated at 28 °C with minimal light exposure.

One mL of distilled water was pipetted onto a 14-day-old culture of *Trichoderma*, and by rubbing the culture with a sterile glass rod, the conidia were released into solution. The conidial suspension was quantified using a haemocytometer and used to inoculate 500 mL of malt extract broth (MEB: 2 % malt extract) to yield a final concentration of 10^5 conidia/mL. The liquid culture was incubated at room temperature with shaking at 120 rpm and 12 h of light per day for 30 d.

2.2.1.3. *Escherichia coli*

Escherichia coli DH5α (Ambion) was spread on Luria Agar plates (LA: 1 % tryptone, 1 % NaCl, 0.5 % yeast extract, 2 % agar) at 37 °C for 16 h. A single colony was used to inoculate 3 mL of Luria Broth (LB: 1 % tryptone, 1 % NaCl, 0.5 % yeast extract) and grown at 37 °C with shaking at 250 rpm until culture reached an optical density at 600 nm (OD_{600}) of $0.4 - 0.6$. Glycerol (15 %) was added to the *E. coli* culture and stored at -80 °C in 1 mL aliquots.

Preparation and transformation of *E. coli* competent cells were performed as described by DeLuca Lab (Brock University). Ten μL of stock culture was inoculated into 3 mL of LB with 20 mM $MgSO_4$ and grown overnight at 37 °C with shaking at 250 rpm. One mL of the saturated pre-culture was inoculated into 50 mL LB + 20 mM $MgSO_4$ and cultured at 37°C until the OD_{600} reached 0.5. Cell growth was suspended by placing the cultures on ice for 5 minutes. Bacterial cells were pelleted by spinning at 1500 x g for 5 min at 4 °C in an IECCentra-R8 centrifuge. The supernatant was removed and the cells were resuspended in 2 mL of transformation buffer (TFB: 1mM MES-KOH, 0.1M KCl, 50mM $MnCl_2$-$4H_2O$, 50mM $CaCl_2$-$2H_2O$, 10% glycerol) and brought to 20 mL with TFB. Incubation on ice for 30 min allows the cells to acclimate to their

new environment before being pelleted by spinning at 1500 x *g* for 5 min at 4 °C. The cells were resuspended in 5 mL of TFB, followed by the addition of 300 µL of dimethyl sulfoxide (DMSO) to help cells survive freeze-thaw cycle. The competent cells were divided into 100 µL aliquots, frozen in liquid nitrogen and stored at -80 °C for up to 12 months.

2.2.2. DNA manipulations

2.2.2.1. Isolation of fungal genomic DNA

Genomic DNA (gDNA) from *A. bisporus* and *T. aggressivum* was isolated as described by Graham, Mayers, & Henry (1994). Fungal aerial hyphae were scraped off of an agar plate, frozen in liquid nitrogen and crushed with a mortar and pestle. The crushed mycelium was resupended in a DNA lysis solution (10 mM EDTA, 10 Mm Tris, 0.5 % SDS, and 0.5 mg/mL proteinase K). The mixture was vortexed for 2 min, followed by 45 min incubation at 37 °C. Following cell breakdown, 1 volume of phenol/chloroform was added and the mixture was vortexed until it appeared milky (15 sec). The sample was microfuged at 13000 x *g* for 10 min, and the top aqueous layer containing the DNA was transferred to a new tube. The DNA was precipitated with 0.25 volume of 7.5 M ammonium acetate and 2 volumes of cold 95 % ethanol, and placed at -20 °C for 1 h. The gDNA was pelleted by microfugation at 13000 x *g* for 10 min, the alcohol was purged, salts were removed by washing with 1 mL of 70 % EtOH and the gDNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). gDNA concentration and purity was measured using a NanoVue Plus Spectrophotometer and stored at -20 °C.

2.2.2.2. Isolation of bacterial plasmid DNA

Plasmids were isolated from *E. coli* overnight cultures as described by Birnboin & Doly (1979). *E. coli* cells from 1.5 mL of an overnight culture were pelleted by centrifugation at 13000 x *g* for 1 min. The supernatant was discarded and the cells were resuspended in a 100 µL resuspension buffer (10 mM Tris pH 7.5, 25 mM EDTA) by vortexing. Cell walls were broken during a 5 min incubation at room temperature (RT) by 150 µL lysis solution (1 % SDS, 0.2 N NaOH). Following cell lysis, the base was quenched with 150 µL of neutralization solution (3 M

potassium acetate) during 20 min incubation at RT. Cell debris was pelleted by centrifugation at

13000 x *g* for 10 min, and the supernatant containing plasmid DNA (pDNA) was collected in a

new tube. DNA was precipitated with 2 volumes of cold 95% for 1 h at -20 °C followed by

centrifugation at 13000 x *g* for 10 min. The alcohol was purged, salts removed by washing with

1 mL of 70 % EtOH and the pDNA was resuspended in TE buffer. pDNA concentration and

purity was measured using a NanoVue Plus Spectrophotometer and stored at -20 °C.

2.2.2.3. Polymerase chain reaction (PCR)

PCR was optimized for each reaction according to the manufacturer's instructions. Primers

(shown in Tables 2, 3) were designed to specifically amplify the target sequence and ordered

from Sigma Genosys Canada. Confirmation experiments were typically done using *Taq*

polymerase, while clones targeted for gene sequencing were generated using *Pfu* polymerase.

Typical PCR reactions using *Taq* polymerase (Fermentas) contained 2.5 µL of 10x *Taq*

buffer (buffer composition: 100 mM Tris-HCl pH 8, 500 mM KCl, 0.8 % (v/v) Nonidet P40), 1.5

µL $MgCl_2$ (stock: 25 mM $MgCl_2$), 0.5 µL dNTPs (stock: 2.5 mM each dNTP), 0.25 µL *Taq*

(stock: 5 units/µL in buffer: 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 100 mM

KCl, 0.5 % (v/v) Nonidet P40, 0.5 % (v/v) Tween 20 and 50 % (v/v) glycerol), 1 µL of the

forward and reverse primers (stock: 5 µM), 0.5 − 1 ηg of DNA template and completed to 25 µL

with dH_2O.

Typical PCR reactions using *Pfu* polymerase (Fermentas) contained 2.5 µL of 10x *Pfu*

buffer (buffer composition: 200 mM Tris-HCl (pH 8.8 at 25 °C), 100 mM $(NH_4)_2SO_4$, 100 mM

KCl, 1 % (v/v) Triton X-100, 1 mg/mL BSA), 3 µL of $MgSO_4$ (stock: 25 mM), 1 µL dNTPs

(stock: 10mM), 0.3 µL *Pfu* (stock: 2.5 units/µL in buffer: 20 mM Tris-HCl (pH 8.2), 1 mM DTT,

0.1 mM EDTA, 100 mM KCl, 0.1 % (v/v) Nonidet P40, 0.1 % (v/v) Tween 20 and 50 % (v/v)

glycerol), 1.5 µL of the forward and reverse primers (stock: 5 µM), 0.5 − 1 ηg of DNA template

and completed to 25 µL with H_2O.

The PCR reactions were performed in an Ericomp Power Block I system using this generalized protocol: initial DNA template melting at 95 °C for 5 min, followed by 30 to 35 cycles of heating: 95 °C for 30 sec, annealing: 5 °C below primer annealing temp (Table 2) for 30 sec, elongation: 72 °C for 1 min/thousand base pair (kbp) for *Taq* and 2 min/kbp for *Pfu*, a final elongation at 72 °C for 5 min before being cooled to 4 °C.

Table 2: PCR primer names and sequences used in verifying strains and generation of clones. Melting temperatures were obtained with the online DNA calculator from Sigma-Genosys (http://www.sigma-genosys.com/calc/DNACalc.asp).

Primer name	Sequence (5'-3')	Melting temp (°C)	Application
ALAN1	CAGCACCCAGAAGAATAAC	57.2	*T. aggressivum* specific
ALAN2	CAGATCATCTTGATGAGACG	58.3	
ITS1	TCCGTAGGTGAACCTGCGG	68.4	Positive control for *A. bisporus* and *T. aggressivum*
ITS4	TCCTCCGCTTATTGATATGC	61.5	
Lcc1a	CGAGTGTAGTTGCGAAAACCA	65.6	Clone partial laccase I gene for standard curve
Lcc1c	TTTGCCTCGGCGAACAT	65.5	
Lcc2a	CGTACTCGCCGATACCAA	62.3	Clone partial laccase II gene for standard curve
Lcc2c	TTTCCTTGCTCGACGTTG	62.6	
B-tub 1	TTTCGCCTCAAACCCTCG	66.2	Clone partial β-tubulin gene for standard curve
B-tub 3	CGCCAAGCGAGTGTGTAA	63.7	
MnP-F	ATGAGGCCTGTTGCGTTC	63.9	Clone partial MnP gene for standard curve
MnP-R	CTCAATTGTGTCCGAAGCT	60.4	

2.2.2.4. Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook et al. (2001). Agarose (BioShop) was added to a final concentration of 0.8 % to 1.2 % in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA). The agarose was dissolved by heating the mixture to a boil. Ethidium bromide was added (0.2 µg/mL) when the solution cooled to about 55 °C, and the solution was poured into a gel tray to solidify. DNA samples were mixed with 6x loading dye (Norgen Biotech) and loaded into the gel. The gel was then placed in an electrical current of 8 V/cm. The DNA was visualized under ultraviolet (UV) light using a Bio-Rad Gel Doc 1000.

2.2.2.5. DNA Gel Extraction

When isolating select DNA fragments, bands were removed from the gel and the DNA was isolated using the NORGEN DNA Gel Extraction Kit according to the manufacturer's instructions.

2.2.2.6. Recombination

2.2.2.6.1. Digestion

All restriction enzymes were purchased from New England Biolabs (NEB), and digestions were performed as manufacture's instruction. DNA digestion was carried out with 1 μg of DNA, 5 units of the restriction enzyme, the recommended buffer, BSA (if required) in a 20 μL reactions at 37 °C. Digestions were incubated at the optimal temperature for 3 h. Some reactions required higher amounts of DNA which corresponded to higher enzyme concentrations and longer incubation times.

DNA substrates requiring digestion with two restriction enzymes were digested via a double digestion. The Double Digest Calculator on the NEB website (http://www.neb.com/nebecomm/DoubleDigestCalculator.asp?) was used to determine conditions that would give high activity for both enzymes. Both enzymes were added to the digestion reaction mixture using the conditions described by the Double Digest Calculator, and incubated for 5 h. If conditions were not possible to perform a double digest, a sequential digest was performed. After the first digestion, the DNA was isolated using the Enzymatic Reaction CleanUp Kit from Norgen, followed by digestion with the second enzyme.

Following all digestions, the pDNA or gDNA fragment was cleaned and concentrated using the NORGEN Enzymatic Reaction CleanUp Kit. Successful digestions were verified by gel electrophoresis.

2.2.2.6.2. Ligation

T4 DNA ligase was purchased from Fermentas and ligations were performed as recommended by the manufacturer. Each reaction contained approximately 100 ηg of linear

54

pDNA, a 3:1 molar ration of insert DNA to pDNA, 2 µL of 10X T4 DNA ligase buffer (Buffer composition 400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP pH 7.8), 1 unit of T4 DNA ligase (stored in: 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA and 50% (v/v) glycerol) for sticky end ligations and 5 units for blunt end ligations. Blunt end ligations also contained 2 µL of 50 % polyethylene glycol (PEG) 4000, in a 20 µL reaction. Sticky end ligations reactions were incubated at RT for 1 h, while blunt end ligations were incubated overnight.

2.2.2.7. Cloning

Competent cells were thawed on ice before addition of approximately 50 ηg plasmid DNA (pDNA). The mixture was incubated on ice for 20 min, heat shocked in a 42 °C water bath for 45 seconds, and placed back on ice for 5 min. 1 mL of LB was added to the culture and incubated at 37 °C for 1 h with shaking. After the incubation, a serial dilution (10^0, 10^{-1}, 10^{-2}) was made using LB; 100 µL of each dilution was spread onto LB supplemented with 100 µg/mL ampicillin and 100 µg/mL X-Gal (LB+amp+X-gal). The plates were inverted and incubated at 37 °C overnight. Transformants containing gene of interest were picked based on blue – white selection and cultured in 3 mL of LB+amp. Plasmids containing gene of interest were isolated as described in 2.2.2.2.

2.2.2.8. Sequencing

Clones were sent to York University and sequenced with BigDye Terminator chemistry on the Applied Biosystems 3130xL DNA Sequencer.

2.2.3. Toxic *Trichoderma* secondary metabolites

2.2.3.1. Isolation

Following a 30 d incubation of *T. aggressivum* in MEB, organic extracellular components were isolated. The mycelium was removed by filtration through Fisherbrand Filter Paper P8 and washed with distilled H_2O. The organic components of the spent medium were collected by liquid – liquid extraction with dichloromethane (DCM). The organic phase was collected and

most of the solvent removed on a Buchi Rotovapor R-205 yielding a dark yellow solution. As a control, the same procedure was performed using sterile MEB. The plate diffusion assay used metabolites dissolved in DCM; while for cultivation with metabolites, the DCM was completely removed and the metabolites suspended in MeOH assay before addition of metabolites to liquid *A. bisporus* culture.

2.2.3.2. Plate diffusion assay

To confirm toxicity of the metabolites harvested from the spent *T. aggressivum* culture medium, crude extract was added to an actively growing *Agaricus* culture. The metabolites were pipette onto a 5 mm disk on Whatman #1 filter paper in 10 µL aliquots. The DCM was given 5 min to evaporate before the next aliquot. The filter paper containing the metabolites was added to a freshly prepared lawn of *Agaricus* and incubated at 28 °C for 10 d. Inhibition zones around the filter paper were measured to quantify toxicity of the crude extract.

2.2.4. Cultivation of *A. bisporus* with toxic metabolites

Liquid cultures (25 mL in sterile Petri plates) of *A. bisporus* were grown without agitation for 20 d before addition of 100 µL of the *T. aggressivum* metabolites. Methanol was added to the controls. To determine *A. bisporus'* response to the toxic metabolites we measured mRNA transcript abundance of laccase and MnP.

2.2.5. Laccase expression – transcript abundance

A. bisporus laccase and MnP activity was measure at the transcriptional level (mRNA transcripts) and post-translational level (enzyme activity – described in chapter 3).

2.2.5.1. Isolation of mRNA

Following cultivation with *T. aggressivum* metabolites, *A. bisporus* mycelium was harvested by filtration through Whatman #4 filter paper, frozen in liquid nitrogen and crushed with a mortar and pestle. Extraction of total RNA was performed using the NORGEN Total RNA Purification Kit. Since RNases are ubiquitous, DEPC water (0.1% Diethylpyrocarbonate water) was used in all steps and solutions involving RNA.

2.2.5.2. Digestion of genomic DNA

Genomic DNA was removed from the RNA samples by digestion with DNase I (Ambion). The entire RNA sample (44 μL) was augmented with 5 μL of 10x DNase buffer (100 mM Tris, pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$), and 2 units of DNase I. The sample was incubated at 37 °C for 30 min, and the RNA was re-isolated using the NORGEN Total RNA Purification Kit.

2.2.5.3. RNA gel electrophoresis

The RNA samples were run in a formaldehyde agarose gel to verify RNA quality and integrity as described by Sambrook et al. (2001). Agarose (1.4 % w/v) was dissolved in 41 mL 1x MOPS buffer (3-(N-morpholino) propanesulfonic acid: 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) by heating the solution to 100 °C. The solution was allowed to cool for 5 min before addition of 9 mL of formaldehyde and poured into the gel tray.

The RNA samples were mixed with NORGEN's RNA loading dye at a 1:1 ratio. The samples were heated at 80 °C for 5 min, allowed to cool and loaded in the gel. The gel was placed in an electrical current of 10 V/cm and RNA was visualized under ultraviolet (UV) light using a Bio-Rad Gel Doc 1000. RNA integrity was verified by the presence of 18S and 28S ribosomal RNA bands on the gel. The concentration and purity of RNA was measured on a NanoVue Plus Spectrophotometer and stored at -80°C.

2.2.5.4. Production of cDNA

Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to generate complimentary DNA (cDNA) from RNA. Five μg of total RNA, 2μL of 50 μM Oligo dT_{18}, 1 μL of 0.1 M dithiothreitol (DTT), 1μL 5x first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), brought up to 33 μL with DEPC H_2O were combined in a 300 μL tube and incubated for 5 min at 65 °C. In a separate tube, 9 μL of 5x first strand buffer, 4 μL 0.1 M DTT, 2 μL of 10 mM dNTPs, 40 units of RNase out, and 200 units of SuperScript III polymerase were combined. Following the incubation, the contents of the tubes were combined and incubated at 50 °C for 3 h. All cDNA samples were tested by PCR, and stored at -20 °C.

2.2.5.5. Quantitative PCR

Quantitative PCR (qPCR) was performed on the Corbett Rotor-Gene 6000 thermocycler.

Each 20 μL reaction contained 2 μL of the cDNA template, 0.2 mM of each primer and 10 μL of

iTaq Universal SYBR green supermix (Biorad). The qPCR reaction was 95 °C for 5 min,

followed by forty cycles of 95 °C for 15 sec and 58 °C for 30 sec. A high resolution melt curve

was performed to verify specific amplification, and cycle threshold (C_T) values were determined

by the Rotor-Gene Q series software.

Table 3: Primers designed to amplify *lcc*1, *lcc*2, *MnP*, and *β-tub* in q-RT-PCR experiments.

Primer name	Sequence (5'-3')	Melting temp (°C)	Amplification
AL1F-RT	CCACGTGATCCACTCAG	58.5	Laccase 1
AL1R-RT	TGAAAAACTCGTTAGTGGC	61.5	
AL2F-RT	CTGAGGATCCACTCGGG	61.9	Laccase 2
AL2R-RT	AGCTGAAGAATTCGTTGTTGA	60.0	
B-tub 1	TTTCGCCTCAAACCCTCG	66.2	β-tubulin housekeeping gene
B-tub 2	TCCCAGAACTTGGCACCA	65.6	
MnP-F-RT	AATGCGGTGAAGAGGTCC	62.7	Manganese peroxidase
MnP-F-RT	GAGAGAACCATCAGCACCAC	62.4	

2.2.5.5.1. Housekeeping gene

To standardize the amount of cDNA between samples, *β-tub* was used as a housekeeping

gene. The housekeeping gene was treated in the same manner as the target genes.

2.2.5.5.2. Standard Curve

The concentrations of the laccase and β-tubulin clones were measured using a Nanovue

photospectrometer. The copy number of the plasmid was based on its concentration and gene

sequence (Hou et al. 2010) using this formula: Copy number = (amount of pDNA (ηg) *

6.022×10^{23}) / (length of pDNA (bp) * 1×10^9 * 650) (taken from

http://www.uri.edu/research/gsc/resources/cndna.html). This calculation is based on the

approximation that 1 pair of nucleotides weighs 650 Daltons, which means 1 mole of bp weighs

650 grams. A dilution series using 10^0 to 10^7 copies of the *lcc*1*, lcc*2*, MnP*, and *B-tub* clones was measured by qPCR to generate a standard curve for each gene.

2.2.5.5.3. Measurement of transcript abundance

The C_T value of each experimental sample was compared to the standard curve to determine number of transcripts for each gene. The numbers of β-tub transcripts were standardized at 1000 transcripts per reaction for each sample to enable comparison of the *lcc*1, *lcc*2, and *MnP* transcripts over time.

2.3. RESULTS AND DISCUSSION

2.3.1. Toxic *T. aggressivum* metabolites

2.3.1.1. *T. aggressivum* specific PCR test

Since *Trichoderma* is ubiquitous and several species are closely related morphologically, *Trichoderma aggressivum* identity was verified periodically by PCR using specific primers.
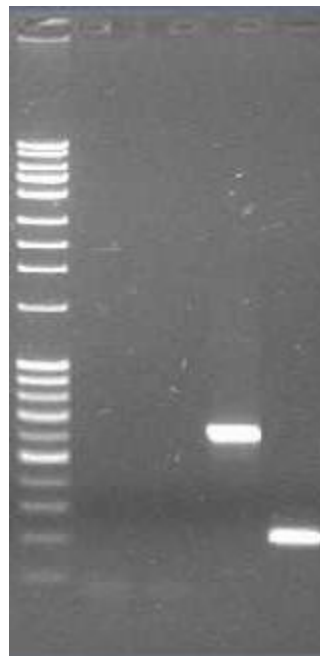
A   B   C   D   E

Figure 9: Gel showing amplification of *Trichoderma aggressivum* specific region, the specific amplicon is 189 base pairs in length. (A) Norgen Highranger Plus Ladder, (B) no template control (NTC) with ITS primers, (C), NTC with Alan primers, (D) gDNA with ITS primers, (E) gDNA with Alan primers.

ALAN1 and ALAN2 primers are specific for *T. aggressivum* (Castle, personal communication) and amplify a 189 bp fragment (Figure 9).

2.3.1.2. Toxicity assay

Trichoderma spp. produce many metabolites that are toxic to competing organisms. Krupke et al. (2003) identified a methylisocoumarin that has increased toxicity towards the growth of commercial white and off-white strains of *A. bisporus* than the commercial brown strains. After isolating organic, extracellular secondary metabolites from *T. aggressivum*, we tested them for toxicity before using them in subsequent experiments (Figure 10). The filter paper containing the *T. aggressivum* secondary metabolites were inhibitory to the growth of *Agaricus* while the paper and solvent (MeOH) controls do not have any effect on the growth of *A. bisporus.* Extractions displaying toxicity were pooled and stored at -20 °C.
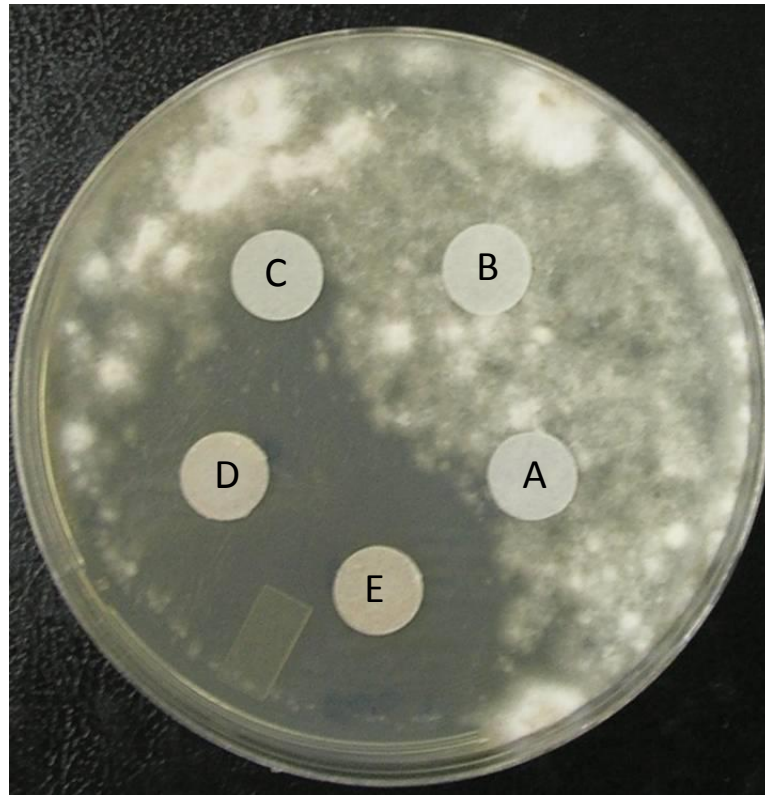
Figure 10: Growth of *A. bisporus* inhibited by the *T. aggressivum* secondary metabolites. (A) 50 µL of methanol (solvent control), (B) paper control, (C) 10 µL of 2° metabolite, (D) 30 µL of 2° metabolite, (E) 50 µL of 2° metabolite.

2.3.1.3. Inhibition of *A. bisporus* by *T. aggressivum* metabolites

The toxicity assay was performed using the off-white (U1) and commercial brown strains (SB65) of *A. bisporus* and the diameter of the inhibition zone surrounding each disc was measured every 10 d for 30 d as shown in Figure 11. The inhibition zone surrounding the filter paper on plates colonized by the commercial brown strain is smaller than the off-white strain at each time point (p<0.001 – Appendix II, Table A1). This observation indicates that the U1 strain
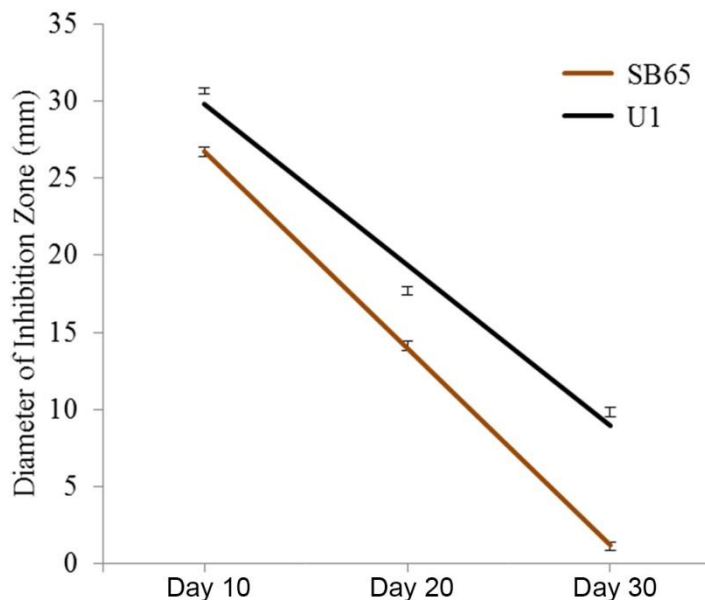
Figure 11: Secondary metabolites produced by *T. aggressivum* are toxic to the growth of *A. bisporus*. SB65 shows greater tolerance toward the metabolites, and greater ability to overcome the toxicity indicating enzymatic activity than U1.

of *A. bisporus* is more sensitive to the toxin than SB65. Krupke et al. (2003) also found that the off-white strain S130 was significantly more sensitive to *T. aggressivum* metabolites than SB65. A second important trend shown by Figure 11 is the negative slope of the trend lines of both U1 and SB65 strains (p<0.001 – Appendix II, Table A1). This indicates that the toxicity of the *T. aggressivum* metabolites is decreasing with time, allowing both *A. bisporus* strains to grow within the inhibition zone. Colonization of the zone was visualized as the appearance of discrete colonies rather than as continued growth from the edge of the non-inhibited mycelium. These observations suggested that the toxic effect is fungistatic rather than fungicidal. The reduction of

toxicity may result from volatility of the toxin, environmental breakdown or enzymatic breakdown. Toxin volatility and environmental breakdown would occur at the same rate in both *A. bisporus* strains, resulting in the same negative slope. However, the rate of growth of the SB65 strain is significantly greater than the U1 (p=0.001 – Appendix II, Table A1), indicating that the SB65 strain is actively changing or degrading the toxin by enzymatic activity. This enzymatic activity allows the SB65 strain to grow into the inhibition zone faster that the U1 strain.

One of the toxic secondary metabolites produced by *T. aggressivum* has been identified as a methylisocoumarin derivative. This phenolic compound may be a substrate for phenol oxidases like laccase and manganese peroxidase. To assess if *T. aggressivum* metabolites have an effect on *A. bisporus* hydrolytic enzyme expression, 20 day-old liquid cultures of the sensitive U1 and resistant SB65 strains of *A. bisporus* were inoculated with 100 µL of the *T. aggressivum* secondary metabolites. The response of *A. bisporus* was measured in terms of transcript abundance of three genes coding for hydrolytic enzymes – laccase 1 (*lcc*1), laccase 2 (*lcc*2), and manganese peroxidase (*MnP*).

2.3.2. Transcriptional activity

Quantitative real time PCR (qPCR) is a powerful technique that provides accurate and reproducible quantification of gene transcripts (Heid et al. 1996). Quantification can be performed "relative" to an internal standard or control, or "absolute" where the number of transcripts can be determined based on a standard curve (Hou et al. 2010). This experiment describes absolute quantification of *lcc*1, *lcc*2 and *MnP* standardized against a housekeeping gene, *β-tub*. To determine absolute quantification, each gene was cloned into a plasmid, and a dilution series of the plasmid yielded a standard curve as modified from Hou et al. (2010). The standard curve allows determination of gene transcript abundance from the threshold cycle generated by the qPCR reaction. After standardizing the transcript number of β-tubulin, we compared the transcript abundance of *lcc*1, *lcc*2, and *MnP*.

2.3.2.1. Clone partial gene sequences

2.3.2.1.1. β-tubulin

Different experimental samples contain different amounts of cDNA depending on the amount of RNA used and efficiency of the RT-PCR reaction. Without a method to standardize between samples, the results from the qPCR reaction may simply show a difference in the concentration of the template rather than expression of a gene. A housekeeping gene is a gene whose expression remains constant through the experiment thus creating a baseline to standardize between samples. A housekeeping gene commonly used for fungi is β-tubulin. Primers β-tub1 and β-tub3 were designed to amplify a 442 bp portion of the *A. bisporus* β-tubulin gene using cDNA as template. The primers were based on a partial gene sequence obtained from the NCBI database (accession # AW324553). The PCR product was visualized via gel electrophoresis to confirm amplification (Figure 12). The β-tubulin PCR products were cloned into pDrive (Qiagen) and sequenced to confirm gene identity.
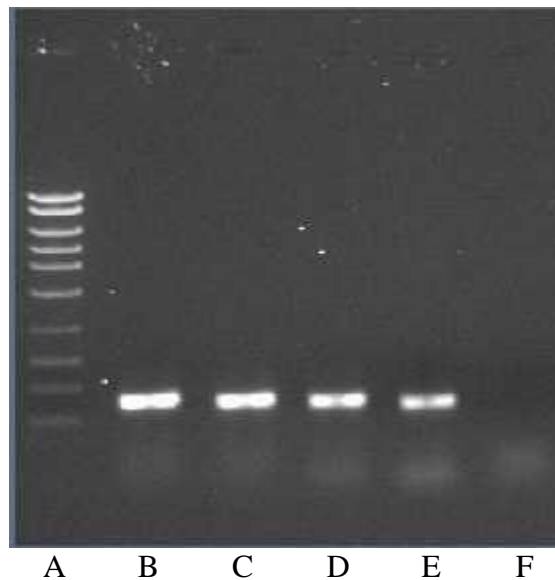
Figure 12: Gel showing amplification of partial clone of *A. bisporus* β-tubulin gene using β-tub1 and β-tub3 primers. Norgen Midranger ladder (A); cDNA template (B-E); NTC (F).

2.3.2.1.2. Laccase 1

The complete *lcc*1 sequence was obtained from the NCBI database (accession # L10664).

Primers Lcc1a and Lcc1c were designed to amplify *lcc*1 using cDNA to avoid introns from

affecting the real time amplification. After repeated attempts, *lcc*1 could not be amplified with

cDNA (example in Figure 13) suggesting that laccase 1 is not expressed under the conditions

used to grow *A. bisporus*. Smith et al. (1998) measured *lcc*1 expression in culture and in

| A | B | C | D | E |
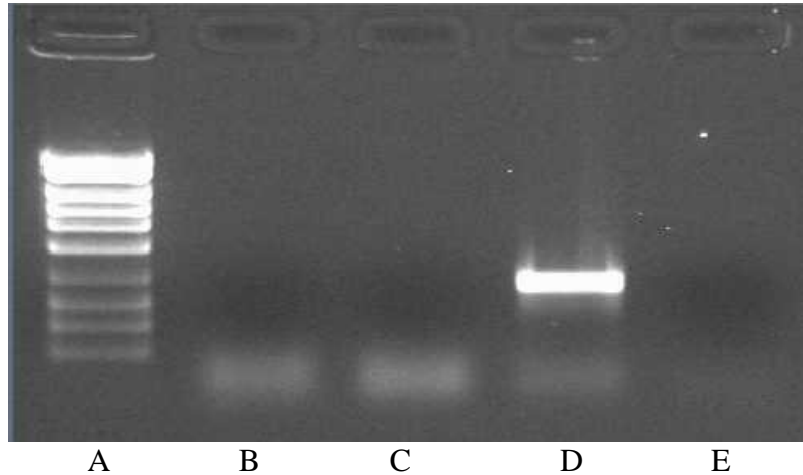
Figure 13: Agarose gel shows PCR amplification of *A. bisporus* laccase 1
gene using Lcc1a and Lcc1c primers. Norgen Midranger Ladder (A);
cDNA template (B & C); gDNA (D); NTC (E).

A   B   C   D   E
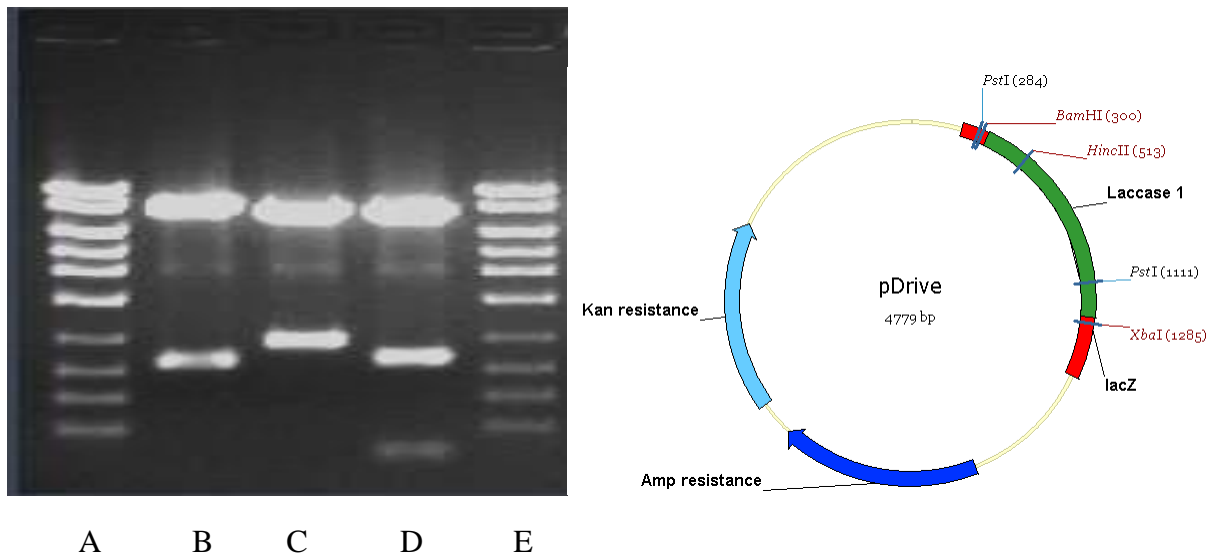
Figure 14: Agarose gel showing the digested pDrive plasmid harbouring
the *lcc*1 gene. Norgen Midranger ladder (A & E); clone cut with *Bam*H1
and *Pst*1 (B); cut with *Bam*H1 and *Xba*1 (C); cut with *Pst*1 and *Xba*1 (D).
The vector with the clone and restrictions sites is shown on the right.

compost, and also found that *lcc*1 is expressed at low levels.   By switching to gDNA a 928 bp

fragment of *lcc*1 was amplified as shown in Figure 13. The amplified laccase 1 gene was ligated

into pDrive and cloned into *E. coli.* The plasmid was isolated from the bacterium and digested

with restriction enzymes to ensure the presence of the desired sequence (Figure 14). Further

confirmation that the clone contained the laccase 1 clone was obtained by having the plasmid

sequenced (Figure 15).

```
Lcc1gDNA    : GGATCAACAATGAGGCTGTCCAACGCTTTAGTATTGGTCGCCGCATGCATTTCGAGTGTAGTTGCGAAAACCAGAACCTTCGACTTCGAC :  540
Lcc1cDNA    : .........ATGAGGCTGTCCAACGCTTTAGTATTGGTCGCCGCATGCATTTCGAGTGTAGTTGCGAAAACCAGAACCTTCGACTTCGAC :   81
Lcc1clone   : ...................................................CGAGTGTAGTTGCGAAAACCAGAACCTTCGACTTCGAC :   38

Lcc1gDNA    : CTAGTCAATACCAGGCTCGCGCCTGATGGGTTTGAAAGGGAGTGAGTATATTTCTATACAGATTCATCGTTAGAGTTTACTGACCTTGAT :  630
Lcc1cDNA    : CTAGTCAATACCAGGCTCGCGCCTGATGGGTTTGAAAGGGA................................................. :  122
Lcc1clone   : CTAGTCAATACCAGGCTCGCGCCTGATGGGTTTGAAAGGGAGTGAGTATATTTCTATACAGATTCATCGTTAGAGTTTACTGACCTTGAT :  128

Lcc1gDNA    : TTAAACTAGCACCGTGGTCATCAATGGAGAATTCCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATAA :  720
Lcc1cDNA    : .........CACCGTGGTCATCAATGGAGAATTCCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATAA :  203
Lcc1clone   : TTAAACTAGCACCGTGGTCATCAATGGAGAATTCCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATAA :  218

Lcc1gDNA    : CAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATTGTGAGTTACTTTTTGAGCCCAGATTGACTTGACTATATAACACAAATTAG :  810
Lcc1cDNA    : CAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATT................................................. :  243
Lcc1clone   : CAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATTGTGAGTTACTTTTTGAGCCCAGATTGACTTGACTATATAACACAAATTAG :  308

Lcc1gDNA    : CATTGGCATGGATTTTTCCAAGCGAGAACTTCTGGTCAGGACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTC :  900
Lcc1cDNA    : CATTGGCATGGATTTTTCCAAGCGAGAACTTCTGGTCAGGACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTC :  333
Lcc1clone   : CATTGGCATGGATTTTTCCAAGCGAGAACTTCTGGTCAGGACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTC :  398

Lcc1gDNA    : ACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAACGTTCGTCCTGATCCCTTAAATACATCTTCAGCGTAAATTGACATTTCTTCAAGTT :  990
Lcc1cDNA    : ACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAACGTTC................................................... :  372
Lcc1clone   : ACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAACGTTCGTCCTGATCCCTTAAATACATCTTCAGCGTAAATTGACATTTCTTCAAGTT :  488

Lcc1gDNA    : ATTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGGTAATTCCTTCCTCTTGCATGCGA : 1080
Lcc1cDNA    : ..TGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACG........................ :  436
Lcc1clone   : ATTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGGTAATTCCTTCCTCTTGCATGCGA :  578

Lcc1gDNA    : AGATATTTTTGCCGACTTTCGTTTAGACCCACGTGATCCACTCAGACATCTCTATGATGTTGATGACGAAAGTACCGTCATCACTCTAGC : 1170
Lcc1cDNA    : ..........................ACCCACGTGATCCACTCAGACATCTCTATGATGTTGATGACGAAAGTACCGTCATCACTCTAGC :  500
Lcc1clone   : AGATATTTTTGCCGACTTTCGTTTAGACCCACGTGATCCACTCAGACATCTCTATGATGTTGATGACGAAAGTACCGTCATCACTCTAGC :  668
                                                     ───────────────▶

Lcc1gDNA    : TGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCGTAAGCTTCTTAAAGGAATATTTATTC : 1260
Lcc1cDNA    : TGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACC........................... :  563
Lcc1clone   : TGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCGTAAGCTTCTTAAAGGAATATTTATTC :  758
                                                     ◀───────────────

Lcc1gDNA    : ATTTTCATAACCCAATTCCTGCAGCGTGCAAGATTCTGGCTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCCTCAGTAAGTAGAT : 1350
Lcc1cDNA    : .........................CGTGCAAGATTCTGGCTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCCTCA.......... :  619
Lcc1clone   : ATTTTCATAACCCAATTCCTGCAGCGTGCAAGATTCTGGCTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCCTCAGTAAGTAGAT :  848

Lcc1gDNA    : CAAAACAGAAAAGGTGATGATGAAAATTTGACTTTACTTGCTTAGCACCATTCGCCGTCGTCAATGTTCGCCGAGGCAAACGTTACAGGC : 1440
Lcc1cDNA    : ............................................CACCATTCGCCGTCGTCAATGTTCGCCGAGGCAAACGTTACAGGC :  664
Lcc1clone   : CAAAACAGAAAAGGTGATGATGAAAATTTGACTTTACTTGCTTAGCACCATTCGCCGTCGTCAATGTTCGCCGAGGCAAA.......... :  928
```

Figure 15: Sequence alignment showing the *lcc*1 gDNA and cDNA
sequences (accession # L10664) from the NCBI database and the *lcc*1
clone sequence. qPCR forward and reverse primers are indicated by
arrows.

The sequence of the cloned gene (*Lcc*1clone) is 100 % homologous to the gDNA and cDNA

sequences obtained from the NCBI database. Since the PCR reaction used gDNA as a template

the clone contains 6 introns. In order to use this clone in qPCR, we designed qPCR primers contained within one exon (shown by arrows in Figure 15).

2.3.2.1.3. Laccase 2

*A. bisporus lcc*2 mRNA sequence was obtained from the NCBI database (accession # L10663).

A   B   C   D
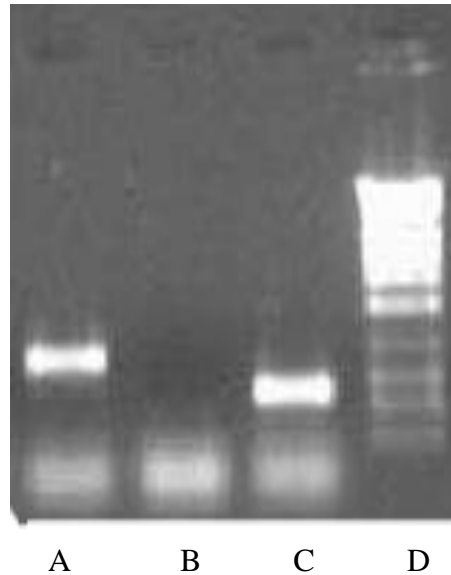
Figure 16: Gel showing amplification of *A. bisporus* laccase 2 gene. Genomic DNA template (A); NTC (B); cDNA template (C); Midranger ladder (D).

A   B   C   D   E
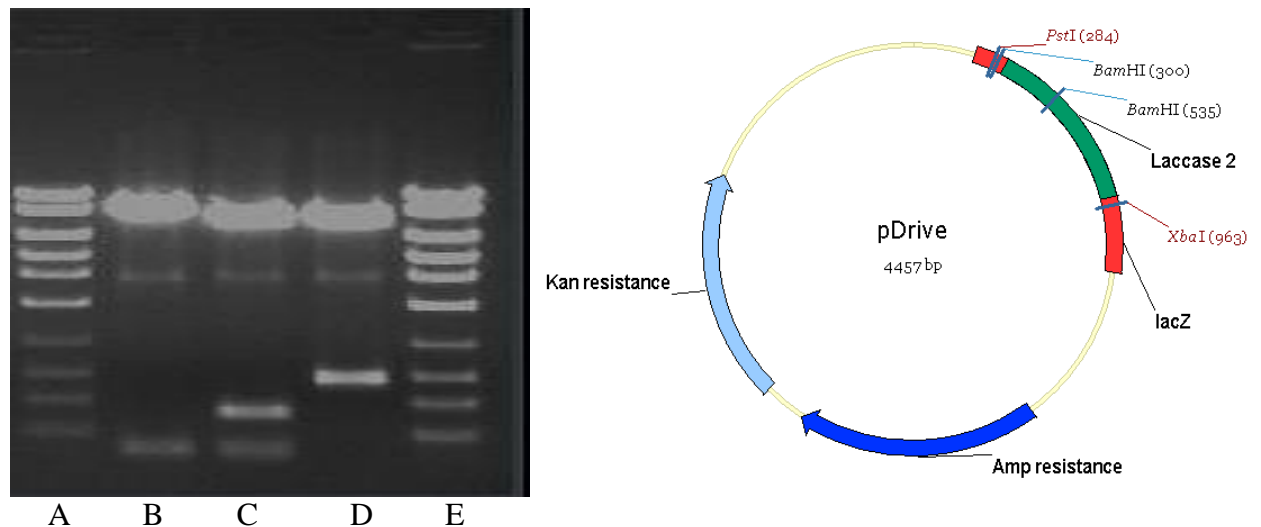
Figure 17: Agarose gel showing the digested pDrive plasmid harbouring the *lcc*2 gene. The vector with the clone and restrictions sites is shown on the right. Norgen Midranger (A & E); cut with *Bam*H1 and *Pst*1 (B); cut with *Bam*H1 and *Xba*1 (C); cut with *Pst*1 and *Xba*1 (D).

Primers lcc2a and lcc2c were designed to amplify 606 bp of *lcc*2 using a cDNA template (Figure

16). The *lcc*2 amplified from cDNA, was smaller than the sequence amplified from gDNA (920

bp) because it lacks introns. The *lcc*2 gene fragment was ligated into the pDrive cloning vector,

cloned into *E. coli* and cultured. The plasmid was isolated and the insert was confirmed by

digestion with restriction enzymes (Figure 17) and sequencing (Figure 18). The sequence of the

*lcc*2 clone was 100 % homologous to the cDNA sequence obtained from the database. To

visualize the introns, the alignment includes the gDNA sequence of *lcc*1. The homology

```
Lcc1gDNA  : GGATCAACAATGAGGCTGTCCAACGCTTTAGTATTGGTCGCCGCATGCATTTCGAGTGTAGTTGCGAAAACCAGAACCTTCGACTTCGAC : 540
Lcc2cDNA  : .........ATGAGGTTTTCCAACGCTTTTTCTACTGGTCGCCGCCTGCATTTCGAGCGTACTCGCCGATACCAAAACCTTCAACTTCGAC :  81
Lcc2clone : .......................................................CGTACTCGCCGATACCAAAACCTTCAACTTCGAC :  34

Lcc1gDNA  : CTAGTCAATACCAGGCTCGGGCCTGATGGGTTTGAAAGGGAGTGAGTATATTTCTATACAGATTCATCGTTAGAGTTTACTGACCTTGAT : 630
Lcc2cDNA  : CTAGTCAATACTAGGCTTGCACCTGATGGGTTTGAAAGGGA................................................. : 122
Lcc2clone : CTAGTCAATACTAGGCTTGCACCTGATGGGTTTGAAAGGGA................................................. :  75

Lcc1gDNA  : TTAAACTAGCACCGTGGTCATCAATGGAGAATTCCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCCGCATCCCTCTGCATAA : 720
Lcc2cDNA  : .........CACCGTGGTTATCAATGGAGAATTCCCTGGAACACTGGTCCAAGTCAACAAGGGCGATAGTGTCCCGATCCCGGTCAACAA : 203
Lcc2clone : .........CACCGTGGTTATCAATGGAGAATTCCCTGGAACACTGGTCCAAGTCAACAAGGGCGATAGTGTCCCGATCCCGGTCAACAA : 156

Lcc1gDNA  : CAAACTGACTAGCCGGACCATGAGGCGAAGTGTATCCATTGTGAGTTACTTTTTGAGCCCAGATTGACTTGACTATATAACACAAATTAG : 810
Lcc2cDNA  : CAAACTGACCAGCTCAACCATGAGGCGAAGTGTATCCATT................................................. : 243
Lcc2clone : CAAACTGACCAGCTCAACCATGAGGCGAAGTGTATCCATT................................................. : 196

Lcc1gDNA  : CATTGGCATGGATTTTTCCAAGCGAGAACTTCTGGTCAGGACGGGCCTTGCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTC : 900
Lcc2cDNA  : CACTGGCATGGATTTTTCCAAGCGAGGACTTCTGGTCAGGACGGGCCTGCATTCGTGAATCAGTGCCCGCAACCACCCAACACAACCTTC : 333
Lcc2clone : CACTGGCATGGATTTTTCCAAGCGAGGACTTCTGGTCAGGACGGGCCTGCATTCGTGAATCAGTGCCCGCAACCACCCAACACAACCTTC : 286

Lcc1gDNA  : ACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAAGCGTTCGTCCTGATCCCTTAAATACATCTTCAGCGTAAATTGACATTTCTTCAAGTT : 990
Lcc2cDNA  : ACCTATGAATTCAGCGTGGCGGACCGAGTCTGGAACTTTC................................................. : 372
Lcc2clone : ACCTATGAATTCAGCGTGGCGGACCGAGTCTGGAACTTTC................................................. : 325

Lcc1gDNA  : ATTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGGTAATTCCTTCCTCTTGCATGCGA : 1080
Lcc2cDNA  : ..TGGTACCATTCACACTTGTCCACCCAATATTGTGATGGCCTTCGTGGTGCCTTCGTCGTTTA........................... : 434
Lcc2clone : ..TGGTACCATTCACACTTGTCCACCCAATATTGTGATGGCCTTCGTGGTGCCTTCGTCGTTTA........................... : 387

Lcc1gDNA  : AGATATTTTTGCCGACTTTCGTTTAGACCCACGTGATCCACTCAGACATCTCTATGATGTTGATGACGAAAGTACCGTCATCACTCTAGC : 1170
Lcc2cDNA  : .....................TGACCCTGAGGATCCACTCGGGCATCTTTACGATGTTGATGATGAAACTACTGTCATCACCCTAGC : 500
Lcc2clone : .....................TGACCCTGAGGATCCACTCGGGCATCTTTACGATGTTGATGATGAAACTACTGTCATCACCCTAGC : 453

Lcc1gDNA  : TGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCGTAAGCTTCTTAAAGGAATATTTATTC : 1260
Lcc2cDNA  : TGAATGGTATCACGTCCTCGCACCCGATATCAACAACGAATTCTTCAGCTCTGGCATTATACC........................... : 563
Lcc2clone : TGAATGGTATCACGTCCTCGCACCCGATATCAACAACGAATTCTTCAGCTCTGGCATTATACC........................... : 516

Lcc1gDNA  : ATTTTCATAACCCAATTCCTGCAGCGTGCAAGATTCTGGCTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCCTCAGTAAGTAGAT : 1350
Lcc2cDNA  : .....................CGTGCAAGATTCTGGTTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCGAGA.......... : 619
Lcc2clone : .....................CGTGCAAGATTCTGGTTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCGAGA.......... : 572

Lcc1gDNA  : CAAAACAGAAAAGGTGATGATGAAAATTTGACTTTACTTGCTTAGCACCATTCGCCGTCGTCAATGTTCGC.CGAGGCAAACGTTACAGG : 1439
Lcc2cDNA  : ........................................CGCCATTCGCCGTCGTCAACGT.CGAGCAAGGAAAACGCTATAGG : 663
Lcc2clone : ........................................CGCCATTCGCCGTCGTCAACGT.CGAGCAAGGAAA.......... : 606
```

Figure 18: Sequence alignment showing the *lcc*1 gDNA (accession #
L10664) and *lcc*2 cDNA (accession # L10663) sequences from the
database and the *lcc*2 cloned sequence. qPCR forward and reverse
primers are indicated by arrow.

between laccase 1 and 2 demonstrates that the genes are well conserved, highlighting the difficulty in selectively amplifying *lcc*1 and *lcc*2 genes. Laccase clones could be differentiated by the presence of a *Pst*1 restriction site at 1111 bp in the *lcc*1 clone (Figure 14) and the *Bam*H1 restriction site at 535 bp in the *lcc*2 clone (Figure 17). The alignment using *lcc*1 and *lcc*2 shows some variation that was exploited in qPCR development. This variable region is the target for specific primers used to amplify *lcc*1 and *lcc*2 in the qPCR reaction (primers underlined in red in Figure 18).

2.3.2.1.4. Manganese Peroxidase

The manganese peroxidase (*MnP*) sequence was obtained from the NCBI website (accession number AJ699058). Primers were designed to amplify a 500 bp portion of the *MnP* gene from cDNA (Figure 19). The *MnP* gene fragment was ligated into pDrive and replicated in *E. coli.*
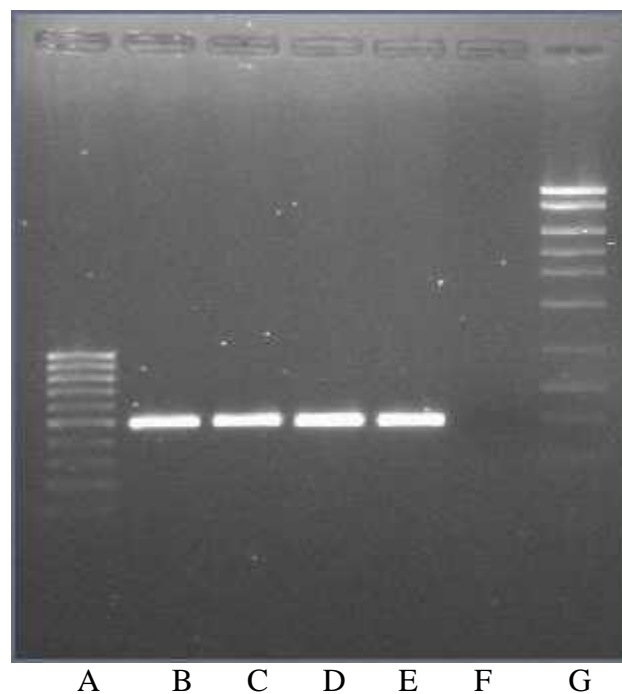
Figure 19: Gel showing amplification of the *A. bisporus* MnP gene. PCRsizer ladder (A); *MnP* amplified from cDNA template (B – D); NTC (F); Midranger ladder (G).

2.3.2.2. Generation of standard curves

Knowing the DNA sequence and concentration of each of the cloned genes and the plasmids, we were able to calculate copy number. Plotting the C_T values generated by qPCR using serial dilutions of the clones provided a standard curve for each of the four genes (Figure 20). The standard curves ranged from 5 orders of magnitude (β-tub) to 7 orders (*lcc*1 & *lcc*2) and included from 10 to over 10 million templates in the reaction. The equation line of each standard curve was used to determine the number of templates of each of the genes in each experimental reaction. Since the range of the standard curve was so large, almost all experimental C_T values fell within the linear range adding to the validity of the results. The average starting copy number of *β-tub* transcripts was approximately 1000 transcripts per reaction. To standardize between the different fungal strains, days, genes and experimental conditions, we based the copy number of other genes relative to 1000 transcripts of β-tubulin.

B-tubulin

$y = -1.464\ln(x) + 31.985$
$R^2 = 0.9969$

Lcc1

$y = -1.489\ln(x) + 33.667$
$R^2 = 0.9957$

Copy number of gene
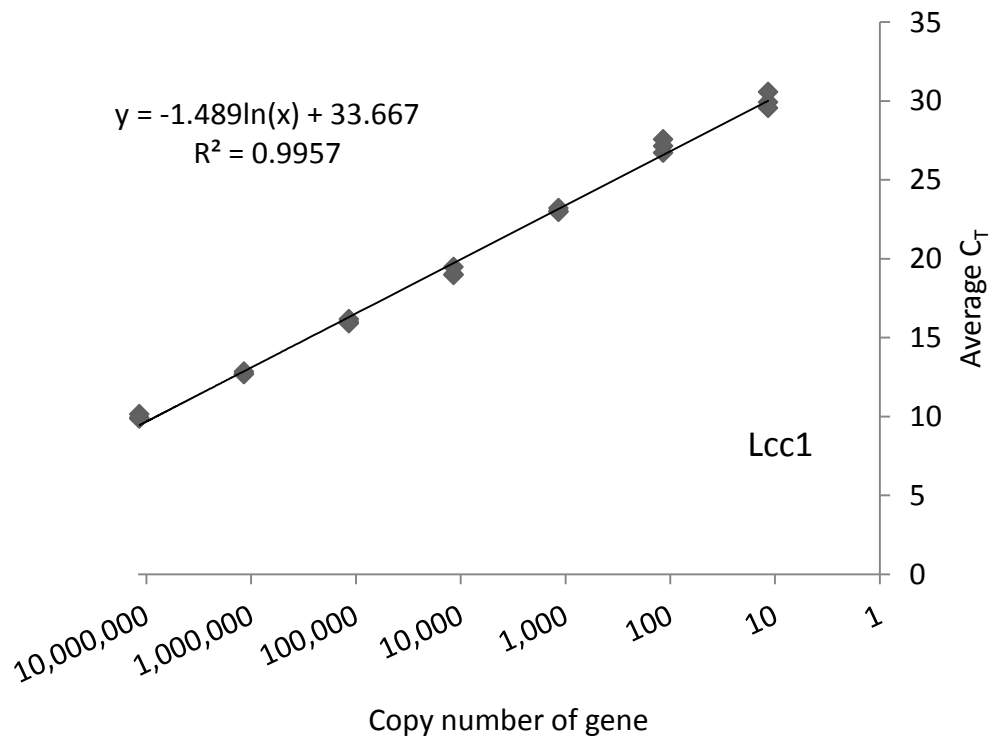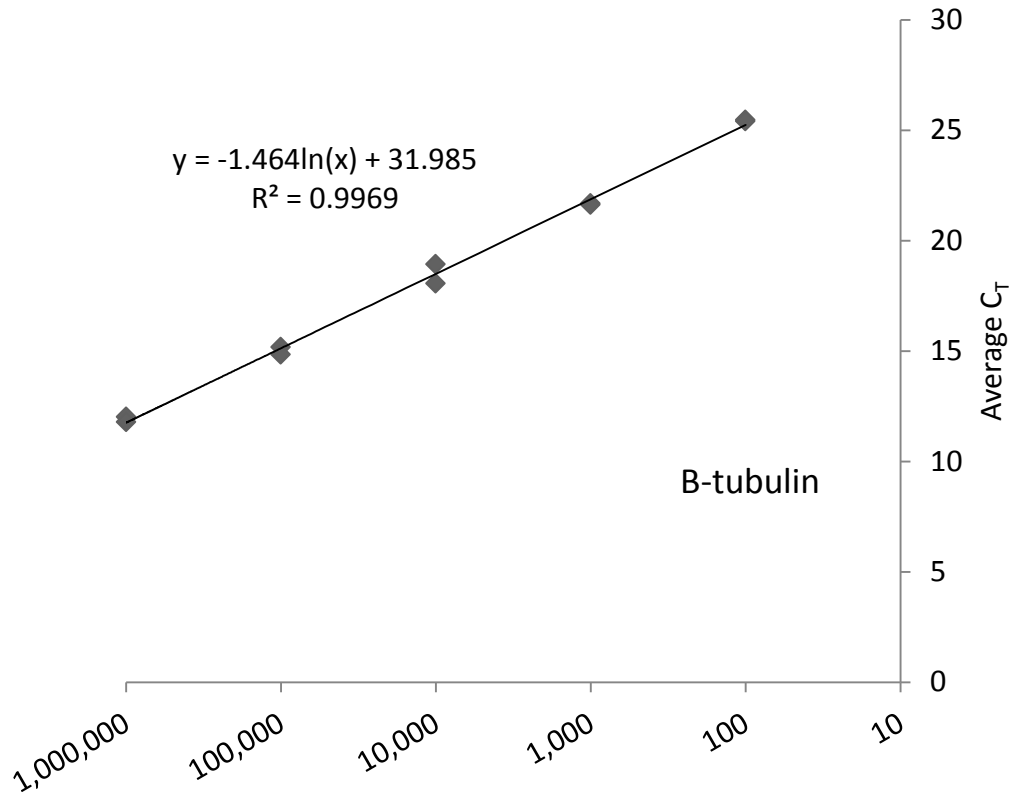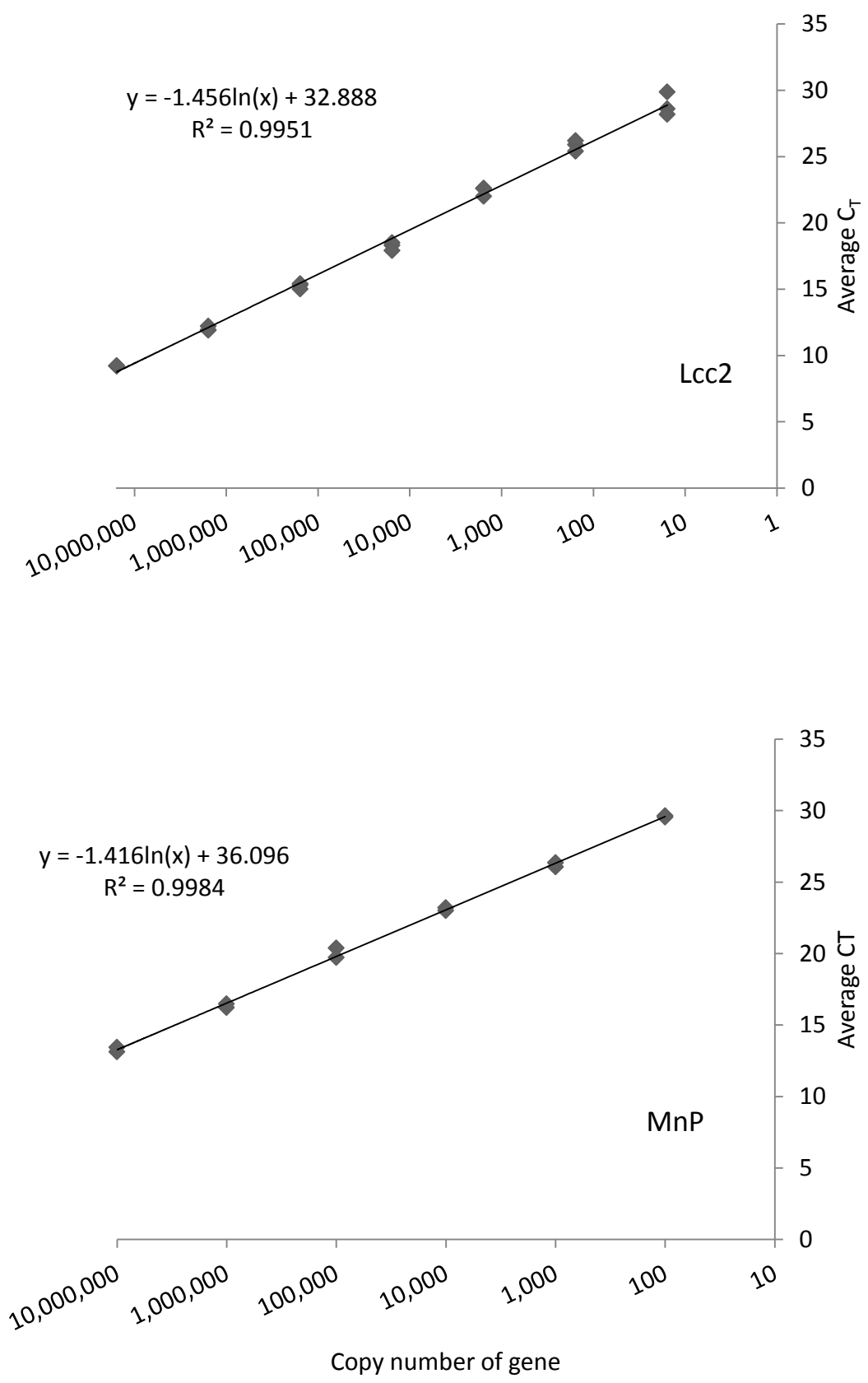
Figure 20: Standard curves for *A. bisporus* β-*tub, lcc*1, *lcc*2, and *MnP*. Each standard curve spanned 5 – 7 orders of magnitude.

2.3.2.3. Primer specificity

Primers were designed to amplify 80-100 bp of the laccase genes in qPCR (underlined in red in Figures 14 & 17).   Since *lcc*1 and *lcc*2 are 91.4 % homologous at the mRNA level (Perry et al. 1993b), great caution was taken to design primers and experimental conditions that would specifically amplify each gene. The amplification plot shown in Figure 21A shows the
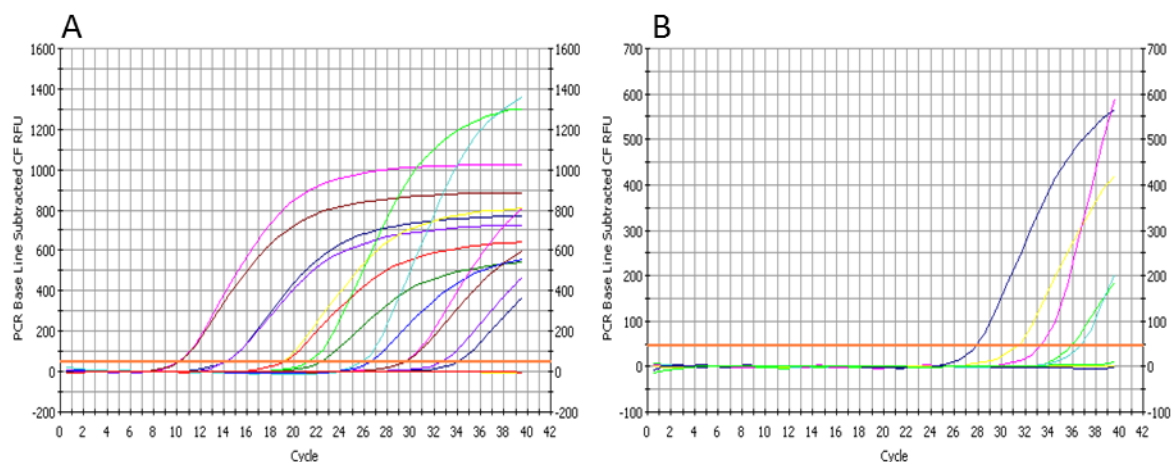
Figure 21: Two amplification plots using a serial dilution of the *lcc*1 clone using *lcc*1 specific primers (A) and *lcc*2 specific primers (B).

*lcc*1 clone being amplified with the *lcc*1 specific primers. As expected, the highest concentration of starting template reached the threshold at lowest cycle in the PCR reaction. Each ten-fold dilution corresponded to an increase in approximately 3 C_T. The amplification plot shown in Figure 21B is the same dilution series of *lcc*1 template amplified with *lcc*2 specific primers. Using *lcc*2 primers resulted in a vast increase in C_T.   The highest concentration of template using the appropriate primer had a C_T value ≈9, while using *lcc*2 primers had a C_T value ≈28. This huge difference proves that the *lcc*1 gene can be specifically amplified. Figure 22 shows the reciprocal amplification plot using the *lcc*2 clone.
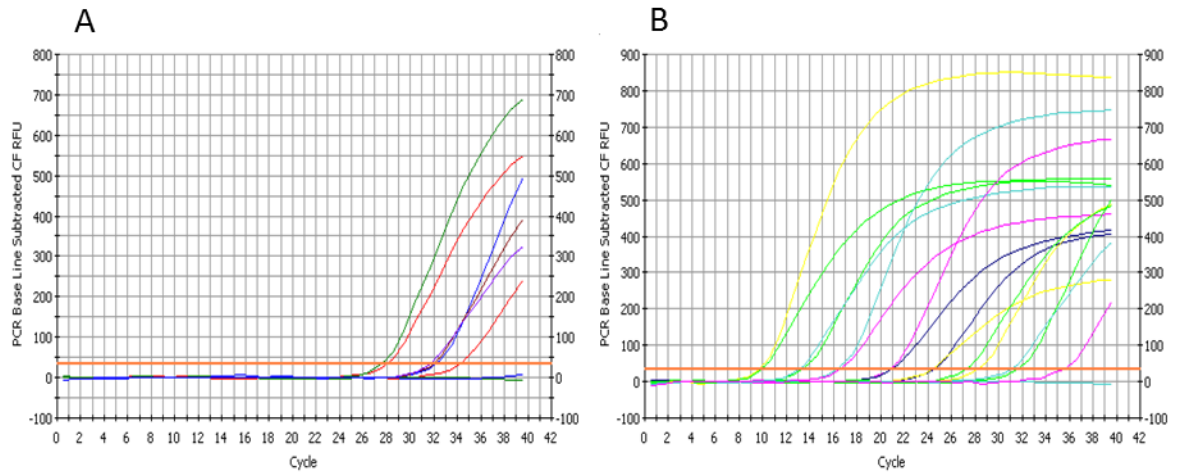
Figure 22: Two amplification plots using a serial dilution of the *lcc*2
clone using *lcc*1 specific primers (A) and *lcc*2 specific primers (B).

Once again, despite the sequence similarities between *lcc*1 and *lcc*2, Figure 22 shows that we can

selectively amplify l*cc*2. This primer specificity experiment was part of the optimization of the

qPCR reaction and was performed using the BioRad iCycler iQ Real-Time PCR Detection

System with 96 well plates and the BioRad iQ SYBR green supermix. All other experiments

(including standard curves and gene expression) were performed on the newer Corbett Rotor-

Gene 6000 thermocycler using BioRad's iTaq universal SYBR green supermix.

2.3.2.4. Transcript abundance

2.3.2.4.1. *Lcc*1

The number of *lcc*1 transcripts was measured using qPCR for the off-white (U1) and

commercial brown strains (SB65) of *A. bisporus* in solitary culture (control) and in the presence

of *T. aggressivum* secondary metabolites (Figure 23). Initially, *lcc*1 transcript was not detected

in either the off-white or brown strains of *A. bisporus*. By day 4, both strains increased

transcription significantly (p=0.002 – Appendix III: Table A2). However, there was no

difference in *lcc*1 transcript abundance between the off-white and brown strains of mushroom

(p=0.408), nor did the presence of *T. aggressivum* metabolites affect transcript abundance

(p=0.117).
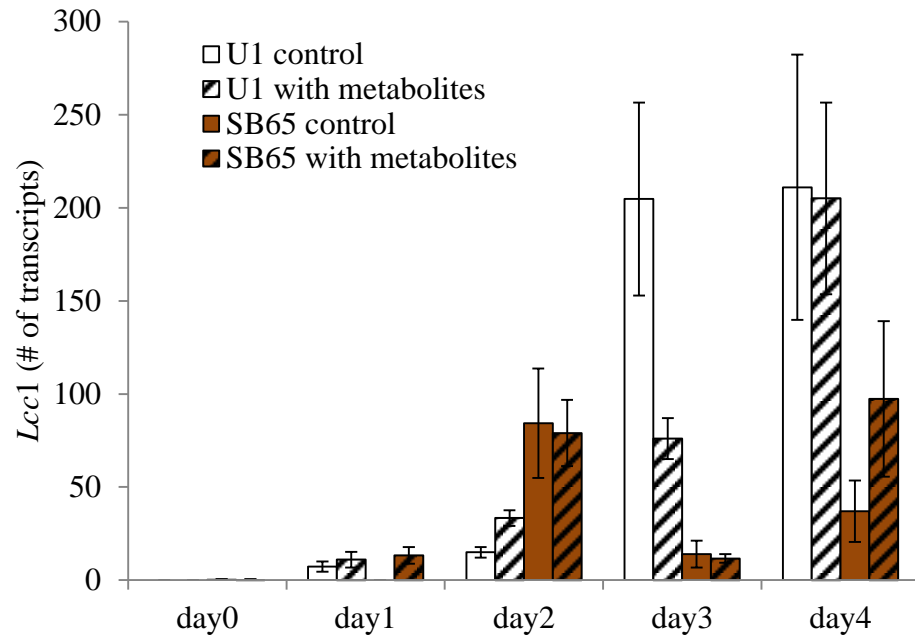
Figure 23: *A. bisporus lcc*1 transcript abundance was measured over 5 d in solitary culture (control) and in the presence of *T. aggressivum* secondary metabolites.

A one-way ANOVA testing for difference in *lcc*1 mRNA expression showed no statistically significant differences ($p > 0.05$ (Appendix III − Table A3) between the two strains of *A. bisporus* or between the control and the culture exposed to the toxin at each of the five time periods.

Several groups have shown that laccase activity fluctuates with *A. bisporus* colonization and production of fruiting bodies (Bonnen et al. 1994; Turner 1974; Turner et al. 1975; Wood & Goodenough, 1977; Wood, 1980a). Laccase enzyme accumulates in the compost until just after the formation of pins (Wood & Goodenough, 1977), followed by rapid decline in activity due to enzyme inactivation and proteolysis (Wood, 1980b). Laccase mRNA was shown to follow the same trend (Ohga et al. 1999). The increasing laccase mRNA and protein reflects increasing *A. bisporus* mycelial growth, and is thought to be a good indicator of mycelial biomass in compost (Matcham, Jordan & Wood, 1985; Bonnen et al. 1994). Our results indicate that *lcc*1 transcript abundance is increasing with time in both the U1 ($p = 0.012$) and SB65 ($p = 0.041$) at a greater rate than mycelial biomass accumulation since we standardized using β-tubulin. While

conditions for inducing *lcc*1 transcription have not been described, it is possible that the increase

shown here is a result of an undefined nutritional or developmental stimulus. Since there was no

difference in *lcc*1 transcript abundance between the off-white and brown strains of mushroom,

and the presence of *T. aggressivum* toxin had no effect on *lcc*1 transcript abundance, *lcc*1 is

likely not involved in commercial brown strains resistance to green mold disease.

2.3.2.4.2. *Lcc2*

The control cultures, grown in the absence of the *T. aggressivum* metabolites, showed no

significant difference in the transcript abundance of *lcc*2 with time (Figure 24). However, by

adding *T. aggressivum* metabolites, both strains of *A. bisporus* increased the number of *lcc*2

transcripts ($p<0.001$ − Appendix III − Table A4) to levels that are much higher than the untreated

control cultures ($p=0.001$). While both strain enhanced *lcc*2 transcript abundance in response to

T. aggressivum metabolites, the increase was significantly greater in the SB65 than in the U1

strain ($p<0.001$).

A one way ANOVA test showed that there was a statistically significant difference

between the groups at all five time points (day 0, $p=0.001$; day 1, $p=0.004$; day2, $p=0.008$; day 3,

$p=0.001$; day4, $p<0.001$ Appendix III −Table A5). The different letters in Figure 24 correspond

to a significant difference in *lcc*2 transcript abundance within that time point using the

Bonferroni post-hoc analysis ($p<0.05$). Initially, *lcc*2 transcript abundance in SB65 was 3 fold

higher than in U1, suggesting that SB65 produces laccase at constitutively higher levels in the

early stages of mycelial growth. The increased level of laccase may procure an advantage to the

SB65 by earlier breakdown of toxic phenolic compounds.

A. bisporus' two laccase gene sequences were first published by Perry et al. (1993b)

which allowed Smith et al. (1998) to measure *lcc*1 and *lcc*2 expression. Smith et al. (1998)

measured *lcc*1 and *lcc*2 expression after 10 d growth on compost or malt extract medium, and

found that *lcc*2 is expressed 300 fold and 7000 fold higher on ME medium and compost

respectively, than *lcc*1. The present study also measured asymmetrical expression of *lcc*1 and
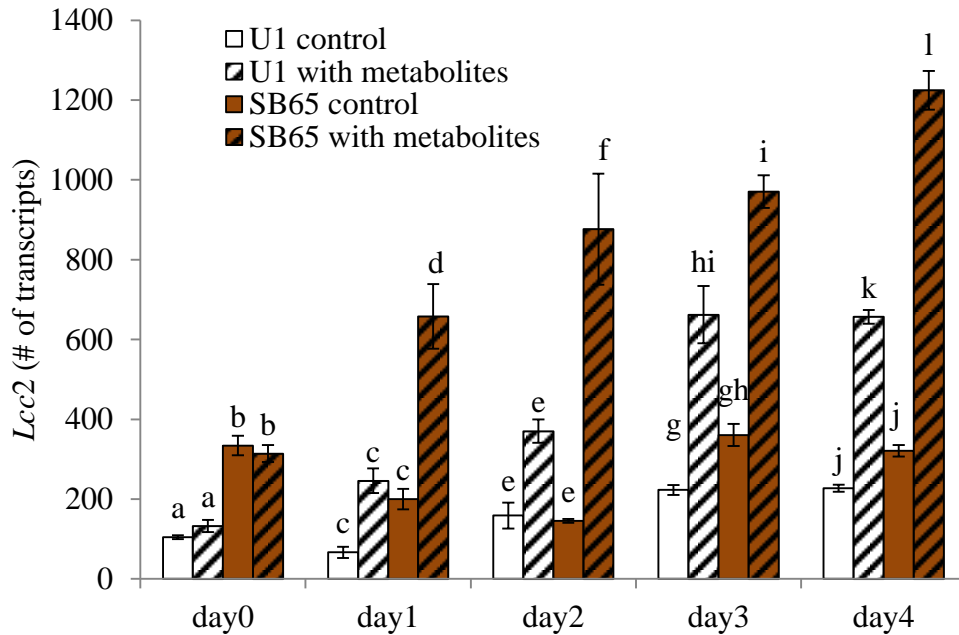
Figure 24: *A. bisporus lcc*2 mRNA transcript abundance was measured by qPCR over 5 days in solitary culture (control) and in the presence of *T. aggressivum* secondary metabolites.

*lcc*2 ranging from 10 to 20 fold more *lcc*2 than *lcc*1. The methodology of this study is

significantly different in terms of culture media, incubation times and quantification, which

likely account for the less dramatic differences in *lcc*1 versus *lcc*2 expression. In particular,

Wood (1980a) showed that growth of *A. bisporus* on malt extract results in 3-4 fold increase in

laccase activity compared to any other single carbon source, while this report used CYM for the

growth of *Agaricus*. Overall, *lcc*1 transcript abundance increased with time and *lcc*2 transcript

abundance remained constant through the duration of this experiment. These data provide

further evidence supporting the idea that laccase 1 and 2 are controlled by different regulatory

circuits which respond to different conditions (Smith et al. 1998).

The addition of *T. aggressivum* metabolites had a significant effect on both *A. bisporus*

strains. The brown strain responded immediately to the presence of the toxin. *Lcc*2 transcript

abundance increased within 24 h, and remained significantly higher than the control. Likewise,

the off-white strain increased *lcc*2 transcript abundance in response to *T. aggressivum* metabolites, but the process was delayed by 48 h. The early response of the commercial brown strains may indicate a better toxin sensing ability, faster detoxification and increased resistance to green mold disease. The delay in the off-white strain's response to the toxin and laccase production may contribute to its increased sensitivity to the toxin and greater losses associated with green mold disease. The changes in *lcc*2 transcripts support the concept of increasing hydrolytic enzyme production in response to the *Trichoderma* toxin. As shown by the toxicity assay, both strains are able to overcome the toxicity of *Trichoderma*`s metabolites, although the brown strain does it more efficiently. Likewise, *lcc*2 transcript abundance increases in both strains in response to the presence of the metabolites, but the timing and level of induction is earlier and higher in the brown strain. These data indicate that *lcc*2 may have a role in the increased resistance of the commercial brown strain of *A. bisporus* to green mold disease.

2.3.2.4.3. *MnP*

The U1 and SB65 strains of *Agaricus* exposed to *T. aggressivum* were also evaluated for differential transcript abundance of the *MnP* gene (Figure 25). Transcript abundance of *MnP* did not increase over the duration of the experiment (p=0.069 – Appendix III – Table A6). There was no significant difference in transcript abundance between the U1 and SB65 strains of *A. bisporus* (p=0.952), and the addition of *T. aggressivum* metabolites did not have an effect on *MnP* transcript abundance (p=0.210).

Laccase is one of the major protein products synthesized by *A. bisporus*, constituting 2.1% of all fungal proteins (Wood 1980a). Therefore it was surprising that we found *MnP* transcript abundance to be higher than both laccases. In *A. bisporus* colonization of compost, *MnP* production parallels the production of laccases and correlates directly with the degradation of lignin (Bonnen et al. 1994). These observations led the authors to suggest that laccase and MnP are similarly and developmentally regulated in *A. bisporus*. However, the induction of *lcc*2, the

predominant laccase gene in *A. bisporus*, in the presence of *T. aggressivum* metabolites suggests differential regulation of these genes.
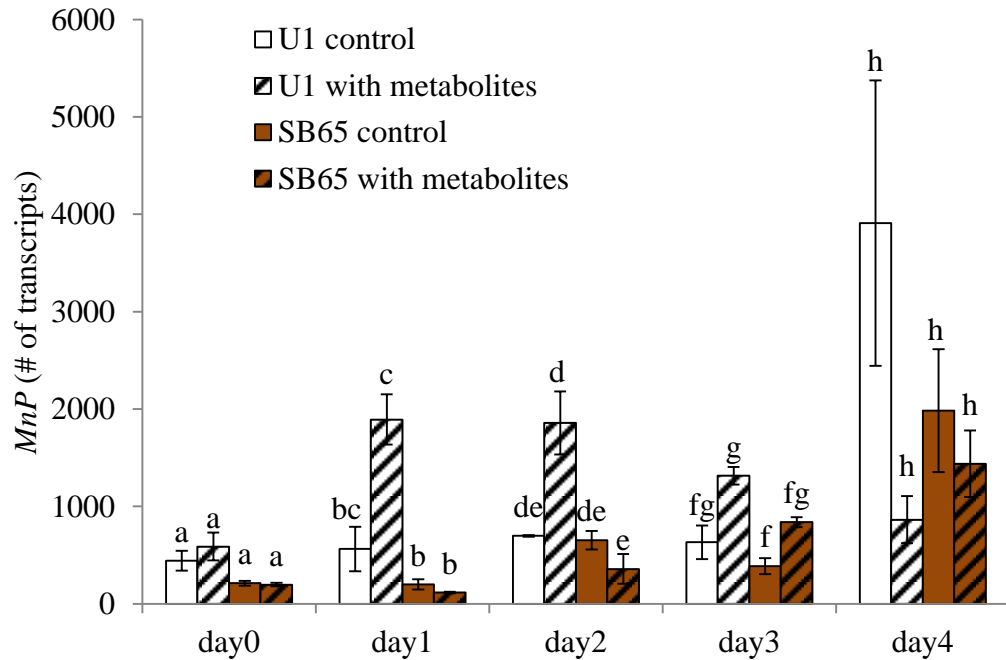
Figure 25: *A. bisporus* RNA transcripts of the manganese peroxidase gene were measured over 5 d in solitary culture (control) and in the presence of *T. aggressivum* secondary metabolites.

MnP likely does not play a direct role in providing resistance to the commercial brown strains of mushroom to green mold disease because there was no significant difference between the expression of *MnP* between the U1 and SB65 strains, nor does the presence of *T. aggressivum* metabolites affect *MnP* transcription.

2.4. Summary

The SB65 strain of *A. bisporus* displays greater resistance to *T. aggressivum* secondary metabolites than the U1 strain. While both strains are able to overcome the toxicity, the SB65 strain does it faster suggesting a role for enzymatic breakdown of the toxins. We measured transcript abundance of three *A. bisporus* genes *lcc*1, *lcc*2 and *MnP* in solitary culture and in response to exposure to toxic secondary metabolites produced by *T. aggressivum*. With time,

*lcc*1 transcript abundance increased in solitary culture suggesting that it may respond to some nutritional or developmental stimuli. The addition of *T. aggressivum* metabolites caused a significant increase in *lcc*2 transcript abundance in both strains of *A. bisporus* suggesting that *lcc*2 has a role in resistance. Increased *lcc*2 transcript abundance at day 0 combined with a more sensitive and dramatic response to the toxins indicate that *lcc*2 may be a contributing factor in the SB65 strain's increased resistance to *T. aggressivum* metabolites and perhaps in compost. Performing high throughput transcriptome-wide sequencing to identify genes that are up-regulated in response to *T. aggressivum* metabolites and mycelium would generate a large pool of genes that are likely involved in the *A. bisporus* defence response.

Chapter 3: *A. bisporus* **response to** *T. aggressivum* **metabolites – laccase activity**

3.1. Introduction

Laccases, also known as large blue copper proteins or blue multi-copper oxidases (BMCO), are polyphenol oxidases containing copper (Baldrian, 2006; Thurston, 1994). Laccase substrates have been difficult to define because their spectrum overlaps with those of other oxidases and because laccases are remarkably unspecific (Thurston, 1994). Their broad substrate range includes polyphenols, methoxy-subsitituted phenols, aromatic diamines and others (Baldrian, 2006; Trejo-Hernandez et al. 2001).

Most fungal laccases are extracellular glycoproteins, 60-80 kDa in size with 15-20% glycosylation (Thurston, 1994). Likewise, *A. bisporus* laccase protein is 65 kDa and forms a 100 kDa dimer where one chain is partially proteolytically cleaved (Perry et al. 1993a; Wood, 1980a; Wood, 1980b). *A. bisporus* laccase is a glycoprotein containing 14.6% carbohydrate and has a pI of 3.4 – 4.0 (Wood, 1980a). During vegetative growth, laccase activity directly correlates with *A. bisporus* biomass resulting in laccase being used as an indicator of mycelial growth (Wood, 1979). Several groups have shown that laccase activity fluctuates with *A. bisporus* colonization and production of fruiting bodies (Turner, 1974; Turner, et al. 1975; Wood & Goodenough, 1977; Wood, 1980a). Laccase enzyme activity increases in the compost until just after the formation of pins (Wood & Goodenough, 1977), followed by rapid decline due to enzyme inactivation and proteolysis (Fermor & Wood, 1981; Wood, 1980b). In the absence of the formation of fruiting bodies, laccase activity peaks at day 25-40 and remains consistently high in compost (Wood & Goodenough, 1977) and on malt extract medium under laboratory conditions (Wood, 1980a).

While laccases have a role in lignin degradation (Leonowicz et al. 2001), data indicate a wide range of functions including defense against stressful conditions (Baldrian, 2004; Mayer & Staples, 2002; Thurston, 1994), melanin synthesis (Nagai et al. 2003) and breakdown of toxic xenobiotic, phenolic compounds and secondary metabolites (Baldrian, 2006; Mayer & Staples).

Intracellular laccases may transform low molecular weight phenolic compounds within the cell (Baldrian, 2006), as described in gill tissue of *Lentinula edodes*, whose intracellular laccase oxidized phenolic compounds contributing to gill browning (Nagai et al. 2003). Similarly, intracellular laccase in *A. bisporus* could be involved in resistance to green mould disease by enzymatic transformation of the toxic phenolic compounds produced by *T. aggressivum*. Wood (1980a) reported that 88.5% of *A. bisporus* laccase is extracellular, 11.5% is present in the soluble intracellular fraction of *A. bisporus*, and is not associated with the cell wall (Sassoon & Mooibroek, 2001). *A. bisporus* extracellular laccase has been well studied and is thought to be primarily involved in lignin degradation, while the significance of intracellular laccase has not been reported. This chapter looks at intracellular laccase activity of the off-white and commercial brown strains of *A. bisporus* resulting from the presence of *T. aggressivum* secondary metabolites, and how laccase may be involved in green mold resistance.

3.2. Methodology

3.2.1. Protein isolation

A. bisporus U1 and SB65 mycelia were cultivated in CYM and exposed to *T. aggressivum* metabolites as described in chapter 2. After exposure to toxic metabolites, intracellular protein was isolated at day 0, 2 and 4 as described by Criquet et al. (1999) with modifications. The mycelium was frozen in liquid nitrogen and crushed with a mortar and pestle. One gram of powered mycelium was combined with 10 mL of extraction buffer. Different extraction buffers used to isolate protein included 0.1 M $CaCl_2$, 0.1 M Na_3PO_4 (total and precipitated with $(NH_4)_2SO_4$), 0.1 M KCl, 0.1 M $CaCl_2$ with 0.025 % polyvinylpolypyrrolidone (PVPP) and H_2O. The mixture was shaken for 1 h at 120 rpm at RT and passed through Whatman #4 filter paper to remove cellular debris. The mixture was centrifuged for 20 min at 12000 x *g* at 4 °C and the supernatant was filtered through 8 μm filter paper (Milli-pore). The protein solution was loaded into dialysis tubing with molecular weight cut-off 12 – 14 kDa (Fisher Scientific) and dialysed

overnight at 4 °C against 2 mM bis-Tris. The tubing was incubated with dry polyethylene glycol to reduce the volume to about 0.25 mL and resuspended into 1 mL of H_2O.

3.2.2 Protein quantification

Concentration of protein was determined using the Bradford assay (Bradford, 1976). A standard curve was generated using Bovine Serum Albumin (BSA) by combining 800 µL of H_2O, 200 µL of protein assay dye reagent (Bio-Rad) and dilution series of BSA. The solution was incubated at RT for 5 min, followed by the addition of 3 mL of H_2O and the absorbance was recorded at 595 nm. The assay was repeated in triplicate for each protein sample and absorbance values within the linear portion of the standard curve were used to calculate protein concentration.

3.2.3 Laccase enzyme activity assay

The assay for the enzymatic breakdown of syringaldazine as described by Criquet et al. (1999) was used to measure laccase activity. Syringaldazine is a substrate that is hydrolyzed by peroxidases and laccases. Peroxidases require hydrogen peroxide for the reaction to occur, so in the absence of hydrogen peroxide syringaldazine will be selectively oxidized by laccase (Harkin, Larsen & Obst, 1974). Laccase activity can be measured with a spectrophotometer at 525nm by the formation of quinone (pink colour) from the enzymatic oxidization of syringaldazine (yellow colour) (Harkin & Obst, 1973; Harkin et al. 1974; Baldrian, 2006).

Each sample included 3 mL of phosphate buffer (pH 6), 100 µL of 2.5 mM syringaldazine (in 90% EtOH, 5% MeOH, 5% isopropanol), and 5 µg of total protein in a cuvette. The reaction was allowed to proceed for 60 sec before reading absorption at 525 nm. Each sample was measured in triplicate, and laccase specific activity was quantified in units where one unit of laccase enzyme will produce a change in absorbance at 530 nm of 0.001 per µg of protein using syringaldazine as the substrate (Ride, 1980; Sigma Aldrich [http://www.sigmaaldrich.com/ technical-documents/protocols/biology/enzymatic-assay-of-laccase.html]). The reaction

conditions described above were optimized for extraction buffer, copper supplementation, reaction time and mass of protein used in reaction.

3.2.4 Toxicity test using glutathione

Glutathione was added to CYM plates to a final concentration of 20 µM before the addition of *A. bisporus* mycelium and *Trichoderma* metabolites as described in the toxicity assay described in section 2.2.3.2. Cultures were incubated and the zone of inhibition was measured every ten days for thirty days.

3.3. Results and discussion

Crude protein extracts were used throughout these studies and protein purification was not necessary because the substrate in the assay is selectively hydrolyzed by laccase. The test does not distinguish between different laccase isoforms, and so results represent total laccase activity.

3.3.1 Bradford assay

The Bradford assay was used to quantify protein concentration in each sample based on a standard curve using known amounts of BSA (Figure 26).
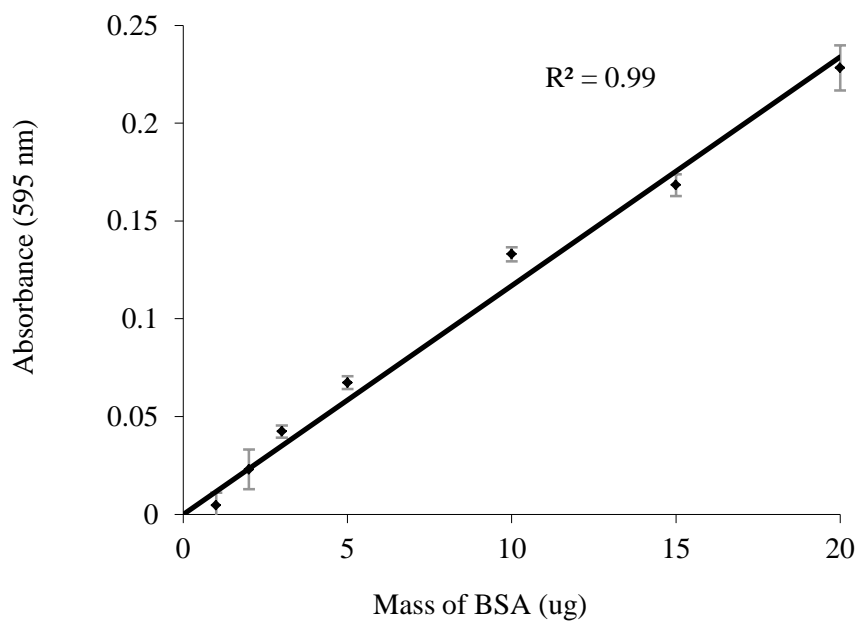
Figure 26: Standard curve generated using BSA in the Bradford assay and used to calculate protein concentration in samples.

3.3.2 Optimization of laccase activity assay

The laccase activity assay was modified from a study on forest litter laccase activity (Criquet et al. 1999). Total protein from *A. bisporus* mycelium was extracted using buffers including $CaCl_2$, $CaCl_2$ with PPVP, KCl, Na_3PO_4, H_2O, and H_2O followed by $(NH_4)_2SO_4$ precipitation. Extraction using 0.1 M $CaCl_2$ resulted in the greatest laccase activity seen spectrophotometrically by increase in absorbance per µg of total protein (Figure 27A). Criquet et al. (1999) also reported recovering the highest laccase activity in samples when the buffer included 0.1 M $CaCl_2$.

A

B
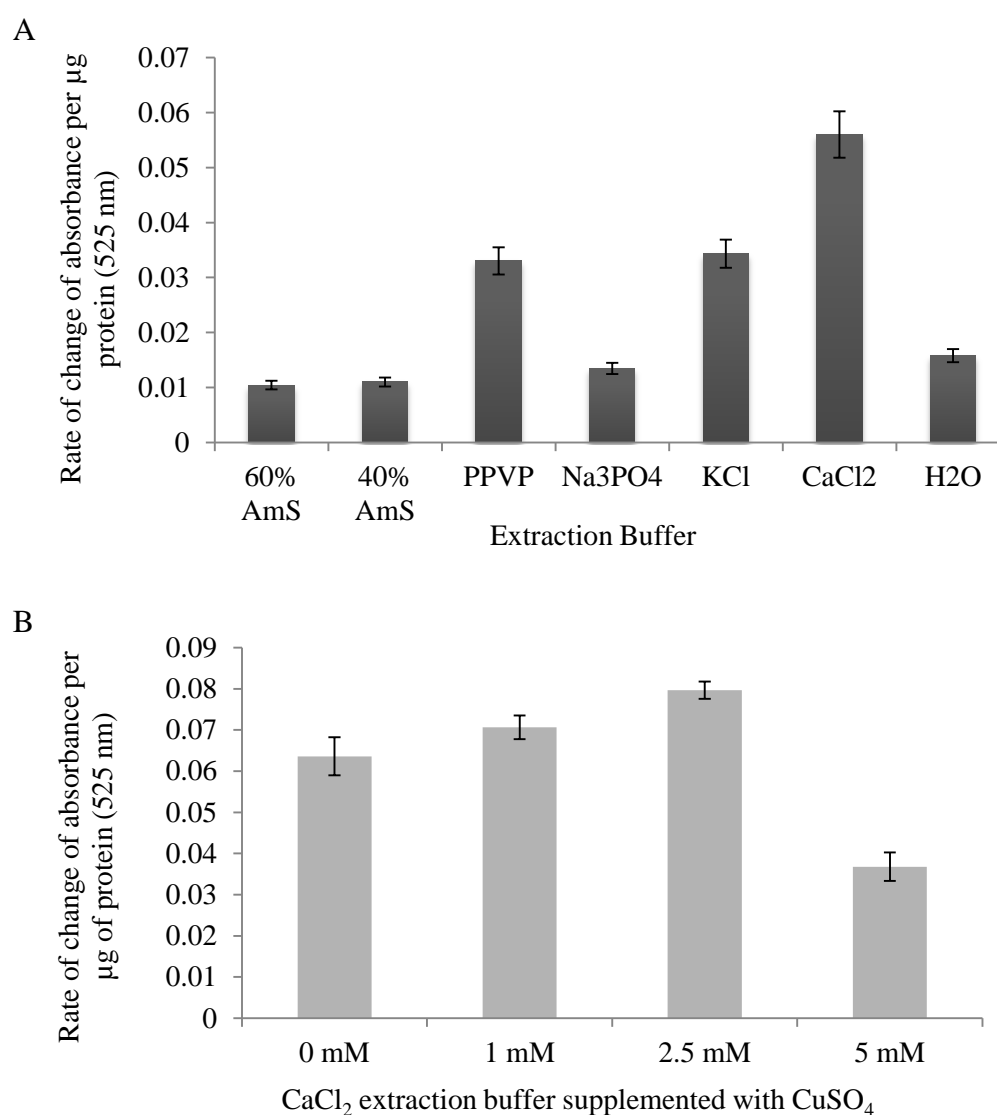
Figure 27: A: Different buffers used to extract total protein from *A. bisporus* affected laccase activity per µg of total protein. B: Adding $CuSO_4$ to the $CaCl_2$ extraction buffer increased laccase activity.

With the exception of the type II center, copper atoms are tightly bound within the laccase protein. Thurston (1994) describes that T2 copper can be stripped from the protein during purification. Since copper is an integral component of laccase active site, it was possible that protein isolation and dialysis would strip laccase of its copper and reduce its activity. Copper can be selectively removed from laccase by chelating agents (EDTA, dimethyl glyoxime, N,N'-dimethyldithiocarbamate, NTA) which results in a loss of catalytic activity (Kunamneni et al. 2007). Supplementation the enzyme with $CuSO_4$ to increase laccase activity was tested to confirm this possibility (Figure 27B) and supplementation with 2.5 mM $CuSO_4$ yielded the highest laccase activity.   This observation is similar with Larrondo et al. (2003) who described the highest activity at 5 mM Cu (II) concentrations in laccase extracts from the white rot basidiomycete *Ceriporiopsis subvermispora*.

The amount of protein to use in the reaction was optimized, as was the time given for the reaction to proceed. Figure 28A displays the different reaction rates using different amounts of protein and all curves showed a linear increase for 75 sec. Figure 28B displays the laccase assay using different amounts of total protein (µg) showed a linear increase in quinone formation. To ensure that the assay would be sensitive to both high and low concentrations of laccase, experimental conditions were selected that would lie in the middle of the range of each experiment.

In summary, the total protein was extracted from *A. bisporus* mycelium using $CaCl_2$ and supplemented it with 2.5 mM $CuSO_4$. We used 5 µg of total protein in the assay, and allowed the reaction to proceed for 60 sec before taking an absorbance reading.

A

B
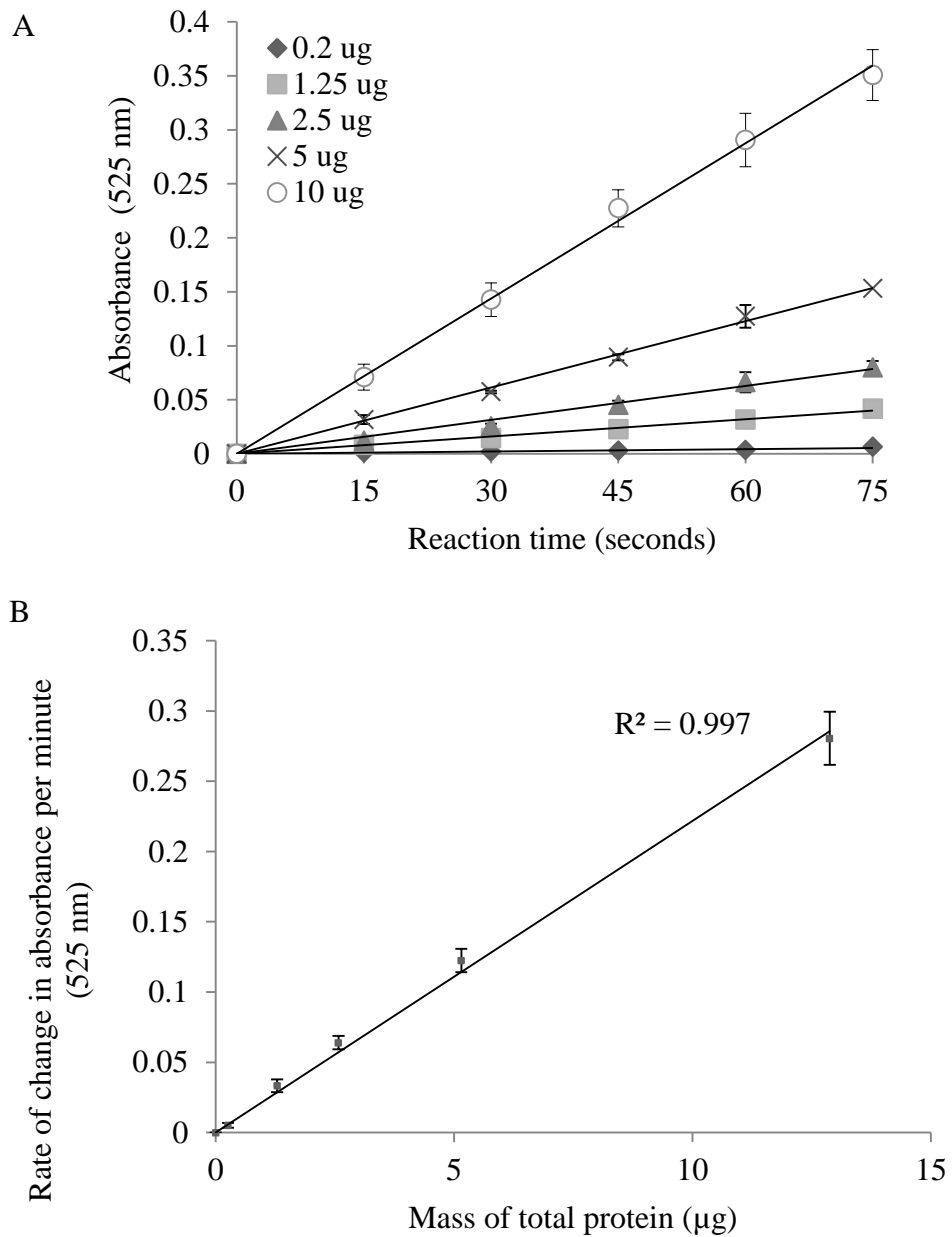
Figure 28 A: Different amount of total protein (μg) yielded different reaction rates for the *A. bisporus* laccase enzyme. B: Increasing amount of total *A. bisporus* protein in assay showed a linear increase in the laccase enzyme activity across the range of the experiment.

3.3.3   Laccase activity

Laccase activities were determined from the commercial off-white and brown strains of *A. bisporus*. We quantified laccase enzyme in control cultures and in cultures exposed to *T. aggressivum* secondary metabolites for a 4 d period (shown in Figure 29).
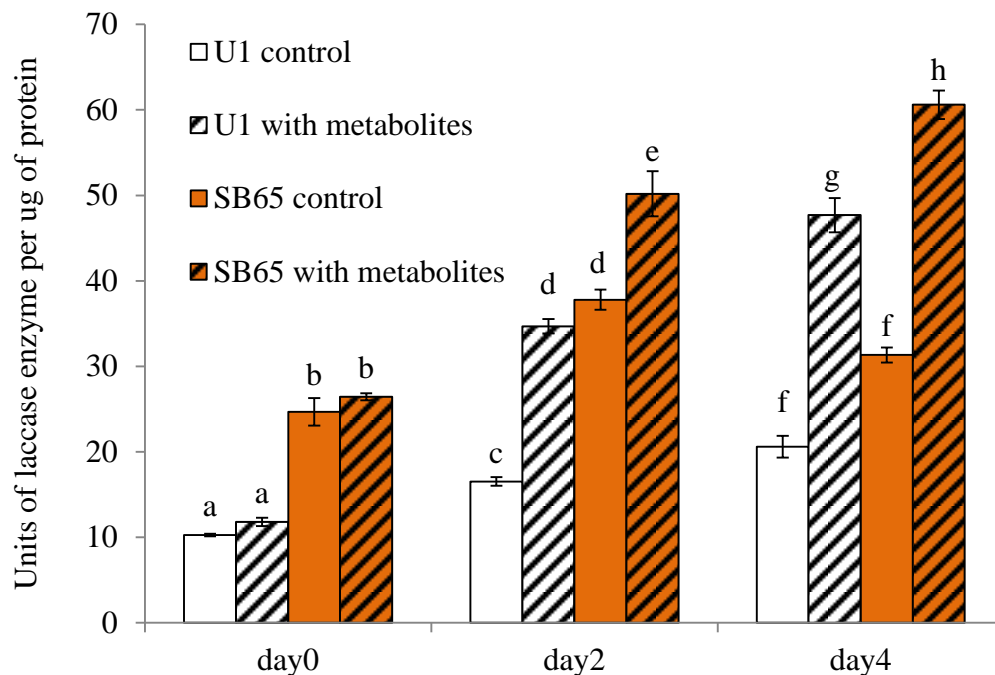
Figure 29: *A. bisporus* laccase enzyme activity isolated from cultures grown in the presence and absence of *T. aggressivum* toxic metabolites. Letters designate statistical differences within each day (ANOVA – Bonferroni Post-Hoc ($p \leq 0.05$).

Laccase activities remained relatively constant in the controls throughout the four days; however the addition of the *T. aggressivum* metabolites caused a significant increase in laccase activity in both strains of *A. bisporus* ($p \leq 0.001$ – Appendix IV: Table A8). There was also a statistically significant difference in the laccase activity between the off-white and brown strains ($p < 0.001$).

One-way ANOVA tests of laccase enzyme activities between the two strains of *A. bisporus* and the control and experimental conditions showed statistically significant differences at all time periods (day0, $p < 0.001$; day2, $p < 0.001$; day4, $p < 0.001$) (Appendix IV: Table A9). The different letters in Figure 31 represent a significant difference in activity within that time point using the Bonferroni post-hoc analysis ($p \leq 0.05$). The activity of the laccase enzyme at day 0 was two-fold higher in SB65 than U1 in the untreated samples. Over the four days, U1 slightly increased activity while SB65 reduced activity and by day four there was no significant difference in activity between the two strains. While the addition of *T. aggressivum* metabolites

caused a significant increase in activities by both strains in day 2 and 4, the level of activity was always significantly higher in SB65 than U1. Laccase activity levels reflected the *lcc*2 transcript abundances reported in chapter 2. The controls had minor changes in *lcc*2 transcript abundances and LCC activities, while the presence of *Trichoderma* metabolites stimulated the formation and accumulation of *lcc*2 transcripts and laccase activities in both mushroom strains. The brown strain SB65 had a greater abundance of *lcc*2 transcripts and laccase activity in the absence of *T. aggressivum* metabolites, and responded faster in the presence of the toxins resulting in a greater abundance of transcripts and activity that the off-white strain U1. These attributes, therefore, may be partially responsible for greater resistance of SB65 to green mold disease.

3.3.4 Toxicity test using glutathione

Glutathione (GSH) is a tripeptide composed of cysteine and glycine attached by a peptide bond, and a glutamate residue attached by the carboxyl group of its side chain to the amine group of the cysteine (Meister & Anderson, 1983). GSH is a ubiquitous intracellular peptide in bacterial, plant, fungal, and animal, especially mammalian liver, cells (Lu, 2009; Rehman & Anjum, 2011). GSH has several functions within cells including serving as a reservoir for cysteine, detoxifying electrophiles and scavenging free radicals (Lu, 2009). Phenolic hydroxyl compound catalyzed by laccase have an electron and proton enzymatically removed, generating a phenoxy radical (Leonowicz et al. 2001). These reactive phenoxy radicals undergo further chemical reactions forming oxidized quinones or polymers. The presence of glutathione may reduce the phenoxy radicals back to the toxic phenolic hydroxyl compound, negating the function of laccase. When glutathione was added to the medium (Figure 30), both the U1 and SB65 strains of *A. bisporus* became more sensitive to the toxic metabolites produced by *T. aggressivum* than cultures grown in the absence of GSH ($p \leq 0.001$ – Appendix V: Table A10)
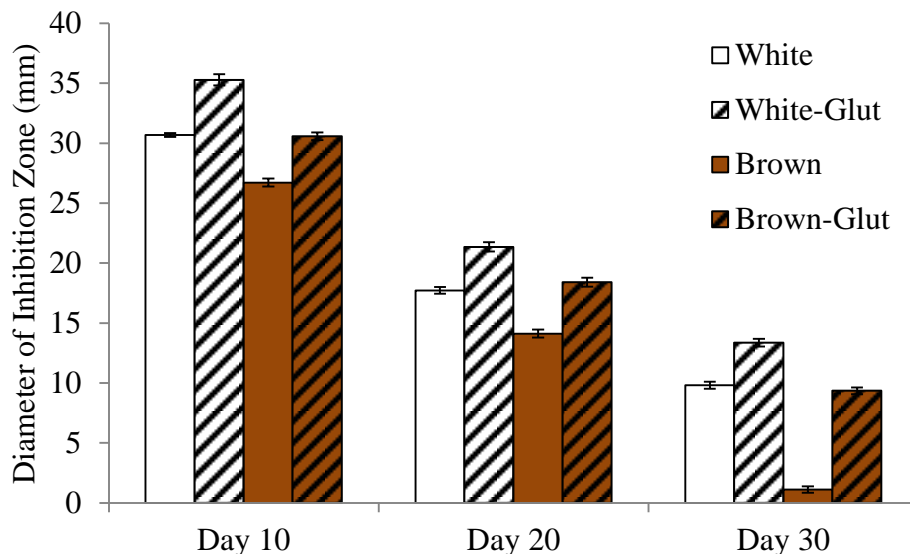
Figure 30: Glutathione increases the toxicity of secondary metabolites produced by *T. aggressivum* to the growth of both off-white and brown strains of *A. bisporus*

Since laccase is produced in response to the presence of *T. aggressivum* metabolites, the metabolites may be a substrate of laccase. Phenolic compounds, like the methyl isocoumarin identified as toxic to *A. bisporus* growth (Krupke et al. 2003), are likely substrates for phenol oxidases like laccase. Laccase binds its substrate and removes a single electron generating an unstable and reactive phenolic radical (Thurston, 1994). These phenolic radicals typically undergo further reactions generating oxidized quinones or polymerize (Lundell et al. 2010). The addition of GSH may reverse the reaction catalyzed by laccase, since one of the most important non-enzymatic functions of GSH is to reduce carbon radicals (Galano & Alvarez-Idaboy, 2011). GSH may function in the medium by reducing the phenolic radicals produced by laccase back into the toxic compound, increasing the sensitivity of *A. bisporus* to the toxic metabolites. GSH is consumed by the reaction resulting in *A. bisporus* being able to overcome the metabolites toxicity, just not as effectively as the control (Figure 30).

3.3.5. Toxicity of Aspinolide B

The major component of the *T. aggressivum* toxic metabolites was purified and identified by LC-UV-MS and NMR as aspinolide B (Dr. David Miller, Carleton University). The purified aspinolide B did not have major inhibitory effects against *A. bisporus* (Figure 31). This supports Krupke et al. (2003) report that the major components of the *T. aggressivum* metabolites visualized by TLC had little toxic effect against the growth of *A. bisporus*. The minor components of the extract, including the methylisocoumarin, possessed the toxicity.
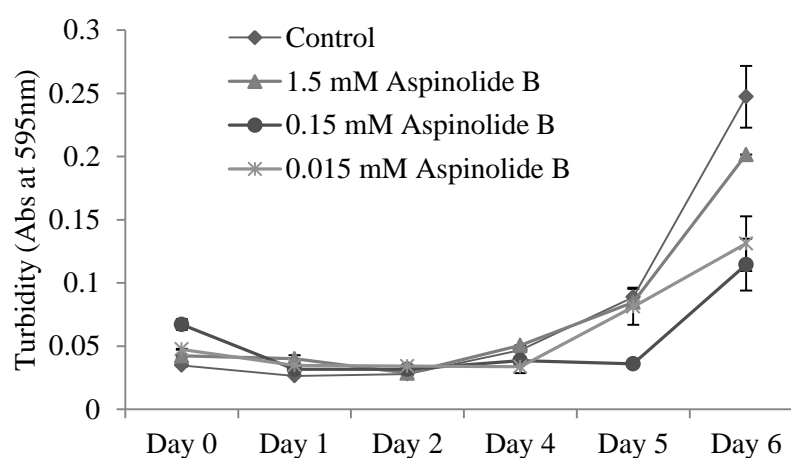
Figure 31: The purified aspinolide B metabolite from *T. aggressivum* shows little toxicity towards the growth of *A. bisporus*.

3.3.6. Effect of exogenous laccase on metabolite toxicity

To determine if laccase is able to use the *T. aggressivum* metabolites as a substrate and reduce their toxicity, we incubated the metabolites with exogenous laccase before adding them to the plate diffusion assay (Figure 32A). There was no significant difference in the toxicity of the metabolites following the addition of laccase. There are several explanations for the maintenance of toxicity of the metabolites, even in the presence of laccase. First, the metabolites are not a substrate of laccase and the laccase induction described in chapter 2 is just a general response to the presence of phenolic compounds. Second, there were problems with solubility of the metabolites into a buffer that would allow laccase activity. Perhaps the solvent

incompatibility was not making the metabolites available to the enzyme. Third, laccases have

been shown to only degrade low redox potential phenolic compounds directly; laccase are unable

to oxidize many recalcitrant aromatics directly (Camerero, et al. 2005). However, laccase are
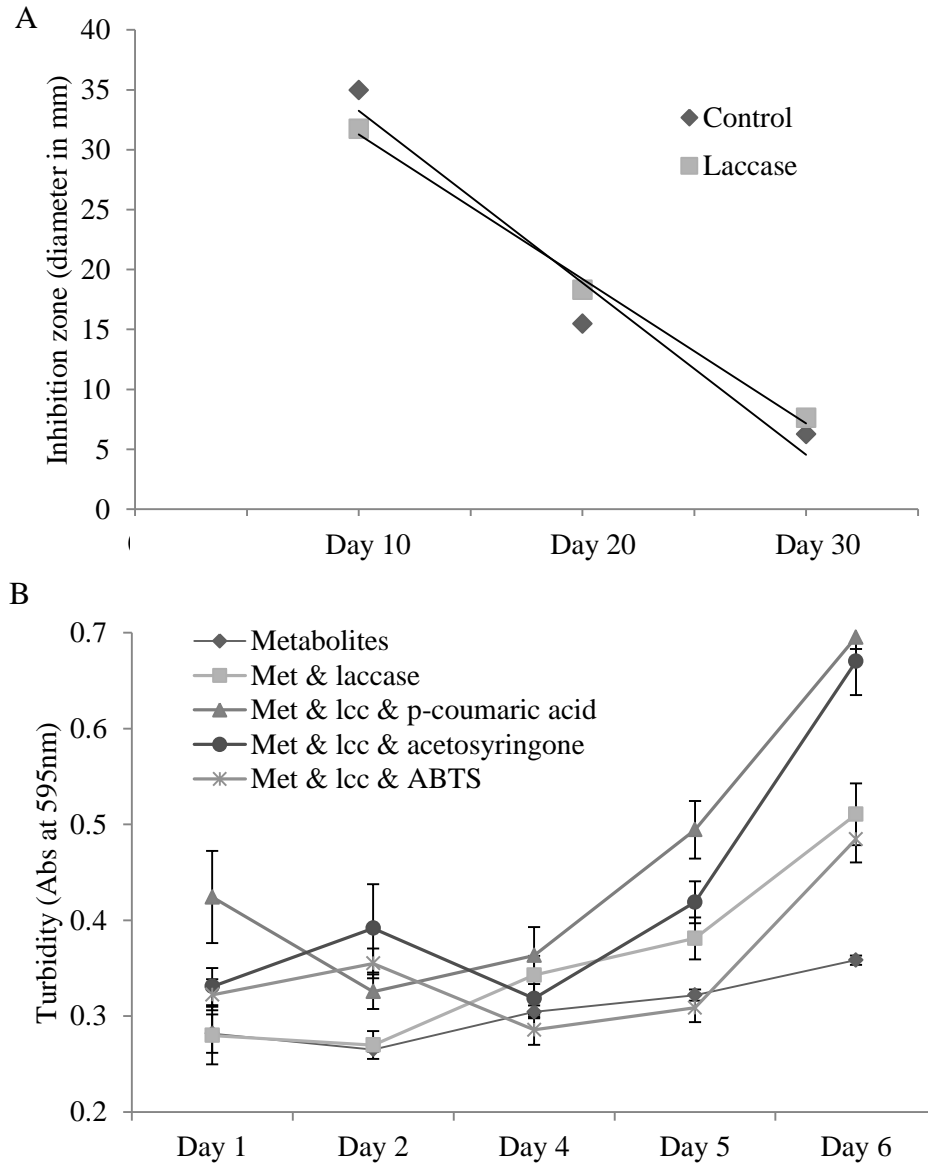
Figure 32: A: Plate diffusion assay measuring the inability of exogenous laccase to reduce the toxicity of *T. aggressivum* secondary metabolites. B: *A. bisporus* growth in liquid culture was measured spectrophotometrically over six day to determine the effect of exogenous laccase in the presence of mediator p-coumaric acid, acetosyringone and ABTS on the toxicity of *T. aggressivum* secondary metabolites.

able to degrade these recalcitrant aromatic indirectly through the application of enzyme

mediators (Camerero, et al. 2005). We tested the ability of laccase to degrade the metabolites

using three common laccase mediators, p-coumaric acid, acetosyringone and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) (Figure 32B). There was very little growth of *A. bisporus* in the presence of the metabolites alone even after six days incubation. When the metabolites were incubated with laccase enzyme with or without ABTS, there is a small increase in culture turbidity indicating a small amount of growth. However, the addition of laccase with p-coumaric acid or acetosyringone resulted in a substantial increase in turbidity, indicating that the toxicity of the metabolites is actively being reduced by the presence of the enzyme and mediator.

3.4. Summary

In solitary *A. bisporus* culture, laccase enzyme activity remains constant since increasing laccase activity reflects increasing biomass as previously described by Wood, 1980a. Similar to *lcc*2 transcript abundance, LCC activity at day 0 is significantly higher in the SB65 strain; and exposure to *T. aggressivum* metabolites caused both strains of *A. bisporus* to increase LCC activity significantly, where the increase was always greater in the SB65 strain. When a reducing agent (glutathione) was added to culture media, the sensitivity of both strains was increased presumably because glutathione reduced phenoxy radicals, generated as intermediate in the laccase catabolism of toxic secondary metabolites, back to the active compound. On the other hand, exogenous laccase in the presence of two mediators, p-coumaric acid or acetosyringone, is able to detoxify the metabolites and *A. bisporus* is able to colonize the medium with greater ability. This data provides further evidence that laccase has a role in the detoxification of *T. aggressivum* secondary metabolites and commercial brown strain resistance may be attributed to increased laccase production.

Chapter 4: Sequence analysis of *A. bisporus* laccase genes

4.1. Introduction

A. bisporus' laccase gene sequence was first reported by Perry et al. (1993b), further described by Smith et al. (1998) and Morin et al. (2012). Perry et al. (1993b) described the genomic sequence of *lcc*1 and the coding sequence of *lcc*2. Smith et al. showed that the genes are found in tandem within the genome and reported the intergenic sequence including *lcc*2 regulatory elements. Morin et al. (2012) reported the genome sequence of one homokaryon (H97) that is presence within the U1 strain of *A. bisporus* and described the presence of 12 genes predicted to be laccases.

The *lcc*1 coding sequence is 1563 bp contained within 15 exons and interrupted by 14 introns (Perry et al. 1993b). Seven putative regulatory elements were identified upstream of the *lcc*1 coding sequence, including two TATA boxes, one CAAT motif, and four sequences that show homology to other putative promoter sequences (Perry et al. 1993b). The *lcc*1 transcript codes for a 520 aa protein including a 19 aa signal sequence cleaved from the active 58 kDa extracellular peptide.

The *lcc*2 coding sequence is 91.4 % homologous to the *lcc*1coding sequence (1428 of 1563 bp) (Perry et al. 1993b). The regulatory sequence of *lcc*2 has one TATA and several unique CAAT boxes which may contribute to the differential expression between the two laccase genes (Smith et al. 1998). The deduced amino acid sequence of LCC2 is identical to LCC1 at 484 of 520 aa (93%) and both proteins have five glycosylation sites, though two are unique to each protein.

The purpose of the research reported in this chapter was to compare sequence information of both *lcc*1 and *lcc*2 genes from both the hybrid off-white (U1) and commercial brown (SB65)

strains of *A. bisporus* with the goal of understanding the roles of regulatory and coding

sequences in differential expression of the two genes in the two strains.

4.2. Methodology

4.2.1. Cloning laccase genes

The entire coding sequence of *lcc*1 and *lcc*2 from the U1 and SB65 strains of *A. bisporus* were

amplified by PCR using primers specific for laccase sequences found in both genes (Table 4).

Primers were designed based on laccase sequence information on the NCBI database (*lcc*1

accession number L10664.1; *lcc*2 accession number L10663.1). All PCR reactions used *Pfu*

polymerase to minimize polymerase induced mutations. PCR products were ligated in pUC19

linearized with *Hinc*II and transformed into *E. coli* DH5α. Plasmids were isolated from

transformants and sent for sequencing.

Table 4: Primers used for cloning of *A. bisporus lcc*1 and *lcc*2 genes.

Primer name	Primer sequence (5' – 3')	Melting temperature (°C)
Lc-Reg-F	TCATGAGCGAACCTAAGGC	62.9
Lc-Reg-R	AAGGTTCTGGTTTTCGCAAC	62.9
Lc-Gen-F	CAACGCTTTAGTACTGGTCGC	63.5
Lc-Gen-R	AATTCAGGCTCGATTGCG	63.9
LF1	CTGATTGAATCTTGTGACATAGCC	63.5
LF4	GCTAGTTTGGTTTTAGTAGATTGAATTGG	64.5

4.2.2. Sequencing

Sequencing reactions typically yield 800 – 1000 bp of sequence information, therefore primers

were designed to amplify different segments of the gene (Table 5). Several primers had similar

binding sites in both genes because of sequence similarities. The clones were sequenced by

York University with BigDye Terminator chemistry on the Applied Biosystems 3130xL DNA

Sequencer.

Table 5: Primers used for sequencing *A. bisporus* *lcc*1 and *lcc*2 genes. The primer location refers to the position of the primer on the entire assembled gene sequence of 6572 base pairs. Forward and reverse primers are designated F and R respectively.

Primer name	Primer sequence	Primer location and direction
pUC19 F	5' CAGCTATGACCATGATTACGC 3'	In plasmid backbone F
Lc-Reg-F	5' TCATGAGCGAACCTAAGGC 3'	39-57 F
Lc-Gen-F	5' CAACGCTTTAGTACTGGTCGC 3'	472-492 F
		4339-4359 F
LF1	5' CTGATTGAATCTTGTGACATAGCC 3'	2593-2616 F
		4339-4359 F
LF3	5' CAATCATCCAGTCCAAATTGC 3'	3326-3346 F
LCM-F	5' CGTCGTCAACGTCGAGC 3'	5270-5286 F
pUC19-R	5' AAGGCGATTAAGTTGGGTAAC 3'	In plasmid backbone R
Lc-Reg-R	5' AAGGTTCTGGTTTTCGCAAC 3'	512-535 R
LCM-R	5' ATGGATGAGCTCCTTCTCCTGG 3'	2218-2239 R
		6081-6102 R
Lc-Gen-R	5' AATTCAGGCTCGATTGCG 3'	2741-2758 R
LF2	5' TATCTGGCAATCTTGTAAGGTATGC 3'	3777-3801 R
LF4	5' GCTAGTTTGGTTTTAGTAGATTGAATTGG 3'	4648-4676 R

4.2.3. Analysis

Laccase sequences were assembled and aligned using Invitrogen's Vector NTI Advance 11 and Gene Doc 2.7.0.

4.3. Results and discussion

4.3.1. Cloning laccase genes

*Lcc*1 and *lcc*2 have very similar sequences, and so primers that would non-selectively amplify both genes were used. As a result, a single PCR product could contain both laccase 1 and 2 genes (Figure 33). DNA in lanes B and C were amplified using Lc-Gen-F and Lc-Gen-R primers and are around 2286 bp. Lanes E and F contain the *lcc*1 regulatory regions amplified with the Lc-Reg-F and Lc-Reg-R primers and are 500 bp.

A   B   C   D   E   F
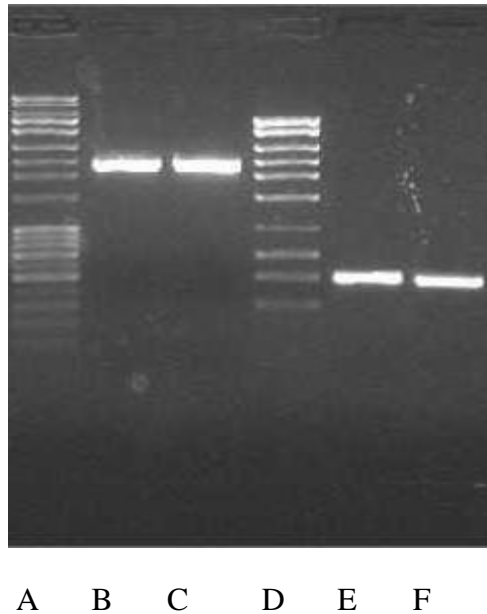
Figure 33: Agarose gel showing laccase gene and regulatory elements amplified by PCR from U1 and SB65. Highranger Plus (A), U1 laccase (2300 bp) (B), SB65 laccase (C), Midranger ladder (D), U1 *lcc*1 regulatory region (500 bp) (E), and SB65 *lcc*1 regulatory region (F).

Since the primers were not selective for *lcc*1 or *lcc*2, the PCR products were cloned into *E. coli*, and all transformants were analyzed by PCR and restriction enzyme digestion to determine identity. PCR verification of amplified plasmid inserts using primers nonselectively amplifying the entire laccase gene (Lc-Gen-F & Lc-Gen-R), and primers selective for *lcc*2 (LCM-F and Lc-Gen-R) (Figure 34). Lanes B through E are amplification products of two different U1 laccase clones. The plasmid amplification products shown in lanes B and C are *lcc*2, because they were amplified with both the nonspecific laccase primers and with the *lcc*2 primers. The plasmid shown in lanes D and E are *lcc*1 because they amplify with the nonspecific primers, but there is no amplification with the *lcc*2 specific primers. The same conclusion can be drawn from the SB65 laccase clones; lanes F and G show amplification of the *lcc*2 gene, while lanes H and I identify the clones as *lcc*1.
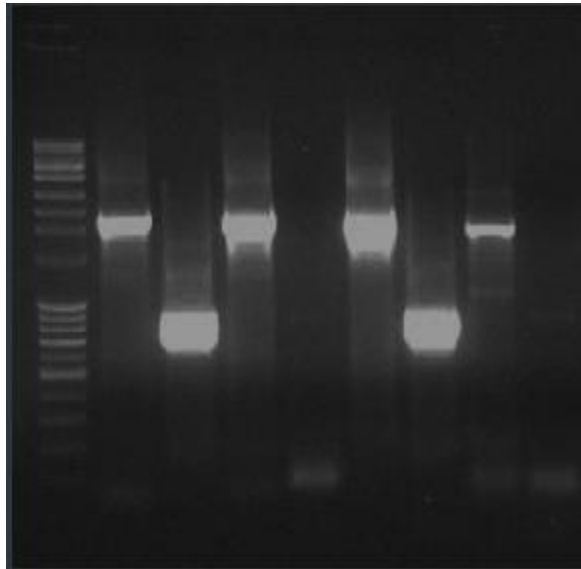
A B C   D E F G   H I

Figure 34: Agarose gel under ultraviolet light identifying *lcc*1 and *lcc*2 clones by PCR. NORGEN's Highranger Plus (A), U1 laccase (B-E) amplified with nonspecific laccase primers (B & D) and with *lcc*2 primers (C & E), SB65 laccase (F-I) amplified with nonspecific laccase primers (F & H) and with *lcc*2 primers (G & I).

Further confirmation of the laccase clones was obtained using specific enzyme restriction sites. *Lcc*1 has one *Ava*1 restriction site and digestion with *Ava*1 will yield products that are 2110 and 177 bp in length. *Lcc*2 has two *Ava*1 restrictions sites which yield three products of 1422, 649 and 208 bp. The U1 laccase clones were amplified by PCR and digested using *Ava*1 restriction enzyme followed by separation and visualized by agarose gel electrophoresis (Figure 35). Identical results were obtained from digestion of SB65 laccase clones with *Ava*1 restriction enzyme (data not shown). The PCR amplification and the restriction enzyme analyses confirmed that *lcc*1 and *lcc*2 were amplified from both the U1 and SB65 strains of *A. bisporus*.

The 1486 bp sequence (including *lcc*2 regulatory elements) that separates the two laccase genes was cloned using the LF1 primer which binds at the 3' end of *lcc*1 and LF4 that binds at the 5' end of the *lcc*2 gene (Figure 36).
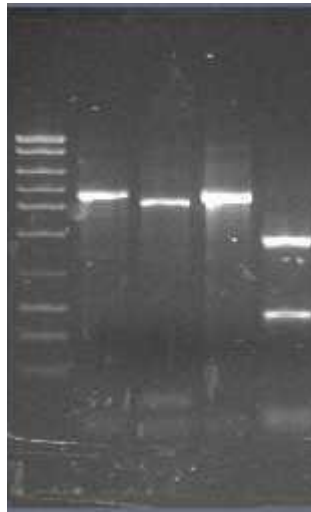
A   B   C   D   E

Figure 35: Agarose gel used to differentiate between *lcc*1 and *lcc*2 clones digestion with *Ava*1 restriction enzyme. NORGEN's MidRanger ladder (A), undigested *lcc*1 clone (B), *lcc*1 clone digested with *Ava*1 (C), undigested *lcc*2 clone (D), and *lcc*2 clone digested with *Ava*1 (E).
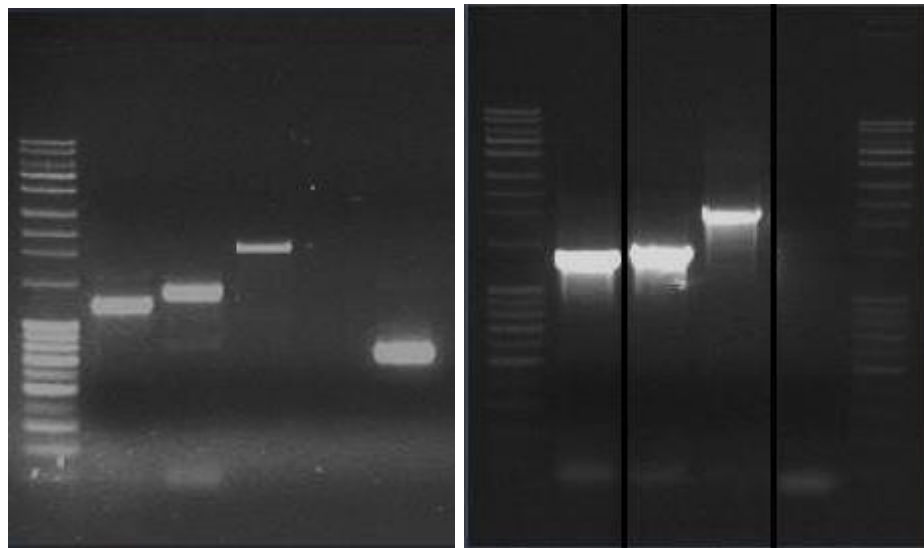
A   B   C   D   E   F   G   H   I   J   K   L

Figure 36: Agarose gels showing amplification of the gDNA region separating the *lcc*1 and *lcc*2 genes in U1(left) and SB65 (right). NORGEN Highranger Plus ladder (A, G, L), first half of spacer (B & H), second half of spacer (C & I), entire spacer (D & J), NTC (E & K), and ITS primer positive control (F).

4.3.2. Laccase sequences

All the clones were sequenced for both the U1 and SB65 strain of *A. bisporus*. The

sequences were assembled and aligned (Appendix VI: Figure A1 & A2). Since the two genes

are found in tandem within the genome (Figure 37), the assembled sequence began

approximately 470 bp upstream of the *lcc*1 transcription start site and ended at the translation

stop codon of *lcc*2 encompassing over 6500 bp.
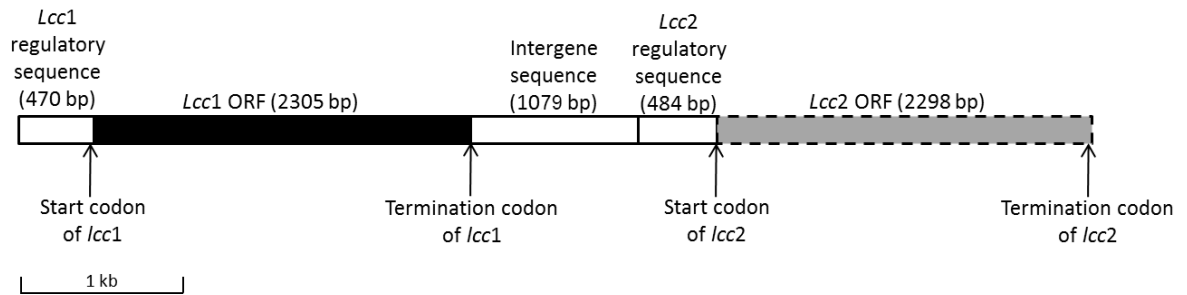
Figure 37. Arrangement of *lcc*1 and *lcc*2 genes in *A. bisporus* genome. The numbers presented are for U1, numbers for SB65 are very similar (modified from Smith et al. 1998).

4.3.2.1. Regulatory region

The "regulatory region" discussed in this section refers to the 470 bp preceding both *lcc*1

and *lcc*2 coding regions. Since the sequence commences 470 bp upstream of the *lcc*1

transcription start site, *lcc*1 regulatory elements further upstream were not analyzed.

A. bisporus appears to be heteroallelic for *lcc*1, as indicated by the different sequence

information upstream of the start codon, but homoallelic for *lcc*2. One allelic form of the *lcc*1

regulatory region (*lcc*1b) was 100% homologous in both *A. bisporus* strains, while *lcc*1a and *lcc*2

regulatory region was 99.7% homologous, each differing in only 1 nucleotide between *A.*

bisporus strains (Figure 38 – single nucleotide substitutions shown by triangle). These

regulatory regions were characterized for general transcriptional factor sites, stress response

elements and nutritional response elements.

4.3.2.1.1. General transcription factor sites

4.3.2.1.1.1. TATA box

The TATA box-binding protein (TBP) is tightly associated with several polypeptides, and together forms a complex that gathers initiation factors and RNA polymerase resulting in transcription of nuclear genes (Nikolov et al. 1996). TBPs are essential for all three eukaryotic RNA polymerases for initiation of transcription of ribosomal, messenger, small nuclear, and transfer RNAs (Nikolov et al. 1996). While the TBP is essential for transcription, over 80% of yeast (and other fungal) promoters do not contain the TATA box consensus sequence (Basehoar, Zanton & Pugh, 2004; Tora & Timmers 2010). Since the TBP binds at both TATA and TATA-less promoters, TATA-less promoters likely contain other factors that stabilize TBP binding which reduces the need for the TATA consensus sequence (Basehoar et al. 2004). Promoters that contain a TATA consensus sequence are often dependent on its presence for transcription, where loss of the TATA sequence results in a loss of expression (Basehoar et al. 2004). The primary function of the TATA box is correct positioning of the polymerase relative to the initiation site rather than controlling transcription rate (Bucher, 1990).

In the laccase promoter sequences of *A. bisporus*, one TATA box preceding the transcription start site of *lcc*1 was found in both strains of *A. bisporus*, and 2 and 3 TATA boxes upstream of U1and SB65 *lcc*2 respectively (Figure 38 & Table 6). The TATA boxes at -54 of *lcc*1 and -53 bp of *lcc*2 have also been described in the U1 strain by Smith et al. (1998). These TATA boxes are likely functional transcription initiators for two reasons: they fall within in the -50 to -200 bp location for the position of a TATA box, and they all have high transcriptional activity (Juo et al. 1996). Perry et al. (1993) described a second TATA box 185 nt upstream of the *lcc*1 start codon (seen in Perry-*lcc*1 sequence in Figure 38) that was not found in our sequence data nor the *A. bisporus* genome sequence published by Morin et al. (2012). The other TATA boxes at -447 in U1 and at -333 and -447 in SB65 are likely TATA-like sequences that

are not involved in gene regulation because they are too far from the promoter and because they

have very low transcriptional activity (Basehoar et al. 2004; Juo et al. 1996). The differential

transcript abundance of laccase in the presence of *T. aggressivum* toxins described in chapter 2

cannot be attributed to TATA box regulation because the TATA box identity is equivalent in

both the U1 and SB65 strains.

Table 6: TATA boxes found in the promoter regions of *lcc*1 and *lcc*2 in the U1 and SB65 strains of *A. bisporus*. Location refers to number of nt upstream of the translation start codon.

Gene	Strain	Location	Sequence	Activity
*Lcc*1	U1	-54	TATAAAG	100
	SB65	-54	TATAAAG	100
*Lcc*2	U1	-53	TATAAAA	100
	SB65	-53	TATAAAA	100
	SB65	-333	TATACTA	3
	U1	-447	TATATTG	21
	SB65	-447	TATATTG	21

The nucleotide substitution 332 bp upstream of the *lcc*2 translation start codon is the only

difference between the U1 and SB65 strains in the promoter regions of the *lcc*2 gene. This

substitution generates a unique TATA box in the SB65 strain which likely has little effect on

laccase transcription because it is too far upstream and has little transcriptional activity.

Therefore, the difference in *lcc*2 transcription between the U1 and SB65 strains in response to *T.*

aggressivum metabolites is most likely not a result of differential regulation due to variation in

the genomic sequence of the laccase promoter within 450 nt of the start codon.

```
H97-lc1    :--------TCCCTTGGCA-------TCAAAGATGCCTACGTCTTGACAGTATGT------CTCTGAAG-C---ACATTGTT-AA----TCATG--AGCAGAC-CTAAGGCC-GATT---TAGCT--ACG--GTGAGT: -385
D649-lc1   :------------------------------------CTACGTCTTGACAGTATGT------CTCTGAAG-C---ACATTGTT-AA----TCATG--AGCGAAC-CTAAGGCC-GATT---TAGCT--ACG--GTGAGT: -383
U1-lc1a    :--------------------------------------------------------------------------------------TCATG--AGCGAAC-CTAAGGCC-GATT---TAGCT--ACG--GTGAGT: -385
U1-lc1b    :--------------------------------------------------------------------------------------TCATG--AGCGAAC-CTAAGGCC-GATT---TAGCT--ACG--GTGAGT: -384
SB65-lc1a  :--------------------------------------------------------------------------------------TCATG--AGCGAAC-CTAAGGCC-GATT---TAGCT--ACG--GTGAGT: -384
SB65-lc1b  :--------------------------------------------------------------------------------------TCATG--AGCGAAC-CTAAGGCC-GATT---TAGCT--ACG--GTGAGT: -384
SB65-lc2   :GGTCGAGTTGGCTGGGCAGGGGCCTTCAAAG------TAC-TC---ACAGTATGAGCAAACCCCTGAAATCAATATATTGGTCAACCGCTCATATTAACGATGGCTACGGCATGATTGGATAGGTGGAGGCCGTGGGA: -382
U1-lc2     :GGTCAAGTTGGCTGGGCAGGGGCCTTCAAAG------TAC-TC---ACAGTATGAGCAAACCCCTGAAATCAATATATTGGTCAACCGCTCATATTAACGATGGCTACGGCATGATTGGATAGGTGGAGGCCGTGGGA: -382
H97-lc2    :GGTCAAGTTGGCTGGGCAGGGGCCTTCAAAG------TAC-TC---ACAGTATGAGCAAACCCCTGAAATCAATATATTGGTCAACCGCTCATATTAACGATGGCTACGGCATGATTGGATAGGTGGAGGCCGTGGGA: -382
D649-lc2   :GGTCAAGTTGGCTGGGCAGGGGCCTTCAAAG------TAC-TC---ACAGTATGAGCAAACCCCTGAAATCAATATATTGGTCAACCGCTCATATTAACGATGGCTACGGCATGATTGGATAGGTGGAGGCCGTGGGA: -382
                                                  ••••••••••••••                                              •••••••


H97-lc1    :--CGTGACCAACGTCCAGTGGCAAAGCGCTGGTTTT-AGCATTAAATCACAAACTGAACCACAGTACAACGCCTGCAAGAC--ACGCGCGTGATGTTCAATACAAACGTTGAACATCTATGCGCCTGTAACGGGGAG: -253
D649-lc1   :--CGTGACCAACGTCCAGTGGCAAAGCGCTGGTTTT-AGTATTAAATCACAAACTGAACCACAGTACAACGCCTGCAAGAC--ACGCGCGTGATGTTCAATACAAACGTTGAACATCTATGCGCCTGTAACGGGGAG: -251
U1-lc1a    :--CGTGACCAACGTCCAGTGGCAAAGCGCTGGTTTT-AGCATTAAATCACAAACTGAACCACAGTACAACGCCTGCAAGAC--ACGCGCGTGATGTTCAATACAAACGTTGAACATCTATGCGCCTGTAACGGGGAG: -253
U1-lc1b    :--CGTGACCAACGTCCAGTGGCAAAGCGCTGGTTTT-AGTATTAAATCACAAACTGAACCACAGTACAACGCCTGCAAGAC--ACGCGCGTGATGTTCAATACAAACGTTGAACATCTATGCGCCTGTAACGGGGAG: -252
SB65-lc1a  :--CGTGACCAACGTCCAGTGGCAAAGCGCTGGTTTT-AGCATTAAATCACAAACTGAACCACAGTACAACGCCTGCAAGAC--ACGCGCGTGATGTTCAATACAAACGTTGAACATCTATGCGCCTGTAACGGGGAG: -252
SB65-lc1b  :--CGTGACCAACGTCCAGTGGCAAAGCGCTGGTTTT-AGTATTAAATCACAAACTGAACCACAGTACAACGCCTGCAAGAC--ACGCGCGTGATGTTCAATACAAACGTTGAACATCTATGCGCCTGTAACGGGGAG: -252
SB65-lc2   :AACTTGACAAATATCCAGTCGCAAAGCACTGATTTCCAGCATTAG-TGTTATACT--ACAGCAG--ACCACCCC-GCAAAAATAAGGCGTGGGATGTCCGACAGGTACGTTGTTCATTCATAGGCCCGTATTGGCCA-: -251
U1-lc2     :AACTTGACAAATATCCAGTCGCAAAGCACTGATTTCCAGCATTAG-TGTTAAACT--ACAGCAG--ACCACCCC-GCAAAAATAAGGCGTGGGATGTCCGACAGGTACGTTGTTCATTCATAGGCCCGTATTGGCCA-: -251
H97-lc2    :AACTTGACAAATATCCAGTCGCAAAGCACTGATTTCCAGCATTAG-TGTTAAACT--ACAGCAG--ACCACCCC-GCAAAAATAAGGCGTGGGATGTCCGACAGGTACGTTGTTCATTCATAGGCCCGTATTGGCCA-: -251
D649-lc2   :AACTTGACAAATATCCAGTCGCAAAGCACTGATTTCCAGCATTAG-TGTTAAACT--ACAGCAG--ACCACCCC-GCAAAAATAAGGCGTGGGATGTCCGACAG-TACGTTGTTCATTCATAGGCCCGTATTGGCCA-: -252
                                                         ▲                                                                            •••••  ▼


H97-lc1    :AGAAATCTCGTGGAACTGCCCTGGATGATAAATTTCATCAGCTTCGAATGTCAGTGACATTGAATGTTATTAAATACCCAGCAGAGAGTGGCTCGTCCTCAATGTGAGCGCGTAAACTCCTGAAAAA-TTTTCG---: -120
D649-lc1   :AGAACCTCGTGAAACTGCCCTGGATGATAAATTTCATCAGCTTCGAATGTCAGTGACATTGAATGTTA-AAATACCCAGCAGAGAGTGGCTCGTCCTCAATGTGAGCGCGTAAACTTTAGAAAA--TTTCCG---: -120
U1-lc1a    :AGAAATCTCGTGGAACTGCCCTGGATGATAAATTTCATCAGCTTCGAATGTCAGTGACATTGAATGTTATTAAATACCCAGCAGAGAGTGGCTCGTCCTCAATGTGAGCGCGTAAACTCCTGAAAAA-TTTTCG---: -120
U1-lc1b    :AGAACCTCGTGAAACTGCCCTGGATGATAAATTTCATCAGCTTCGAATGTCAGTGACATTGAATGTTATTAAATACCCAGCAGAGAGTGGCTCGTCCTCAATGTGAGCGCGTAAACTTTAGAAAA--TTTCCG---: -120
SB65-lc1a  :AGAAATCTCGTGGAACTGCCCTGGATGATAAATTTCATCAGCTTCGAATGTCAGTGACATTGAATGTTATTAAATACCCAGCAGAGAGTGGCTCGTCCTCAATGTGAGCGCGTAAACTCCTGAAAA--TTTTCG---: -120
SB65-lc1b  :AGAACCTCGTGAAACTGCCCTGGATGATAAATTTCATCAGCTTCGAATGTCAGTGACATTGAATGTTATTAAATACCCAGCAGAGAGTGGCTCGTCCTCAATGTGAGCGCGTAAACTTTAGAAAA--TTTCCG---: -120
SB65-lc2   :ACAAGTCTCGCACAATTACCTCGGACGGTAAACGCCATCAGCTTCCAAAATCA----------ATGTTGTCAACTCCCTTGCGAAGAGTGGCTCGTCCTCTATGTGAACCCGCGAACTCCTGAAAAATTTCGGGCC-: -124
U1-lc2     :ACAAGTCTCGCACAATTACCTCGGACGGTAAACGCCATCAGCTTCCAAAATCA----------ATGTTGTCAACTCCCTTGCGAAGAGTGGCTCGTCCTCTATGTGAACCCGCGAACTCCTGAAAAATTTCGGGCC-: -124
H97-lc2    :ACAAGTCTCGCACAATTACCTCGGACGGTAAACGCCATCAGCTTCCAAAATCA----------ATGTTGTCAACTCCCTTGCGAAGAGTGGCTCGTCCTCTATGTGAACCCGCGAACTCCTGAAAAATTTCGGGCC-: -124
D649-lc2   :ACAAGTCTCGCACAATTACCTCGGACGGTAAACGCCATCAGCTTCCAAAATCA----------ATCTTGTCAACTCCCTTGCGAAGAGTGGCTCGTCCTCTATGTGAACCCGCGAACTCCTGAAAAATTTCGGGCC-: -125
                ••••                                    •••••••••••••                        ══════


H97-lc1    :-----TGGATTGTGGTCTCAGGCATATCGCTGGTGGTTTCTCCTTGTTGATAAAAA-TACGTATCTTCAGGCTATAAAGATGGGGCAGAGGACCGTG-GATTGACTCTCCAGC------GGTAG-GATCAACAATG: 3
D649-lc1   :-----TGGATTGTGGTCTCAGGCATATCGCTGGTGGTTTCTCCTTGTTGATAAAAA-TATGTATCTTCAGGCTATAAAGACGGGGCAGAGGACCGTG-GATTGACTCTCCAGC------GGTAG-GATCAACAATG: 3
U1-lc1a    :-----TGGATTGTGGTCTCAGGCATATCGCTGGTGGTTTCTCCTTGTTGATAAAAA-TACGTATCTTCAGGCTATAAAGATGGGGCAGAGGACCGTG-GATTGACTCTCCAGC------GGTAG-GATCAACAATG: 3
U1-lc1b    :-----TGGATTGTGGTCTCAGGCATATCGCTGGTGGTTTCTCCTTGTTGATAAAAA-TATGTATCTTCAGGCTATAAAGACGGGGCAGAGGACCGTG-GATTGACTCTCCAGC------GGTAG-GATCAACAATG: 3
SB65-lc1a  :-----TGGATTGTGGTCTCAGGCATATCGCTGGTGGTTTCTCCTTGTTGATAAAAA-TACGTATCTTCAGGCTATAAAGATGGGGCAGAGGACCGTG-GATTGACTCTCCAGC------GGTAG-GATCAACAATG: 3
SB65-lc1b  :-----TGGATTGTGGTCTCAGGCATATCGCTGGTGGTTTCTCCTTGTTGATAAAAA-TATGTATCTTCAGGCACGGGGCAGAGGACCGTG-GATTGACTCTCCAGC------GGTAG-GATCAACAATG: 3
SB65-lc2   :ATACATGAATCGTGGTCCCAAGCATAGCGTCGGCGGTTTCTCATAGCGGATAAAAA-TAAATAACTTCGCGTTATAAA-A-G-----CTCTAGACATTTTGTTTG-CTCTCCAGCCAACCAGCAAGTGTT--ACAATG: 3
U1-lc2     :ATACATGAATCGTGGTCCCAAGCATAGCGTCGGCGGTTTCTCATAGCGGATAAAAA-TAAATAACTTCGCGTTATAAA-A-G-----CTCTAGACATTTTGTTTG-CTCTCCAGCCAACCAGCAAGTGTT--ACAATG: 3
H97-lc2    :ATACATGAATCGTGGTCCCAAGCATAGCGTCGGCGGTTTCTCATAGCGGATAAAAA-TAAATAACTTCGCGTTATAAA-A-C-----CTCTAGACATTTTGTTTG-CTCTCCAGCCAACCAGCAAGTGTT--ACAATG: 3
D649-lc2   :ATACATGAATCGTGGTCCCAAGCATAGCGTCGGCG-TTTCTCATAGCGGATAAAAAATAAATAACTTCGCGTTATAAA-AAG-----CTCTAGACATTTTGTTTG-CTCTCCAGCCAACCAGCAAGTGTT--ACAATG: 3
                                                                                   ══════
```

102

Figure 38: Alignment of regulatory regions of *lcc*1 and *lcc*2 from the U1 and SB65 strains of *A. bisporus* and sequences published on the database. Black highlights consensus between all 10 sequences, grey highlights consensus between 6-8 sequences. Single nucleotide differences between strains of *A. bisporus* are shown by triangles. Putative regulatory elements are identified for *lcc*1 (above sequence) and *lcc*2 (below sequence) include solid lines for TATA boxes and dotted lines for CAAT boxes.

4.3.2.1.1.2. CCAAT box

Another common and important regulatory element of gene expression is the CCAAT box (Benoist et al. 1980), found in approximately 30% of eukaryotic promoters. The CCAAT can be found at variable distances, preferentially -212 to -57 bp, and most commonly 75 – 80 bp, upstream of the transcription start site (Bucher, 1990; Chowdhary et al. 2010; Singh et al. 2013). The nucleotide CAAT core is well-conserved and typically the preceding nucleotide is a cysteine residue, so CAAT is used synonymously with CCAAT box (Singh et al. 2013). In addition, CCAAT boxes function in both orientations so the complementary sequence ATTGG acts as a CCAAT box (Bucher, 1990).

Two CCAAT boxes were found in the *lcc*1 regulatory region and six in the *lcc*2 regulatory region (Figure 38, Table 7). All CCAAT boxes described by Smith et al. (1998)

Table 7: CCAAT boxes found in *lcc*1 and *lcc*2 promoter regions of U1 and SB65 strains of *A. bisporus*. Location is relative to the translation start codon.

Gene	Location	Sequence
*Lcc*1	-153	CAAT
	-292	CAAT
*Lcc*2	-200	CAAT
	-239	CAAT
	-259	ATTGG
	-406	ATTGG
	-444	ATTGG
	-450	CAAT

were present in the U1 and SB65 sequences, however, they did not consider the complementary sequence ATTGG that can be found thrice in the *lcc*2 regulatory region.

*Lcc*2 has numerous unique CCAAT boxes which may contribute to the increased expression of the *lcc*2 gene.

4.3.2.1.1.3. Mammalian response element (Sp1)

Specificity protein 1 (SP1), is a human transcription factor and described in the regulatory sequence of the fungus, *Pleurotus sajor-caju*, laccase 1 gene (Soden & Dobsen, 2003). Activation of transcription by Sp1 functions bidirectionally in the presence of a well-defined upstream element 5' GGGCGG 3' (Dynan & Tjian, 1985; Everett, Baty, & Chambon, 1983). The Sp1 consensus sequence is not present before either laccase gene in *A. bisporus*.

4.3.2.1.2. Stress Response
4.3.2.1.2.1. Heat shock element (HSE)

In an unstressed cell, the Heat Shock Factor (HSF), a transcription factor, exists as a monomer with little DNA binding affinity. When a eukaryotic cell is exposed to stress conditions (heat shock), the HSF assembles into a trimer, localizes to the nucleus and binds the heat shock element (HSE) (Morimoto, 1993). The HSE is an array of a repeating 5bp sequence nGAAn assembled in alternating orientation: nGAAnnTTCn (Sorger, 1991). There are conflicting reports in literature concerning the number of nGAAn repeats required for the HSF trimer to bind DNA with high affinity. Some reports describes high affinity binding in the presence of only two repeats (Soden & Dobson, 2003; Sorger, 2001) while most claim that high affinity binding requires the presence of at least three repeats (Guertin & Lis, 2010; Mager & Druijff,1995; Rabindran et al. 1994; Vuister, et al. 1994).

Smith et al. (1998) describe a HSE in the promoter region of both *lcc*1 and *lcc*2 genes with the consensus sequence nGAnnn. This consensus sequence is not supported in literature, as it is clear that the consensus sequence is the well-conserved pentamer nGAAn (Sorger, 1991). Analysis the promoter regions of the laccase genes yielded several putative

HSE different than the one described by Smith et al. (1998). Four putative HSE were seen within the *lcc*1 promoter, and three of the four have three or more repeats. Five putative HSE were found upstream of the *lcc*2 transcription start site, but only one had more than two repeats (Table 8).

```
                                                                                          MRE
lcc1a : ---------------------------------------------------------------------------tca:-420
lcc1b : ---------------------------------------------------------------------------tca:-419


              MRE
lcc1a : tg--agcgaac-ctaaggcc-gatt---tagct--acg--gtgagt--cgtgaccaacgtccagtggcaaagcgct:-357
lcc1b : tg--agcgaac-ctaaggcc-gatt---tagct--acg--gtgagt--cgtgaccaacgtccagtggcaaagcgct:-356
              MRE

                                                     MRE              XRE
lcc1a : ggtttt-agcattaaatcacaaactgaaccacagtacaacgcctgcaaga--cacgcgcgtgatgttcaatacaaa:-284
lcc1b : ggtttt-agtattaaatcacaaactgaaccacagtacaacgcctgcaaga--cacgcgcgtgatgttcaatacaaa:-283
                                                     MRE              XRE

                        MRE                     --> <-- HSE --> <---
lcc1a : cgttgaacatctatgcgcctgtaacggggagagaaatctcgtggaactgccctggatgataaatttcatcagcttc:-208
lcc1b : cgttgaacatctatgcgcctgtaacggggagagaaactcgtgaaactgccctggatgataaatttcatcagcttc:-207
                        MRE                     <-- <-- HSE --> <---

                                                              MRE MRE MRE
lcc1a : gaatgtcagtgacattgaatgttattaaatacccagcagagagtggctcgtcctcaatgtgagcgcgtaaactcct:-132
lcc1b : gaatgtcagtgacattgaatgttattaaatacccagcagagagtggctcgtcctcaatgtgagcgcgtaaacttta:-131
                                                              MRE MRE MRE

          HRRE  ▼                 Nit2                Nit2                   Nit2
lcc1a : gaaaaa-ttttcg--------tggatggtggtctcaggcatatcgctggtggtttctcctgttgataaaaatacg: -65
lcc1b : gaaaa--tttccg--------tggatggtggtctcaggcatatcgctggtggtttctcctgttgataaaaatatg: -65
                                  Nit2                Nit2                   Nit2

          Nit2                                       CreA_____  ACE1
lcc1a : tatcttcaggctataaagatgggggcagaggaccgtg-gattgactctccagc------ggtag-gatcaacaatg:  3
lcc1b : tatcttcaggctataaagacgggggcagaggaccgtg-gattgactctccagc------ggtag-gatcaacaatg:  3
          Nit2                                       CreA        ACE1
```

Figure 39: *Lcc*1 regulatory regions of *A. bisporus* U1. Black highlight homology between both *lcc*1 alleles and *lcc*2, while grey highlights homology between two of the three sequences. The triangle designates the nt absent in the SB65 sequence. Putative regulatory elements are identified as follows: HSE (heat shock response element) where arrows indicate the orientation of the 5 bp units of a heat shock element with the consensus nGAAn sequence; solid arrows indicate perfect matches and dashed lines denote imperfect matches (Sorger, 1991); MRE (metal response element); ACE1 (activation of cup1 expression); XRE (xenobiotic response element); HRRE (humic response regulatory element); CreA (cAMP mediated glucose repression); Nit2 (Nitrogen repression response element).

Table 8: Heat shock responsive elements (HSE) in the promoter regions
of *A. bisporus'* two laccase genes. The location of the HSE is number of
base pairs relative to the translation start codon. Uppercase letters
indicate alignment with GAA consensus sequence.

Gene	# of repeats	Location	Sequence
*lcc*1a	4	-142 to -122	gtAAacTcCtgaAAaaTTtt
	3	-193 to -178	tGAAtgTTattaAAt
	2	-225 to -215	atAAatTTCa
	4	-253 to -233	aGAAatcTCgtGgAacTgCc
*lcc*1b	4	-141 to -121	gtAAacTTtagaAAatTTCc
	3	-192 to -177	tGAAtgTTattaAAt
	2	-225 to -215	atAAatTTCa
	4	-252 to -232	aGAAccTCgtGAAacTgCc
*lcc*2	2	-124 to -114	aTaCatGAAt
	2	-146 to -136	acTCctGAAa
	2	-210 to -200	cTTCcaaAAt
	2	-350 to -330	tTTCcaGcAt
	4	-385 to -365	gGAAacTTgacaAAtaTcCa

The heat shock response occurs when a cell is exposed to elevated temperatures
and/or a wide variety of chemical inducers (Rabindran et al. 1994). Upon induction, the
HSFs aggregate, localize to the nucleus, bind the HSE and induce expression of the target
gene. The HSE includes the nGAAn consensus sequence occurring multiple times in
opposite orientations. Mager & Druijff (1995) describe that two adjacent 5-bp units are
required for stable binding *in vitro*, while a trimer represents a complete binding site for the
HSF molecule. In addition, imperfect repeats have lower binding affinity than perfect
repeats (Carranco, Almoguera, & Jordano1999), therefore, the putative *lcc*1 HSE and the
four *lcc*2 HSE with only two repeats are likely going to demonstrate low binding affinity for
the HSF because each has one imperfect repeats leaving only one consensus sequence with
high binding potential. Furthermore, within the nGAAn sequence the G at position two is
absolutely conserved while the As at positions three and four are highly conserved.
Substitution of the G-2 results in a fivefold decrease in the binding affinity of the HSF in
yeast cells (Mager & Druijff, 1995). Re-examination of the HSE elements present in the

laccase regulatory elements with the idea that a functional HSE has two or more perfect

repeats, or one perfect with at least two imperfect repeats, and eliminating those which do not

```
                    STRE                                              STRE
gttggctgggcaggggccttcaaagtactcacagtatgagcaaaccccctgaaatcaatatattggtcaaccgctca:-428

                                        CreA
tattaacgatggctacggcatgattggataggtggaggccgtgggaaacttgacaaatatccagtcgcaaagcact:-352

                        ▼                     CreA
gatttccagcattag-tgttaaact--acagcag-accaccccc-gcaaaaataaggcgtgggatgtccgacaggta:-281

cgttgttcattcataggcccgtattggcca-acaagtctcgcacaattacctcggacggtaaacgccatcagcttc:-206

                                                            MRE
caaaatca----------atgttgtcaactccccttgcgaagagtggctcgtcctctatgtgaacccgcgaactcct:-140

    HRRE
gaaaaaatttcgggccatacatgaatcgtggtcccaagcatagcgtcggcggtttctcatagcggataaaaataaa: -64

                                        MRE CreA
taacttcgcgttataaa-a-----gctctagacattttgtttg-ctctccagccaaccagcaagtgtt--acaatg:   3
```

Figure 40: *Lcc2* regulatory region of *A. bisporus* U1. Black highlight homology with both *lcc1* alleles, while grey highlights homology between two of the three sequences. The triangle designates the nt substitution in the SB65 sequence (a = t). Putative regulatory elements are identified as follows: box: MRE (metal response element); STRE (stress response element); HRRE (humic response regulatory element; CreA (cAMP mediated glucose repression).

have the conserved G (or C) at position 2 revealed only one putative HSE in both *lcc1*

sequences (≈ -252 to -232 bp) (Figure 39) while *lcc2* does not have any. The effect that heat

shock has on *A. bisporus'* laccase expression has not been described; however, this data

indicates that *lcc1* transcription may be affected by heat shock.

4.3.2.1.2.2. Metal responsive element (MRE)

MRE for all laccases have the consensus sequence TGCRCNC, and the presence of

one MRE is usually indicative of organisms ability to response to heavy metals including

zinc, copper, cadmium, and others (Eastwood et al. 2011; Faraco, Giardina & Sannia, 2003;

Janusz et al. 2013; Piscitelli et al. 2011). Allowing for one bp mismatch from the

TGCRCNC consensus sequence (Eastwood et al. 2011), six putative MREs were found in the

5' region of *lcc*1, four which are inverted and three with overlapping sequences, and two

putative MREs were found preceding *lcc*2 (Figure 39 & 40; Table 9).

Table 9: Metal response elements (MRE) in the promoter regions of *A. bisporus'* two laccase genes. The location of the MSE is number of base pairs relative to the translation start codon.

Gene	Location	Orientation	Sequence
*Lcc*1	-418	reverse	gagcgaa
	-317	reverse	gcctgca
	-270	forward	tgcgcct
	-148	forward	tgagcgc
	-147	reverse	gagcgcg
	-145	reverse	gcgcgta
*Lcc*2	-157	forward	tgaaccc
	-29	forward	tgctctc

4.3.2.1.2.3. ACE1 factor

The activation of cup1 expression (ACE1) response element is often present in the

promoter regions of some multi-copper oxidase encoded genes and responds to the presence

of copper (Canessa et al. 2008; Soden & Dobson, 2003; Galhaup et al. 2002). The consensus

sequence for binding of the ACE1 transcription factor is $TCN_{4-6}GCTG$ (Cordoba Cañero &

Roncero, 2008; Janusz et al. 2013). The *lcc*2 gene of *A. bisporus* did not have an ACE1

binding within the promoter region. *Lcc*1 has an ACE1 binding site in the reverse orientation

just 17 bp upstream from the translation start codon (Figure 39). Position of an ACE1

binding site between the TATA box and the ATG site of the laccase 2 gene has been

described in *Gaeumannomyces graminis var. tritici* (Litvintseva & Hensen, 2002), though the

authors did not determine the role of the ACE1 transcription factor in the induction of LAC2

in response to copper. The unique presence of an ACE1 binding site suggests that *lcc*1 may

be induced by the presence of copper. This supposition is consistent with the observation that

laccase transcription levels have been shown to regulated by copper in many fungi including

Trametes versicolor (Collins & Dobson, 1997), *T. pubescens* (Galhaup et al. 2002),

Ceriporiopsis subvermispora (Karahanian et al. 1998), *Pleurotus ostreatus* (Palmieri et al, 2000), *P. sajor-caju* (Soden & Dobson, 2001).

4.3.2.1.2.4. Xenobiotic responsive element (XRE)

Laccases have been shown to be induced by phenolic and aromatic compounds, likely as a defense against toxic aromatic compounds (Piscitelli et al. 2011), though the role of the xenobiotic responsive element (XRE) has not been very well defined in this response (Janusz et al. 2013). Common consensus sequences for XREs are 5' TCACGS 3' (Janusz et al. 2013; Soden & Dobson, 2003) and 5' CACGCW 3' (Fan et al. 2011; Rushmore et al. 1990). One XRE sequence was found in the 5' region of *lcc*1, 302 bp upstream of the transcription start site in the reverse orientation (Figure 39). The presence of the XRE sequence suggested that *lcc*1 transcription may be activated by aromatic compounds, but apparently not by those present in the *T. aggressivum* extracellular extract.

4.3.2.1.2.5. Antioxidant response element (ARE)

The antioxidant response element was first described by Pickett and co-workers in 1990 (Rushmore et al. 1990; Rushmore & Pickett, 1990). The ARE core sequence 5' GTGACNNNGC 3' or the complementary 5' GCNNNGTACAC 3' are required for transcriptional activation by phenolic and aromatic compounds (Rushmore, Morton & Pickett, 1991). The *A. bisporus* laccase regulatory regions were searched for the consensus sequence described by Rushmore et al. (1991), as well as the similar 5' TGACNNNGC 3' described by Soden & Dobson (2003). Four likely ARE consensus sequences were observed in the *lcc*1 regulatory region and three unique ARE sequences in *lcc*2 (Figure 39 & 41; Table 10). We considered these regulatory elements to be not important in gene regulation for several reasons. First, none of the seven putative AREs matched the consensus sequence described by Rushmore et al. (1991), all were homologous with the shorter consensus with one mismatch described by Soden & Dobson (2003). In addition, important regulatory

elements would be expected to be conserved in both genes (as seen with the TATA box) whereas *lcc*1 and *lcc*2 each have several unique AREs. Finally, the frequency of occurrence is comparable in the promoter (1 in 115 bp), the intergene space (1 in 71 bp) and in the coding region of the gene (1 in 74 bp). This suggested that the ARE sequence in the promoter was due to random chance rather than as an important, functioning regulatory element.

Table 10: Antioxidant response elements (ARE) in the promoter regions of *A. bisporus'* two laccase genes as described by Soden & Dobson (2003).

Gene	Location	Orientation	Sequence
*Lcc*1	-369	forward	gtggcaaagc
	-243	forward	gtgaaactgc
	-199	forward	gtgacattga
	-25	forward	tgactctcc
*Lcc*2	-417	reverse	gctacggca
	-158	forward	gtgaacccgc
	-64	forward	taacttcgc

4.3.2.1.2.6. Stress response element (STRE)

The general stress response element (STRE) responds to nitrogen starvation, heat shock, and a variety of chemical stressors (Treger, Magee & McEntee, 1998). The STRE consensus sequence is 5' CCCCT 3' in either orientation (Treger et al. 1998) and has been described upstream of several yeast genes (Mager & Drujff, 1995) and fungal laccase promoters (Galhaup et al. 2002; Janusz et al. 2013; Xiao et al. 2006). We found two STRE in the 5' coding region of *lcc*2, one 460 bp upstream of the translational start codon in the same orientation as the TATA box (Figure 40), and a second 493 bp upstream in the inverse orientation (Figure 40 & 41). There were no STRE consensus sequences upstream of the *lcc*1 gene. A single STRE is sufficient for modest increase in gene transcription, but two or more copies result in a greater than additive level of transcription (Treger et al. 1998).

Therefore, the presence of two STRE copies may account for the increase in *lcc*2 transcription in response to the chemical stresses of *T. aggressivum* secondary metabolites.

4.3.2.1.3. Nutritional regulation

Cells are able to sense the nutritional status of their environment and produce appropriate enzyme to utilize specific substrates. While response element that sense nutritional status of the fungi would not have direct effects on the disease sensitivity of the U1 strain, inducing or repressing hydrolytic enzymes during different nutrient conditions undoubtedly have secondary effects on disease resistance. Since *A. bisporus* laccases have been shown to be induced by malt extract (Wood, 1980a), broken down compost (Kerrigan, Challen & Burton, 2013; Morin et al. 2012; Smith et al. 1999), and rye bran (Hilden et al. 2013) we analyzed the regulatory region for the presence of nutritional response elements.

4.3.2.1.3.1. Humic response regulatory element (HRRE)

The humic response element was recently described by Morin et al. (2012), after the discovery that the majority of compost-induced genes shared the motif TCMKGWDAMAATCTC in the 5' region of the gene. Morin et al. (2012) reported the presence of two humic response sequences in 5' region of *lcc*1, once in *lcc*2, and once in two separate *lcc*2 sensu stricto genes. We found the humic response sequence 144 bp upstream of the *lcc*2 translational start codon, and 136 nt upstream of the start codon in one of the alleles for *lcc*1 (Figure 39 & 40). Since Morin et al. (2012) sequence the H97 homokaryon, they would have sequenced one genome contained in the U1, which is the same as one of the clone sequenced in this study. The other *lcc*1 allele presented in this work is very similar to the D649 strain sequenced by Perry et al. (1993) which lacks the HRRE consensus sequence (Table 11). The relative importance of each nt in the HRRE is not known, but conceivably the alleles with higher identity with the described HRRE consensus sequence would have higher activity. Therefore, likely only one *lcc*1 allele is able to respond with high activity to

the presence of inducers of the HRRE. Several clones of *lcc*2 and multiple database

sequences all show the same *lcc*2 sequence, indicating that *lcc*2 is likely homoallelic; and

both *lcc*2 alleles would be under the control of HRRE. Therefore, the difference in

expression between *lcc*1 and *lcc*2 in compost, and potentially from phenolic metabolites, may

result from the different number of HRRE controlling laccase transcription.

Table 11: Humic response regulatory element consensus sequence in *lcc*1 and
*lcc*2 in different strains of *A. bisporus*.

HRRE (Morin et al. 2012)	T	C	C	T	G	A	A	A	A	A	A	T	C	T	C	# match
D649 *lcc*1 (Perry et al. 1993)	T	T	T	A	G	A	A	A	A	-	-	T	T	T	C	9
H97 *lcc*1 (Morin et al. 2012)	T	C	C	T	G	A	A	A	A	A	T	T	T	T	C	13
U1 *lcc*1a	T	C	C	T	G	A	A	A	A	A	T	T	T	T	C	13
U1 *lcc*1b	T	T	T	A	G	A	A	A	A	-	-	T	T	T	C	9
SB65 *lcc*1a	T	C	C	T	G	A	A	A	A	-	T	T	T	T	C	12
SB65 *lcc*1b	T	T	T	A	G	A	A	A	A	-	-	T	T	T	C	9
D649 *lcc*2 (Smith et al. 1998)	T	C	C	T	G	A	A	A	A	A	A	T	T	T	C	14
H97 *lcc*2 (Morin et al. 2012)	T	C	C	T	G	A	A	A	A	A	A	T	T	T	C	14
U1 *lcc*2	T	C	C	T	G	A	A	A	A	A	A	T	T	T	C	14
SB65 *lcc*2	T	C	C	T	G	A	A	A	A	A	A	T	T	T	C	14

4.3.2.1.3.2. cAMP mediated glucose repression (CreA)

The CreA (cAMP mediated glucose repression) site, originally described in *Aspergillus nidulans*, acts by repressing transcription of a gene in the presence of glucose (Strauss et al.1999). The yeast homologue of CreA is called Mig1 (Ostling, Carlberg, & Ronne 1996). The consensus sequence for the CreA (Mig1) binding is SYGGRG (Nehlin, Carlberg & Ronne, 1991; Strauss et al. 1999). There was one CreA site 20 bp upstream of the *lcc*1 ATG site (Figure 39), and 3 CreA sites at -397, -317, and -25 in *lcc*2 (Figure 40). Since *lcc*2 is the predominant form of laccase produced by *A. bisporus* (Smith et al. 1998), it is advantageous to maintain regulation over *lcc*2 transcription during glucose rich times.

4.3.2.1.3.3. Nitrogen repression response element (Nit2)

Preferred nitrogen sources for filamentous fungi include ammonia, glutamine, glutamate and asparagine (in yeast), but when these are limited fungi will turn to catabolism of secondary nitrogen sources including amino acids, amides, proteins, purines, nitrate and

nitrite (Marzluf, 1997; Xiao & Marzluf 1997). The transcription factor NIT2 activates

expression of genes during conditions of nitrogen starvation (Janusz et al. 2013). A single

GATA sequence represents a weak binding site for NIT2 and most NIT2 bindings sites

contain at least two GATA sequence that are within 30 bp of each other, facing the same or

opposite direction (Fu & Marzluf, 1990; Marzluf, 1997). Mutation of any one of the bases in

the GATA core terminates NIT2 binding with the exception of mutation to GATT which

retains 50% binding affinity (Marzluf, 1997). *A. bisporus* laccase promoters contain several

GATA and GATT motifs as shown in Table 12. The two laccase genes in *A. bisporus* have

similar number of NIT2 binding sites, 9 in *lcc*1 and 8 in *lcc*2, but they vary in strength and

location. In particular, *lcc*1 has 3 GATA and 1 GATT sequences in tandem with fewer than

30 bp between each of them forming a potential binding site with high affinity for NIT2

(Figure 39). There are no sites with two or more GATA consensus sequence in close

proximity in *lcc*2. This suggested that *lcc*1, but not *lcc*2, may be induced during times of

nitrogen starvation.

Table 12: Nitrogen repression response elements in the promoter regions
of *A. bisporus'* two laccase genes

Gene	Motif #	Sequence	Motif strength	Location	Within 30 bp of motif #
*Lcc*1	1	AATC	Weak	-424	2
	2	GATT	Weak	-402	1
	3	AATC	Weak	-333	
	4	GATA	Strong	-226	
	5	GATT	Weak	-118	6
	6	TATC	Strong	-101	5, 7
	7	GATA	Strong	-77	6, 8
	8	TATC	Strong	-65	7
	9	GATT	Weak	-28	
*Lcc*2	1	AATC	Weak	-453	
	2	GATT	Weak	-407	3
	3	GATA	Strong	-402	2
	4	TATC	Strong	-371	5
	5	GATT	Weak	-352	4
	6	AATC	Weak	-203	
	7	AATC	Weak	-117	
	8	GATA	strong	-76	

4.3.2.2. Other non-coding sequences

4.3.2.2.1. Spacer separating *lcc*1 and *lcc*2

Immediately following the translation stop codon of the *lcc*1 gene and up to -460 bp of the *lcc*2 translation start codon lays a spacer of 1079 bp. This spacer was different at 18 nucleotides (1.68%) between the U1 and SB65 strain. Since regulatory elements have been found further upstream then the -460 bp analyzed in section 2.1, we also analyzed the intergene spacer for all the same elements (Figure 41). This analysis was considered to be important for determination of reasons for differential expression of *lcc*2 between the U1 and SB65 strains, but not for the difference in expression between *lcc*1 and *lcc*2 because of the lack of sequence upstream of the *lcc*1 promoter.

The spacer was examined for general transcription factors including the CCAAT box (underlined in Figure 41) and the Sp1 consensus sequence. In the U1 strain, we found 12 CCAAT boxes (1 CCAAT, 2 ATTGG, 9 CAAT), three which are unique to U1 at positions 111, 560 and 700. In the SB65 strain we found 10 CCAAT boxes (1 CCAAT, 2 ATTGG, 7 CAAT), one which is unique to SB65 at position 442. Neither *A. bisporus* strain had the Sp1 consensus sequence. We detected several stress response elements within the intergene space including HSE, MRE, ACE1, XRE, ARE and STRE (Figure 41). We found four HSE consensus sequences based on the criteria that a HSE binding site required at least two perfect nGAAn repeats, or three or more repeats with at least one having a perfect nGAAn repeat. All four HSE are the same in both strains of *A. bisporus*. There are 15 putative MRE similar to the consensus sequence TGCRCNC (Eastwood et al. 2011), but they are all conserved between the U1 and SB65 strains. Both strains have an ACE1 binding site and a XRE consensus sequence at 1055 bp and 324 bp respectively. Sixteen ARE (5' TGACNNNGC 3') sites as described by Soden and Dobson (2003) were seen within the 1079 bp intergenic region, a frequency that is the same in the coding region of the gene, suggesting that these sites were due to random chance and likely non-functional. One ARE

```
                                          Nit2 Nit2        MRE
tttattcaaatatttatattcctctcacaaggaaatctatctctgtgtacaagtaggaattttaaacaatgttta:75

                                        ♦
ccctaaaacaaaatgatcgtaagtattaagtggcggcaataacgaggcaggtagtaatgataacaaacccaggca:150
                                        t

         MRE                       MRE              MRE   MRE
cttgatgcgctttgaattgaagtcttaagggcccatgtgtaacaagaatgcattcactgacacgaccatgagact:225

                       ARE                MRE
ccgaacggtctatatggcactgtcacaaggtgatgagtgaagatagaacaagtttacccagatctgctgaagttg:300

                        XRE
ctgatcctggcagactgtcgattctcacggagccttaaattcttcccatgctcattggcatcagttcccactgtc:375

          MRE                 MRE          --> <--- --->              Nit2
actcgcacgattcacacatgcgagatgacattgttgagcgtatgaaagtacttccaggaagtaatataaatcata:450
        c                                              a              c
                                                            Nit2 ♦
                                                             ♦
       Nit2       --> <--- ---> <---
ttagcgagataatattgacaatgtttgggaaattttctcatagtgagtttcacatccggctctaactgaagatag:525
                     caat                                         g
                                                                  a
                           ♦
gcaggtactccaatgagatgcctccaaattaccttcaatcatccagtccaaatgcactgcaaagtattttgcgc:600
             ccaat                     c                   MRE            MRE

                          ♦
                         Nit2           Nit2
ttccacggcacgtccctcaacaatatactaaataaatgattcc.ttaatgattcggcggtatggccgccttattg:675
                       g           g   aga
                                   Nit2
                                    ♦

              Nit2
       MRE      -->  <---    ♦       Nit2        MRE
aaggtcggcggcgcattggaatcttcaatttttggcaatctatatggtccacgcctctccgtgcttactgcttagt:750
     ct              tg                               a            a

gaagcggcgaaggaagcccacagtctaaaatagcatcagtttttagacgcaattcttgttattctgtagtgcctgg:825

                                            MRE
gccatattgtctcttattgaatatatattacgtatagtcatggcgccggcacgcggacttacatcaaactcttca:900

                                                      Nit2 Nit2
gaaactcttgcttgacgacaggacagatgtatgtcagaattctcatgaggacaggctgtttatcggattgctcgg:975

MRE                                  Nit2    Nit2          MRE
gcgcctaccctcgtaccactgccaactcaccgaactagcataccttacaagattgccagatacccgacgcgcgaa:1050

       ACE1        STRE
ttcggtcaagttggctgggcaggggcctt                                          :1079
      g
```

Figure 41: Intergene sequence separating *lcc*1 and *lcc*2 in *A. bisporus* U1 genome. The nucleotides that differ in SB65 are highlighted and indicated below the line. Putative regulatory elements are identified as: general transcription factors sites including the CCAAT box; stress response elements in bold including the HSE (heat shock response element; 5 nt repeats are indicated by arrows, solid arrow for perfect repeat, dashed arrow for imperfect repeat); MRE (metal response elements); ACE1 (activation of cup1 expression); XRE (xenobiotic response element), ARE (antioxidant response element); STRE (stress response element); and nutrition response elements in italics include the humic response element and NIT2 binding sites (Nitrogen repression response elements). Elements unique to *lcc*1 and *lcc*2 are indicated by a diamond.

consensus sequence as described by Rushmore et al. (1991) occurred within spacer in the same location in both *A. bisporus* strains. The STRE consensus sequence CCCCT was found once, conserved in both *A. bisporus* strains.

Lastly, we searched for nutritional response elements and describe four NIT2 sites that are homologous between the two strains, one where a substitution generates a GATA site in SB65 and a GATT site in U1 (637 – 654 bp) and a substitution at 442 gives SB65 an additional fifth GATA site upstream of two sites that are conserved.

4.3.2.2.2. Introns

Both laccase genes from both strains of *A. bisporus* have 15 exons divided by 14 short introns from 48 to 60 bp in length (Appendix VI – Figure A1 & A2). Every intron starts with the consensus sequence GTNNK and every intron except intron 3 in *lcc*2 end with the consensus HAK. Within 20 bp of the 3' end of the intron, an ACT or CTNA consensus sequence was found in 11 of 14 *lcc*1 introns and 12 of 14 *lcc*2 introns. Those lacking the internal ACT (CTNA) sequence include intron 1 – *lcc*2, intron 3 – *lcc*1, intron 5 – both genes and intron 7 – *lcc*1. These intron structures are in agreement with previously describe laccase introns (Perry et al. 1991b) and the structure of the *cel*1 and glyceraldehyde-3-phosphate dehydrogenase introns of *A. bisporus* (Harmsen et al. 1992; Raguz et al. 1992). The presence of large number of introns provides potential more many different splice variants, yet alternative splicing in laccase has never been shown (Janusz et al. 2013).

4.3.2.3. Gene coding sequence

The *lcc*1 mRNA is highly conserved between the two strains of A. bisporus. The *lcc*1 alleles in the U1 strain show small variation at the 5' end resulting in a protein that is different in 4 aa (99.2% homology). Each of the substitutions likely has little effect on protein function,

activity or localization. There are two substitutions within the signal sequence, phenylalanine to leucine at position 7 and leucine to valine at position 18. These four aa are all hydrophobic, so this substitution will not affect protein trafficking and would be cleaved from the mature, active protein. The substitution at the 20th position from an aspartic acid residue to a lysine is a bigger substitution, from a negative to a positive aa; however it also likely has little affect because they are both hydrophilic aa and because it is at the very end of the mature peptide. In addition, LCC1 is produced in much smaller quantity than LCC2, and therefore a minor contributor in terms of overall laccase function in *A. bisporus*. The fourth substitution at position 22 from a lysine to an arginine is another conservative substitution from one positively charged aa to another. SB65 *lcc*1 alleles are even more conserved, resulting in a protein that is different in only one aa. The conservative substitution of a phenylalanine to leucine at position 7 also would not affect laccase function.

Lcc2 mRNA is 99.7% homologous between the U1 and SB65 strains (1559 of 1563 bp) coding for an identical protein.

LCC1 and LCC2 are remarkably similar in amino acid sequence, and 489 of the 520 amino acids (94%) are homologous (Figure 42). The different allele present in U1 results in the U1a protein which has an additional 3 aa substitutions close to the N-terminus. The laccase signature sequences are well conserved in both proteins, and the copper binding sites show perfect homology with other fungal laccases (Perry et al. 1993b; Giardina et al. 2010). The consensus sequence for N-glycosylation, Asn XxxSer/Thr/Cys (Medzihradszky, 2008), is present six times within each laccase gene. As described by Perry, et al. (1993), the loss of a glycosylation site at Asn239 in LCC2 creates a new glycosylation site at Asn241.
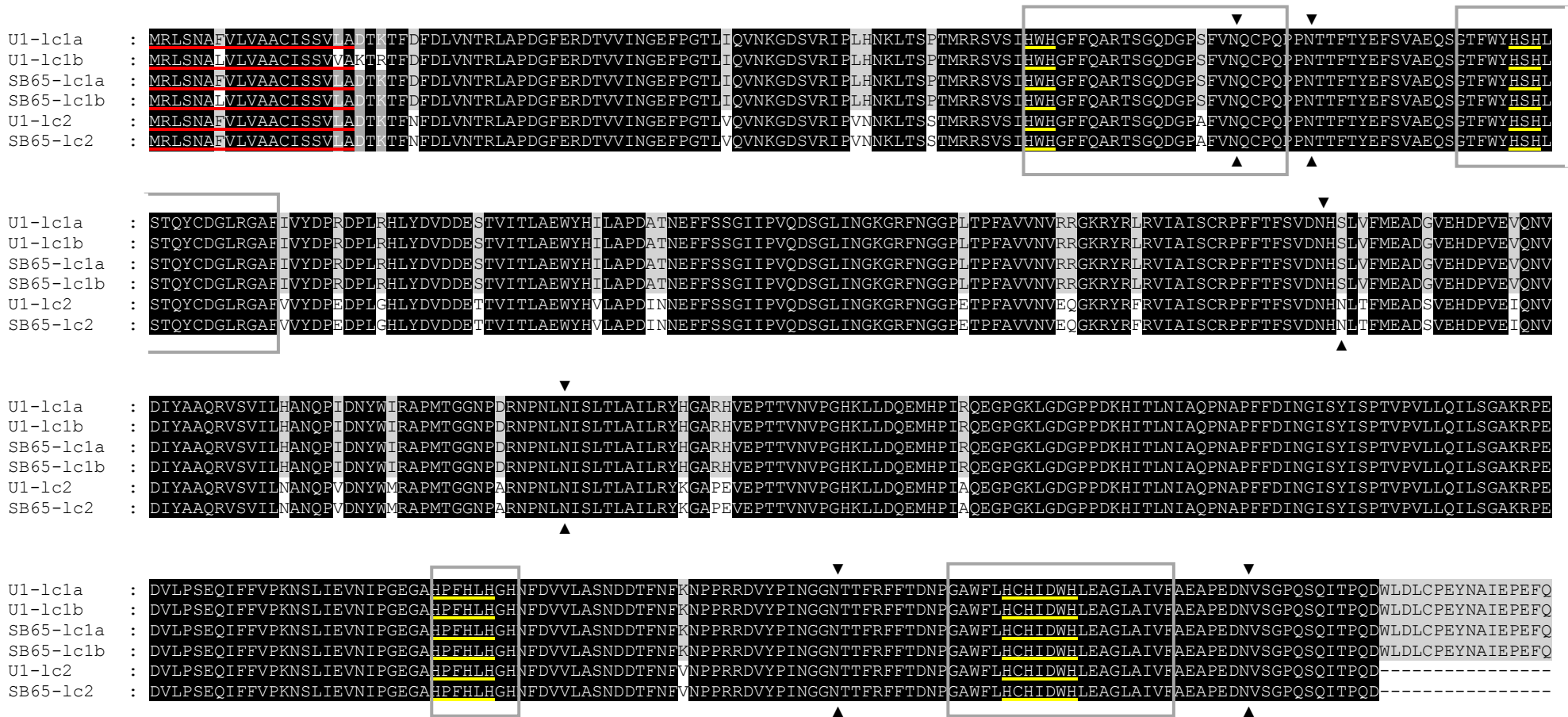
Figure 42: Alignment of predicted laccase 1 and laccase 2 protein sequences from *A. bisporus* U1 and SB65 strains based on genomic DNA sequences. The first 19 amino acids underlined in red are the protein signal sequence. The four laccase signature sequences are boxed and copper binding regions underlined in yellow. Putative N-glycosylation sites are shown by triangles.

4.4. Summary

We sequenced both laccase genes with their regulatory regions of both genes from the U1 and SB65 strains of *A. bisporus* anticipating some mutations or substitution in the coding sequence would explain the differences in the level of laccase expression between the two strains. However, the only difference in the regulatory region was preceding the *lcc*2 gene in SB65 which formed a unique TATA-like sequence with little transcriptional activity. A single deletion in SB65 preceding *lcc*1 resulted in a HRRE element that shared identity with 12 nt compared with 13 nt in the U1 strain. Several nucleotide substitutions within the gene coding sequence resulted in predicted protein with 4 amino acid substitutions in LCC1; however, all these amino acid substitutions are conservative and likely have little effect on protein activity.

Comparison of *lcc*1 and *lcc*2 regulatory elements and coding sequences has been previously described by Perry et al. (1993b) and Smith, et al. (1998). The intensive analysis of transcriptional response elements described in this paper may better describe conditions for the different expression of the genes. . As described by Smith et al. (1998) l*cc*2 has a greater number of CCAAT sites which may result in higher constitutive expression of *lcc*2. Interestingly, *lcc*1 has several unique stress responsive elements including HSE, ACE1, and XRE that are not present in *lcc*2 as well as triple the number of putative MREs indicating that the right stress condition may cause *lcc*1 to be expressed at high levels. Alternatively, the only stress response element unique to *lcc*2 is the general STRE. Since *lcc*2 expression is increased as a result of *T. aggressivum* metabolites, the induction may occur through the STRE. The two genes also appear to be under different nutrition regulation, *lcc*1 being induced under nitrogen starvation conditions (Nit2) while *lcc*2 is repressed under glucose rich conditions (CreA). Unexpectedly, we found that *lcc*1 is heteroallelic, each genome containing its own, unique copy

of *lcc*1, while *lcc*2 is homoallelic. The effect of heteroallelic character in *lcc*1 is most important when examining the regulatory elements for the HRRE. The homoallelic *lcc*2 has both genes under the control of the strong HRRE, while only one of the *lcc*1 alleles has a strong consensus with the HRRE sequence. As a result, a similar response to a condition may result in a different dosage of that response. The higher activity, expression and induction of *lcc*2 on compost may be a result of the homoallelic nature of the gene. It is plausible the phenolic metabolites produced by *T. aggressivum* are inducers of HRRE since they share properties with humic acids. If defence to toxic metabolites and adaptation to colonizing compost share the HRRE regulatory pathway, it is logical that the homoallelic *lcc*2 responses faster and more dramatically to the presence of *T. aggressivum* metabolites than the heteroallelic *lcc*1.

Chapter 5: *A. bisporus* **transformation with laccase RNAi cassette and lactonase gene**

5.1. Introduction

Traditional methods of transformation including electroporation of protoplasts or a combination of $CaCl_2$ and polyethylene glycol are inefficient for many filamentous fungi (de Groot et al. 1998). Transformation using *Agrobacterium tumefaciens* has opened the field of molecular genetics for fungi that are difficult to transform by traditional methods (Michielse et al. 2005). *A. tumefaciens* mediated transformation (ATMT) is easy to use, generates a large number of stable transformants and usually integrates as a single copy within the genome (Michielse et al. 2005). During co-cultivation with a fungus, *A. tumefaciens* can be stimulated to transfer DNA (tDNA) from its tumour inducing (Ti) plasmid to the recipient fungal cell. The tDNA is transported into the host nucleus and integrates randomly into the host genome. The first transformation of *A. bisporus* (de Groot et al. 1998) used germinating spores and suffered from very low transformation efficiency, but efficiency can be increased by seven orders of magnitude using gill tissue of the fruiting body rather than basidiospores (Chen et al. 2000; Mikoscha et al. 2001).

ATMT has been used to silence genes by mutagenesis or targeted gene disruption. These approaches to gene silencing are predicted to be unsuccessful in *A. bisporus* because of their multinucleate heterokaryotic hyphae and basidiospores (Eastwood et al. 2008). RNA interference (RNAi) was first described in *Caenorhabditis elegans* by Fire et al. (1998). By injecting the cells with dsRNA, Fire et al. were able to selectively knockdown expression of the homologous endogenous gene. The dsRNA can also be formed when coding and complementary DNA sequences are introduced in tandem into the genome because the transcript forms a dsRNA with hairpin loop (hpRNA) (Eastwood et al. 2008). The dsRNA is a substrate for the RNase-III-like Dicer enzymes, which cuts the dsRNA into 21-25 nt fragments called silencing RNA

(siRNA). The siRNA is incorporated into the RNA-induced silencing complex (RISC) which contains an Argonaute protein with slicer activity. One strand of the siRNA is removed from the RISC complex, and the remaining strand will bind homologous endogenous mRNA. When the RISC complex binds mRNA it leads to mRNA degradation or translational repression resulting in silencing of the gene (Chang, Zhang & Liu, 2012). Originally, RNAi was thought to be a eukaryotic cell defense against RNA viruses, but studies have shown a role of the RNAi pathway in regulating endogenous gene expression, transposon silencing, programmed DNA elimination, resistance to viruses and other cellular, developmental and physiological pathways (Chang et al. 2012; Salame et al. 2011). The RNAi phenomenon is unique to eukaryotic cells and was first described in Kingdom Plantae in *Petunia hybrid* and termed co-suppression (Napoli, Lemieux & Jorgensen, 1990), Kingdom Fungi in *Neurospora crassa* and termed Quelling (Romano & Macino, 1992) and Kingdom Animalia in *Caenorhabditis elegans* and termed RNAi (Fire et al. 1998).

The two technologies, ATMT and RNAi, were combined in *A. bisporus* by Eastwood et al. (2008) who reported that the gene argininosuccinate lyase could be down-regulated in mycelia expressing an RNAi cassette that generated hpRNA. This chapter describes the development of RNA silencing cassette for laccase and transformation of the cassette into *A. bisporus* using ATMT. The purpose of generating hpRNA was to down-regulate laccase transcripts to further assess the relationship between *A. bisporus* laccase expression and toxicity of *T. aggressivum* metabolites. Krupke et al. (2003) also proposed that the methylisocoumarin produced by *T. aggressivum* should be susceptible to degradation by lactonases because of the presence of a lactone ring in the structure. Therefore, transgenic *A. bisporus* containing a lactonase gene from *Bacillus subtilis* was produced and transformant susceptibility to *T. aggressivum* toxins was assessed.

5.2. Methodology

5.2.1. Growth and manipulation of *A. tumefaciens*

5.2.1.1. Cultivation

Agrobacterium tumefaciens AGL-1 (obtained from DeLuca Lab – Brock University) culture was grown for 48 h at 28 °C on LB plates supplemented with 100µg/mL carbenicillin (LB +Carb). A single colony was selected and grown in 3 mL LB +Carb overnight and grown at 28 °C with shaking at 250 rpm until culture reached an OD_{600} of 0.4 – 0.6. Glycerol (15 %) was added to the *A. tumefaciens* AGL-1 culture and stored at -80 °C in 1 mL aliquots.

5.2.1.2 Competent cells

Ten µL of stock *A. tumefaciens* was inoculated into 5 mL LB +Carb and grown at 28 °C for 48 h with shaking at 250 rpm. One mL of the culture was added to 25 mL of fresh LB+Carb and shaken at 250 rpm until the OD_{600} reached 0.5 (about 5 h). The optimally growing culture was chilled on ice for 30 min and pelleted by centrifugation at 1500 x *g* for 5 min at 4 °C. The supernatant was removed and the cells resuspended in 1 mL of ice-cold 20 mM $CaCl_2$. The cells were dispensed in 100 µL aliquots, froze in liquid nitrogen and stored at -80 °C.

5.2.1.3 Transformation with binary vector

Competent cells were thawed on ice and diluted four-fold with distilled water before the addition of 200 ɳg of pDNA. The *A. tumefaciens* cell culture and pDNA suspension were added to an electroporation tube with an electrode distance of 2 mm (Fisherbrand). Electroporation was performed on the BioRad Gene Pulser by applying a single electric pulse of 2.5 kV using the 25 µF capacitor (Mattanovich et al. 1989). Following electroporation, 1 mL of LB was added to the cells and incubated overnight at 28 °C. A serial dilution was prepared and 100 µL of the 10^0, 10^1, and 10^2 dilutions were plated on LB supplemented with 100 µg/ml carbenicillin and 50 µg/mL chloramphenicol (LB +Carb +Chl) and incubated at 28 °C for 48 h. Transformants were

sub-cultured in LA+Carb +Chl broth for 48 h. Successful incorporation of the binary vector was confirmed by PCR.

5.2.2 Construction of binary vectors

Both the U1 and SB65 strains of *A. bisporus* were transformed with two constructs, a portion of the laccase gene with its complementary sequence which generates an hpRNA for knock-down of laccase expression and a lactonase gene from *Bacillus subtilis*.

5.2.2.1 Binary vector

The pFGC1008-GPD pro vector (Figure 43) contains several origins of replication, a chloramphenicol resistance gene (CHL) and the tDNA region contained between the left and right borders (LB & RB) (Abubaker, 2010). The tDNA sequence contains the hygromycin B resistance gene (HYG) under the control of the mannopine synthase (MAS) promoter and terminator, and a portion of the β-glucuronidase (GUS) sequence under the control of glyceraldehyde-3-phosphate dehydrogenase II (GPD) promoter and poly A tail terminator.
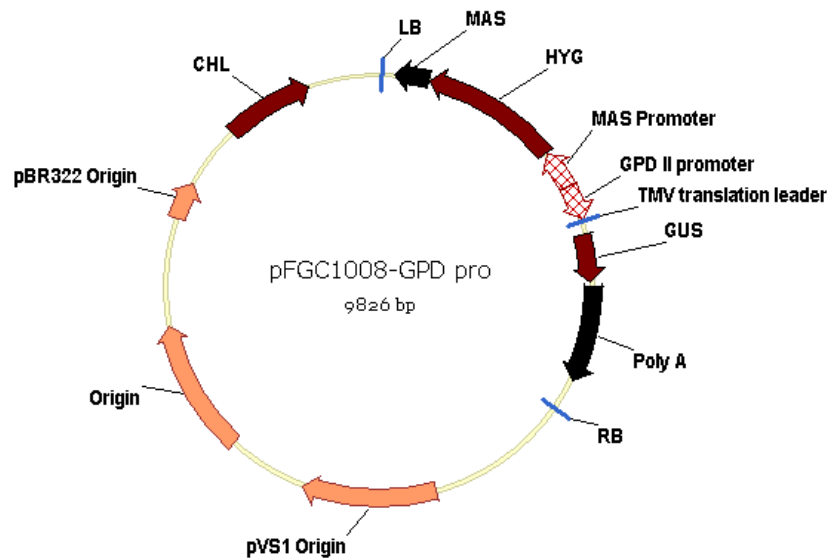
Figure 43: The pFGC1008-GPD pro binary vector used for silencing and transgene constructs to be transformed to *A. bisporus* via *A. tumefaciens*.

5.2.2.2 RNAi construct for knockdown of *A. bisporus'* laccase

The region within the tDNA on the binary vector that can be used to carry transgenes is shown in Figure 44. On either side of the GUS region are restriction sites that can be used to

RNAi bare construct
1481 bp
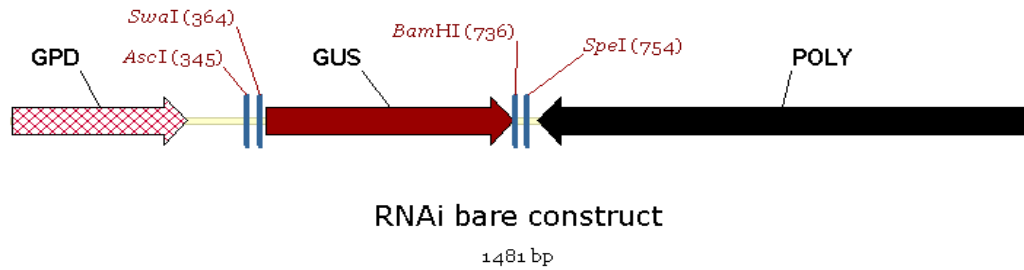
Figure 44: The region of the binary vector where the transgenes can be inserted. By using *Swa*I and *Asc*I restriction sites a DNA fragment can be inserted upstream of the GUS region, and using *Bam*HI and *Spe*I downstream of the GUS region.

insert foreign DNA. The appropriate restriction sides were also incorporated into PCR primers (Table 13) designed to amplify a region of the laccase gene. Digesting the plasmid and the PCR fragment with the same combination of restriction enzymes allows for the integration of the sense and antisense fragments in the correct orientation into the binary vector, as shown in Figure 45. The presence and orientation of the sense and antisense sequence were confirmed by

RNAiconstruct
2406 bp

Lactonase construct
1754 bp
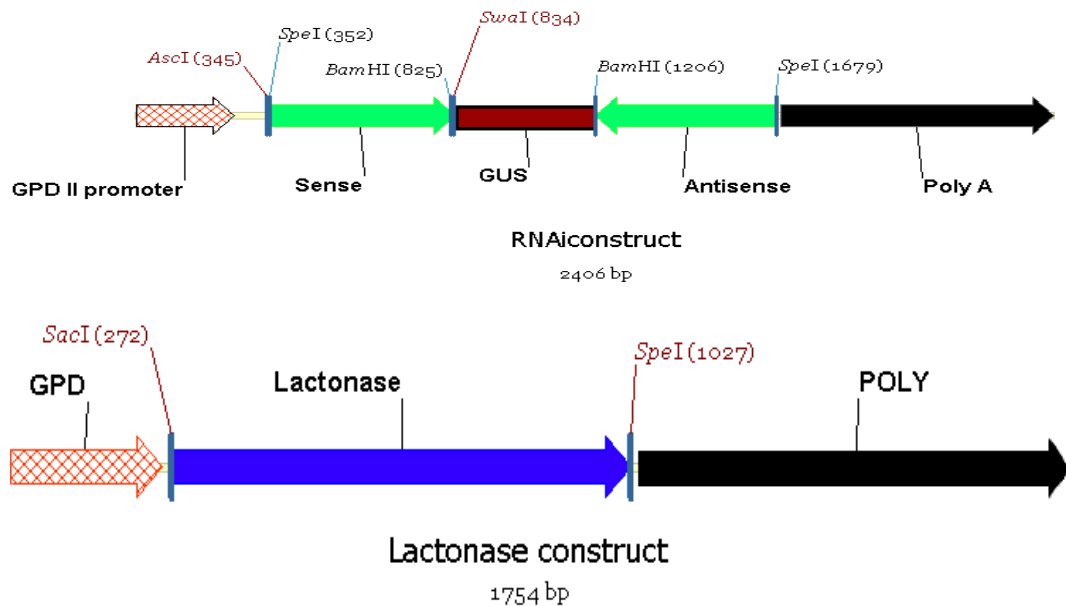
Figure 45: Construct for hpRNA knock down of laccase expression and lactonase expression in *A. bisporus*.

125

Table 13: PCR primers used for construction and confirmation of RNA silencing and lactonase cassettes. Melting temperatures were obtained using the online DNA calculator from Sigma-Genosys.

Primer	Sequence (5'-3')	Melting temp (°C)	Application
Lcc-RNAi-F	TCAGGCGCGCCACTAGTCGA-TAAGCACATCACCCTCAAC	85.6	Primers containing restriction sites for cloning sense and anti-sense sequence for laccase RNAi
Lcc-RNAi-R	CTCATTTAAATGGATCCCTTGA-GGAGTGATCTGAGACTGG	78.5	
GPD-F	AGCGTTGAGGATCTAGAGCTG	62.6	Primers specific for tDNA to test for the desired RNAi sequences
GUS-F	TCAGCAAGCGCACTTACAG	63.0	
GUS-R	TGTAAGTGCGCTTGCTGAG	63.0	
POLY-R	TCGGTGTGTCGTAGATACTAG	60.7	
Bac-LtonF	CAGAGCTCATGACAGTAAA-GAAGCTTTATTTCG	70.8	Primers containing restriction sites for cloning lactonase gene
Bac-LtonR	CGACTAGTCTATATATA-TTCCGGGAACGCTC	68.9	

PCR and sequencing. This construct was transformed into *A. tumefaciens* which was used to transform *A. bisporus*.

5.2.2.3 *B. subtilis* lactonase gene

The lactonase gene from *B. subtilis* was amplified by PCR, ligated to the pUC19 plasmid and cloned into *E. coli*. The primers were engineered with restriction sites (as shown in Table 13) for subsequent integration into the *A. tumefaciens* binary vector. The lactonase gene and the binary vector were digested with *Sac*I and *Spe*I, separated by agarose gel electrophoresis, and the corresponding band was isolated from the gel before ligation to generate the construct shown in Figure 45. The presence and orientation of the gene were confirmed by PCR and sequencing. This construct was transformed into *A. tumefaciens* which was the used to transform *A. bisporus*.

5.2.2.4 Sequencing

The *Bacillus* lactonase gene construct was sequenced by York University with BigDye Terminator chemistry on the Applied Biosystems 3130xL DNA Sequencer. The RNAi constructs were sequenced at The Centre for Applied Genomics at the Hospital for Sick Children, Toronto

using the Illumina HiSeq 2000 and HiScan-SQ, Applied Biosystems SOLiD 5500xl and Roche 454 GS FLX Titanium systems.

5.2.3 ATMT of *A. bisporus*

5.2.3.1 *A. tumefaciens* starvation

A. tumefaciens harbouring the binary vector was grown in LB+Carb, Chl at 28 °C for 48 h. One mL of the actively growing culture was inoculated into 50 mL of AB-sucrose minimal medium (MM +Carb +Chl) (20mM K_2HPO_4, 10 Mm NaH_2PO_4, 20 mM NH_4Cl, 1 mM $MgSO_4 \cdot 7H_2O$, 2 mM KCl, 65 µM $CaCl_2$, 1µM $FeSO_4 \cdot 7H_2O$, 0.5% sucrose) and grown overnight at 28 °C with shaking at 250 rpm until the OD_{600} reached 0.5.

5.2.3.2 Induction of *A. tumefaciens* virulence genes

The starved *A. tumefaciens* cells were pelleted by centrifugation at 2500 x *g* for 5 min and resuspended in 10 mL of induction medium (20 mM NH_4Cl, 1 mM $MgSO_4 \cdot 7H_2O$, 2 mM KCl, 65 µM $CaCl_2$, 1µM $FeSO_4 \cdot 7H_2O$, 2mM $NaPO_4$, 50 mM MES pH 5.6, 0.5% glucose, 200 µM acetosyringone). The cells were grown overnight at 28 °C with gentle shaking at 100 rpm. The cells were pelleted again, and resuspended in 15 mL of CYM.

5.2.3.3 Preparation and transformation of *Agaricus*

Different transformation protocols of *A. bisporus* in literature shows varying transformation efficiencies depending on the growth stage of *A. bisporus*. This project used three different methods for ATMT of *A. bisporus*, two with vegetative hyphae and one with gill tissue from the fruiting body.

In the first method, the medium from an *A. bisporus* liquid culture plate was removed and the mycelium was suspended in 50 mL of fresh CYM medium. The culture was homogenized with a Sorvall Omni-mixer, divided into two equal volumes in sterile flasks and the final volume brought to 50 mL with CYM. The flasks were incubated at RT for 7 d until transformation. The one week culture was centrifuged in a 50 mL falcon tube for 5 min at 2500 x *g* and the

supernatant removed. Fifteen millilitres of the induced *A. tumefaciens* suspension was added to the *A. bisporus* pellet, and the co-cultivation mix was poured into a sterile petri dish and incubated at RT for 72 h.

The second method involved the same 7-d *A. bisporus* culture, followed by a modified transformation. One ball of mycelium (approx. 1-3 mm in diameter) was transferred to a microfuge tube along with 15 µL of the induced *A. tumefaciens* suspension. The tubes were pulse microfuged to pellet the *A. tumefaciens* on the fungal mycelium, the supernatant was removed, and the mycelium coated with *A. tumefaciens* was placed on a black filter paper on CYM plates. These plates were incubated at RT for 72 h.

Transformation of vegetative tissue was very low, so the third method used fruiting body tissue rather than vegetative hyphae. Off-white (to represent U1) and crimini mushrooms (to represent SB65) with unopened caps were purchased and sterilized by dipping in 5% sodium hypochlorite (Clorox bleach), followed by washing in dH_20 and dipping in 70% EtOH. The alcohol was allowed to evaporate before mushrooms were dissected.   The mushrooms were dissected with a sterile scalpel, and thin layers of gill tissue were removed. These gill tissue explants were placed into a sterile petri dish, covered with the induced *A. tumefaciens* suspension and incubated at RT for 72 h.

5.2.3.4   Elimination of *A. tumefaciens*

After 72 h, all *A. bisporus* cultures were transferred aseptically to CYM + Carb +Cefo (100 µg/mL carbenicillin, 200 µg/mL cefotaxime) plates to eliminate *A. tumefaciens.* The plates were incubated at RT for 7 d.

5.2.3.5   Selection of transformants

After 7 d, all the growing *A. bisporus* cultures were transferred to a fresh CYM +Hyg plate (25 µg/mL hygromycin) and incubated for 21 d. Cultures growing on the selective medium identified were selected as putative transformants and transferred to fresh CYM +Hyg plate (50

μg/mL hygromycin) and incubated for another 21 d. Finally, viable colonies were transferred to non-selective CYM plates, and the presence of the transgenes was confirmed by PCR.

5.2.4. Analysis of transformants

5.2.4.1. Laccase expression

A. bisporus transformants containing laccase RNAi cassette colonized CYM media for 20 days before addition of *T. aggressivum* toxic metabolites (section 2.2.4). After four day incubation with metabolites, *A. bisporus* mycelium was harvested. Extracted mRNA was used as template to generate cDNA, and *lcc*1 and *lcc*2 transcript abundance was measured by qPCR (2.2.5). Laccase expression was compared to untransformed white (WU) and brown mushrooms (BU) handled the same as transformants.

5.2.4.2. Toxicity assay

A. bisporus transformants containing laccase RNAi cassette and lactonase cassette were analyzed for sensitivity to *T. aggressivum* secondary metabolites as described in section 2.2.3.2.

5.3. RESULTS AND DISCUSSION

5.3.1. RNA silencing of laccase genes

5.3.1.1 Generation of construct

The RNA silencing construct was based on a region that is homologous in both *lcc*1 and *lcc*2 in both U1 and SB65 (Appendix VI – Figure A3). The primers also contained two restriction enzyme cut sites at the 5' end to aid in specific orientation when ligating into the plasmid. Using cDNA as a template to circumvent introns, an amplicon of 467 bp was generated (Figure 46). The different PCR reactions included RNA without the reverse transcription step, to assess genomic DNA contamination of the RT-PCR reaction, cDNA as the template for the target sequence, and genomic DNA to verify the size of the target is smaller than gDNA because of intron exclusion. The PCR product was cloned into
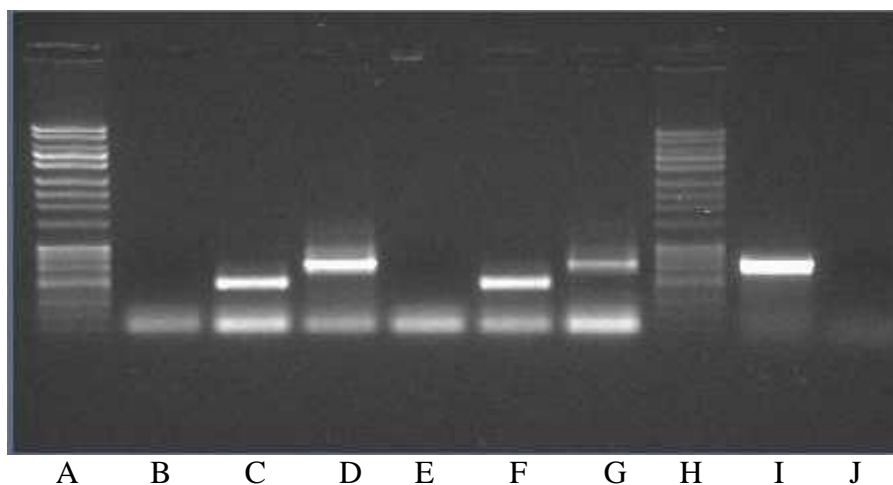
Figure 46: Agarose gel showing amplification of DNA to be used as sense and antisense sequence in RNA silencing. Norgen's Highranger plus ladder (A & H); RNA template (B & E); cDNA template (C & F); gDNA template (D & G); ITS +ve control (I); NTC (J).

pUC 19 and confirmed by restriction digestion (data not shown). To confirm that the identity of the pFGC1008-GPD pro binary vector, we digested with specific enzymes to determine correct size fragments (Figure 47). The size of pFGC1008-GPD is 9826 bp and contains one *Bam*H1 site, which corresponds to the single band just under 10000 bp in lane C. The plasmid has 4 *Hinc*II sites, which will yield four bands in the gel with sizes of 1402, 1799, 1911, and 4889 bp (lane D). The pFGC1008-GPD and the laccase reverse (antisense) sequence in pUC 19 were cut with *Spe*1and *Bam*H1, ligated together and transformed into *E. coli*. The presence of the antisense sequence was confirmed by PCR using the GusF and PolyR primers found in the plasmid backbone (Figure 48).
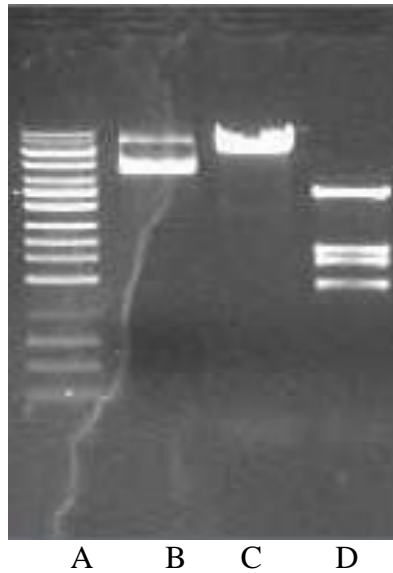
Figure 47: The identity of binary vector pFGC1008-GPD was confirmed by restriction analysis and visualizing fragments by agarose gel electrophoresis. Highranger ladder (A); uncut binary vector (B); binary vector digested with *Bam*H1 (C); and *Hin*cII (D).
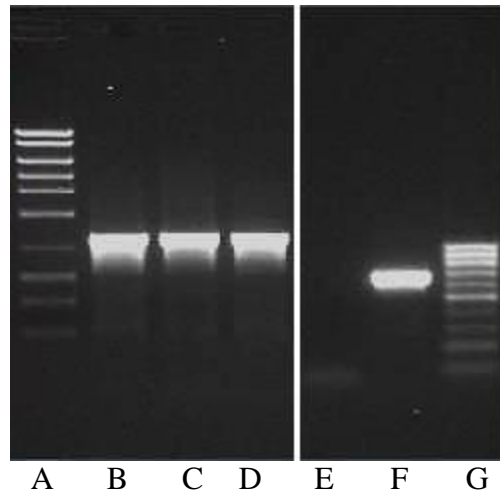
Figure 48: Agarose gel showing PCR amplification of binary vector pFGC1008-GPD with laccase antisense sequence. Midranger ladder (A); successful ligation of antisense sequence (B-D); NTC (E); vector without the insert (F); PCRsizer ladder (G).

The PCR products were digested with four restriction enzymes, *Eco*R1, *Eco*RV, *Hin*cII, and *Sac*I which yielded fragments of the predicted sizes indicating the presence and correct orientation of the laccase antisense sequence (Figure 49).
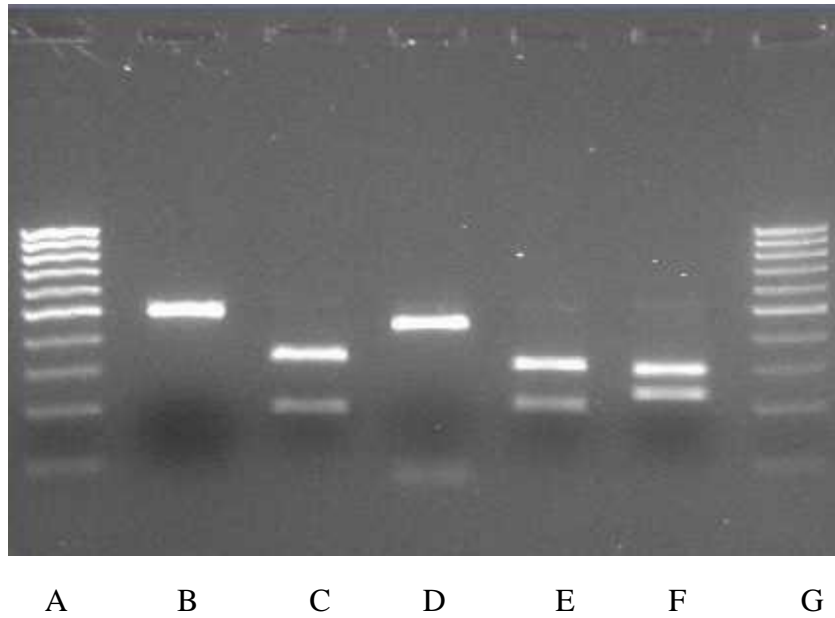
Figure 49: Restriction enzyme confirmation of PCR amplification of laccase antisense sequence within binary vector. Midranger ladder (A & G); uncut PCR fragment (B); PCR fragment digested with *Eco*R1 (C); *Eco*RV (D); *Hin*cII (E); and *Sac*I (F).
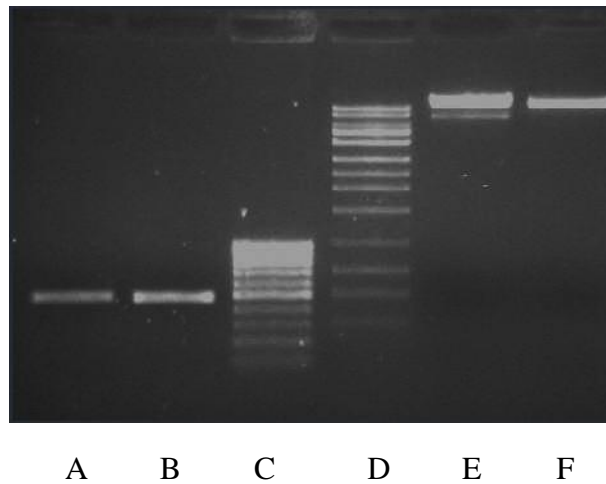
Figure 50: Agarose gel showing digestion of binary vector and laccase sense sequence with *Asc*1 and *Sac*1. Sense sequence (A & B); pFGC1008-GPD containing antisense sequence (E & F); PCRsizer ladder (C); and Highranger (D).

The laccase construct originally isolated using PCR (Figure 46) and the pFGC1008-GPD

plasmid bearing the antisense sequence were cut with the other restriction enzymes, *Asc*1 and

132

*Sac*1, contained on the primers (Figure 50). The laccase sense sequence and linear binary vector were ligated together and cloned into *E. coli*. The plasmid was re-isolated and the incorporation of sense sequence was confirmed by PCR (Figure 51). Table 14 shows which plasmids and primers were used in the PCR reaction, and the expected size of the PCR product. The RNAi construct 3 (lanes F and K in Figure 51) was sequenced to verify that the sense and antisense sequences had been inserted in the correct orientation and this construct was used in the transformation of *A. bisporus*.

5.3.1.2.   Transformation

The RNAi construct was transformed into *A. tumefaciens* by electroporation and transformants were selected by growth in the presence of chloramphenicol. Transformation of the t-DNA into *A. bisporus* was performed in three ways: liquid co-cultivation with vegetative *A. bisporus*, co-cultivation by centrifugation of *A. tumefaciens* with vegetative *A. bisporus* and cultured on solid agar, and liquid co-cultivation with fruiting body tissue of *A. bisporus.* All transformations were followed by selection on CYM plates with 25 µg/mL hygromycin B. Transformation using vegetative tissue was unsuccessful, resulting in zero putative transformants. Transformation using fruiting body tissue was successful in 12.5 % of off-white mushroom gill tissue slices, and 7.5% of brown mushroom gill tissue slices. The transformation efficiency of this experiment is lower than the $30 - 40\%$ efficiency reported by Chen et al (2000) and the 95% efficiency reported by Romaine & Schlagnhaufer (2006) likely due to oxidation of acetosyringone resulting from using a stock solution stored in ethanol (Romaine & Schlagnhaufer (2006). However, these results confirm that the use of gill tissue is superior to vegetative tissue for A. bisporus transformation (Chen et al. 2000; Mikoscha et al. 2001).
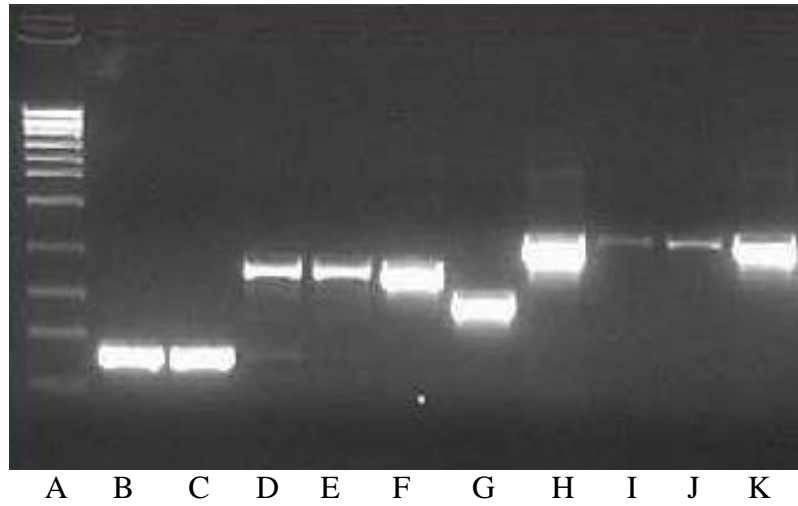
Figure 51: Agarose gel showing PCR confirmations of final RNAi construct. The contents of each lane are described in Table 14.

Table 14: Primers and plasmids used in each PCR reaction (Figure 51) to confirm the desired structure of the RNAi construct (Figure 52)

Lane Letter	Primers	Plasmid	Size (bp)
A	- - - - - NORGEN's MidRanger Ladder - - - - -		
B	GPD-F & GUS-R	pFGC1008-GPD	402
C	"	pFGC1008-GPD+antisense	402
D	"	RNAi construct 1	897
E	"	RNAi construct 2	897
F	"	RNAi construct 3	897
G	GUS-F & POLY-R	pFGC1008-GPD	667
H	"	pFGC1008-GPD+antisense	1146
I	"	RNAi construct 1	1146
J	"	RNAi construct 2	1146
K	"	RNAi construct 3	1146
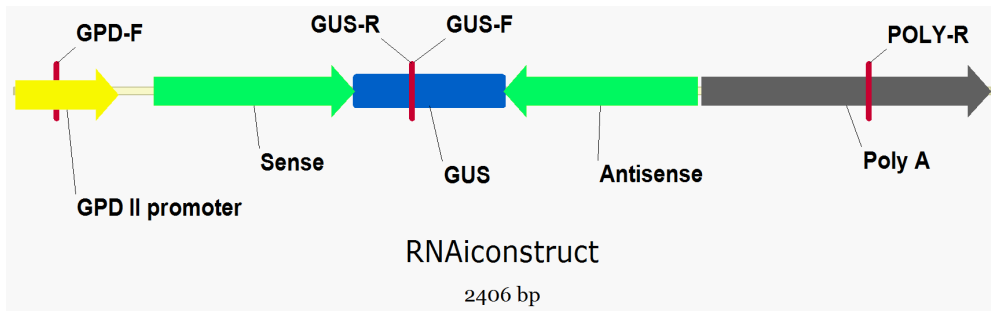
Figure 52: Representation of RNAi construct demonstrating the position of the primers (Table 14) and the size of the pCR products (Figure 51). Primers are indicated by red line perpendicular to the construct.

134

Transformants were transferred to plates containing 50 µg/mL hygromycin B, where the

growth rate diminished noticeably. Colonies that displayed hygromycin resistance were

transferred to non-selective CYM plates where growth rate returned to normal, indicating that

slow growth was due to hygromycin inhibition rather than inclusion of the cassette. DNA was

extracted from all putative transformants, and the presence of the transgene was verified by PCR

(Figure 53). Three different colonies of SB65 that were hygromycin resistant were recovered and

all contained the RNA silencing cassette as confirmed by PCR. Five colonies of U1 were

obtained and four had the RNAi cassette. One transformant of the white strain did not contain

the RNAi construct, and may have resulted from an unstable integration of the RNAi cassette.
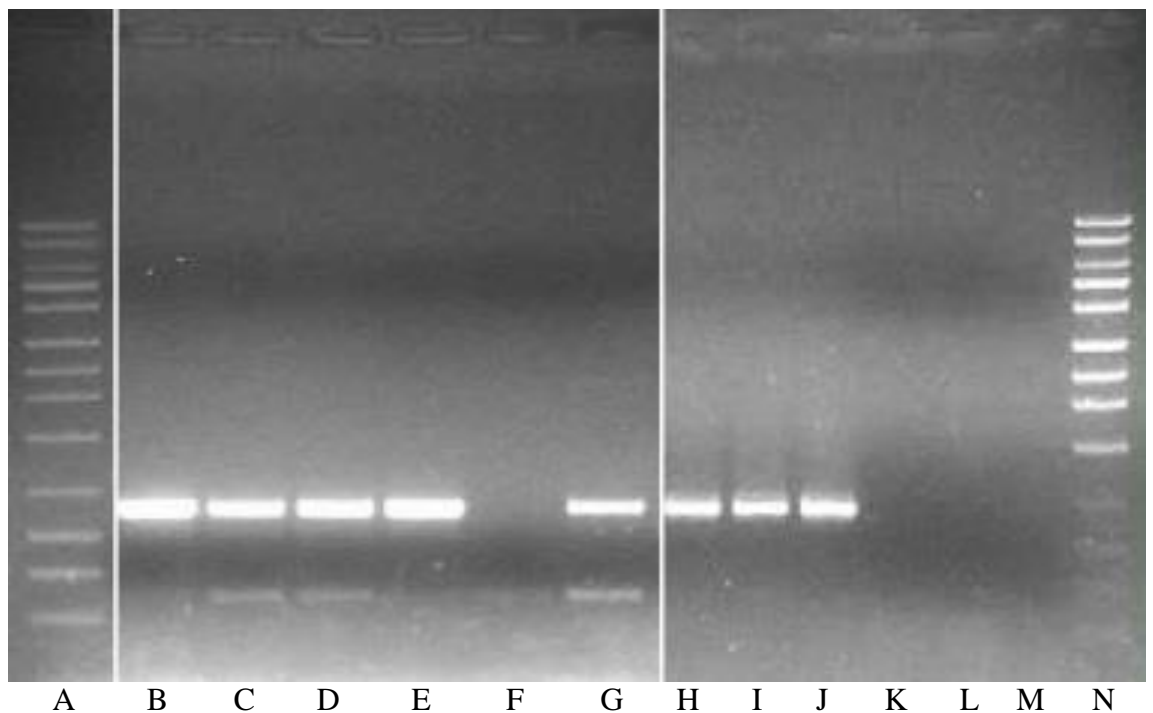
Figure 53: Agarose gels of PCR products demonstrating successful transformation of *A. bisporus* with laccase RNAi construct. HighRanger ladder (A & N); positive control (B); transformed off-white (C-G); transformed brown (H-J), untransformed white (K); untransformed brown (L); NTC (M).

5.3.1.3 Laccase expression

The cultures obtained from off-white and brown mushrooms were handled the same as the

day 4 U1 and SB65 cultures described in section 2.3.2.4. The *lcc*1 and *lcc*2 transcript abundance

of the untransformed off-white (WU) and brown mushroom (BU) is very similar to abundance

135

described for U1 and SB65 (Figures 23 & 24: Day 4, with metabolites). The RNAi constructs

had varying effects on *lcc* transcript levels (Figure 54). Two of the brown strain transformants

(B6 and B21) had significant increases in expression of both laccase genes, while B12 was

similar to the untransformed control. In the U1 background, transformant W1 displayed efficient

knockdown of both *lcc*1 and *lcc*2, W32 and W34 had slightly reduced *lcc*2 expression, and W9

showed no difference from the untransformed control. Variable expression in RNAi

transformants including up-regulation is routinely observed in shRNA transformations (Costa et

al. 2009; Eastwood et al. 2008; Bailey et al. 2013). The reason for up-regulation of target genes

is not clear, but over expression can be just as useful as a knockdown in determining the function

of a gene.
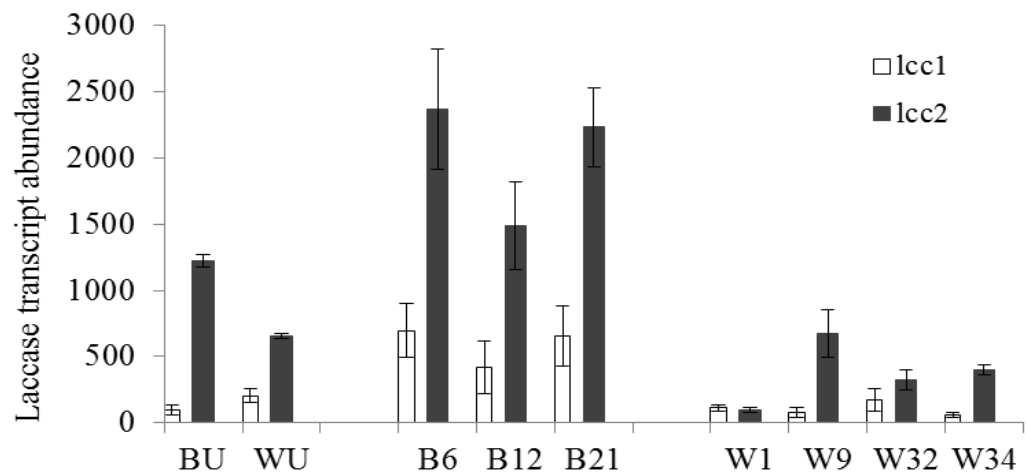
Figure 54: Number of *lcc*1 and *lcc*2 transcripts measured by q-RT-PCR
in brown and white strains of mushroom untransformed (BU & WU) and
transformed with the construct for RNA silencing in liquid culture
containing toxic metabolites from *T. aggressivum.*

The use of antisense mediated silencing has been shown to be slightly more effective at

silencing than hpRNA (87.5% and 55% silencing respectively) as well as mush easier to

construct (Heneghan et al. 2007).

5.3.1.4   Toxicity of *T. aggressivum* metabolites

To determine if a difference in sensitivity to the *T. aggressivum* secondary metabolites

exists between *A. bisporus* displaying different levels of laccase transcripts, the toxicity was

measured on all transformants. A mycelia lawn for each transformant had a filter paper disk containing *T. aggressivum* secondary metabolites in the center. The size of the inhibition zone was determined every ten days for 30 d. (Figure 55).
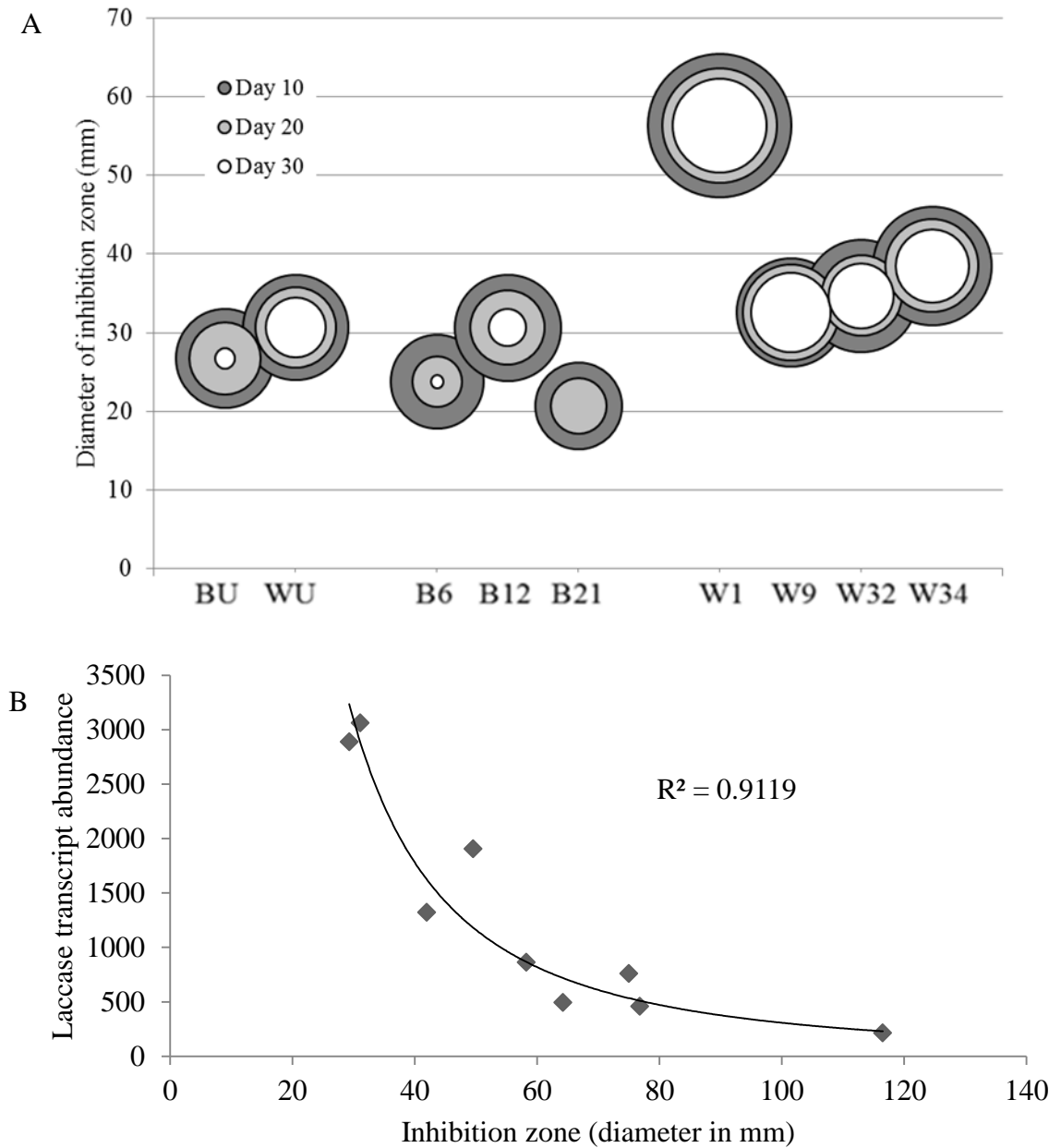
Figure 55 A: Inhibition zones of *A. bisporus* off-white and brown strains of mushroom controls (BU & WU) and those containing the RNA knockdown construct resulting from the presence of toxic secondary metabolites from *T. aggressivum*. B: Sum of inhibition zones over time course (day 10 + day 20 + day 30) displays an exponential correlation to total laccase transcript abundance (*lcc*1 + *lcc*2).

The level of laccase transcription had an inverse, exponential correlation with the

sensitivity of the transformed *A. bisporus* to *T. aggressivum* metabolites. The strains B6 and B21

that overexpressed the laccase genes were more resistant to the toxin than untransformed SB65,

and strain W1 with very low number of laccase transcripts was more sensitive to the toxin than

the progenitor U1. The strains with laccase expression similar to the controls (B12 and W9) had

toxin sensitivity that was similar to the controls. As the quantity of laccase enzyme drops, the

rate at which peptides can dimerize to form functional laccase would decrease dramatically.

Therefore, it is logical that the ratio of laccase transcript abundance to sensitivity to metabolites

would be greater than linear (Figure 55B). The correlation of increased laccase transcription and

increased resistance to *T. aggressivum* toxins provided further evidence for a role of laccase in

commercial brown strain resistance to green mold disease.

5.3.2 Lactonase

In 2003, Krupke et al. identified one of the toxic compounds produced by *T. aggressivum*

as 3,4-dihydro-8-hydroxy-3-methylisocoumarin and the authors speculated that the other,

unidentified toxic metabolite may have a similar structure. This research, in part, led to

registration in Europe of Serenade®, which includes the lactonase producing *Bacillus subtilis* as

an active ingredient, for control of green mold disease (Largenteau and Savoie, 2010). U1 and

SB65 were transformed with a lactonase gene from *B. subtilis* to determine if lactonase could

confer resistance to *T. aggressivum* secondary metabolites.

5.3.2.1 Construct

The lactonase gene was amplified from *B. subtilis* by PCR, and visualization of the PCR product

showed a specific amplicon around 700 bp, the appropriate size for the lactonase gene (Figure

56). The fragment was excised from the gel, ligated into pUC-19 and cloned into *E. coli*.

Successful transformants were selected based on white or blue screening, the plasmid was

isolated and presence of lactonase gene confirmed by PCR (Figure 57). The lactonase gene was

removed from pUC-19 with the *Spe*1 and *Sac*1 restriction enzymes and ligated into pFGC1008-

GPD linearized with the same restriction enzymes. The binary vector was transformed into *E.*

coli and selected for by chloramphenicol resistance. The binary vector was isolated from *E. coli*

and sequenced to confirm the presence and orientation of the lactonase gene within the plasmid

FGC1008-GPD.
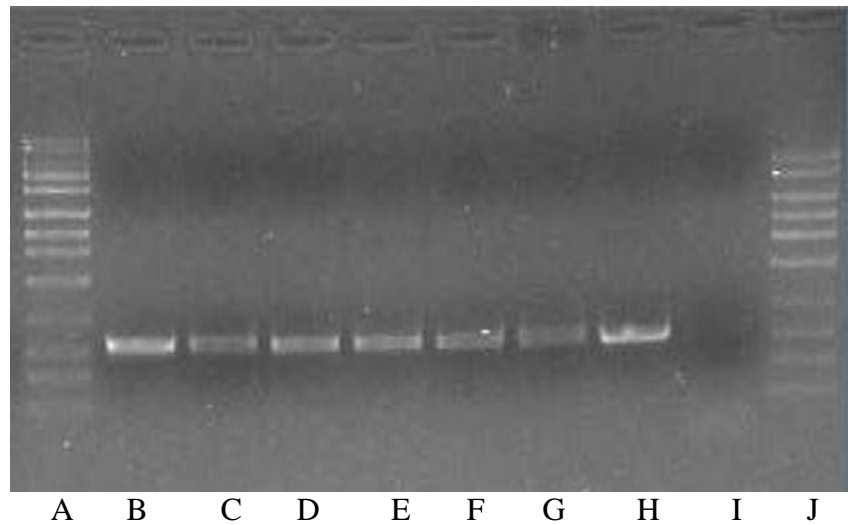
Figure 56: Agarose gel showing amplification of lactonase gene from *B. subtilis*. Highranger ladder (A); lactonase gene (B-H); NTC (I); Midranger ladder (J).
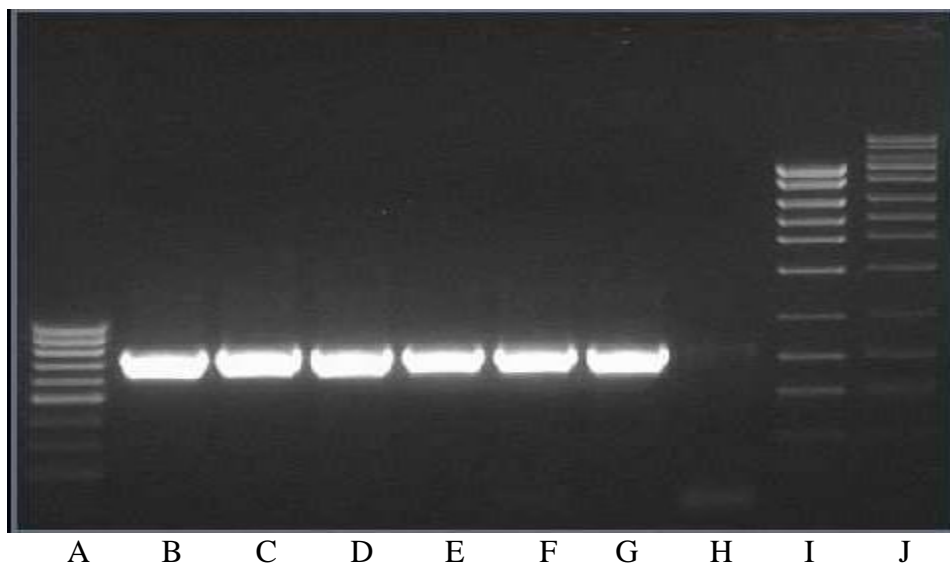
Figure 57: Agarose gel of PCR products confirming the successful cloning of the lactonase gene in pUC19. PCRsizer ladder (A); lactonase clones (B-G); NTC (H); HighRanger (I); UltraRanger (J).

139

5.3.2.2 Transformation

The binary vector was transformed into *A. tumefaciens* by electroporation and the tDNA

containing the lactonase gene was transformed into *A. bisporus* by ATMT. Transformation

using vegetative tissue was extremely unsuccessful, only yielding one transformant (0.28%)

(data not shown). Transformation using fruiting body tissue was successful in 12.5 % of off-

white mushroom gill tissue slices, and 10% of brown mushroom gill tissue slices. We recovered

four SB65 and five U1 colonies that were hygromycin B resistant and all contained the lactonase

gene as confirmed by PCR (Figure 58).
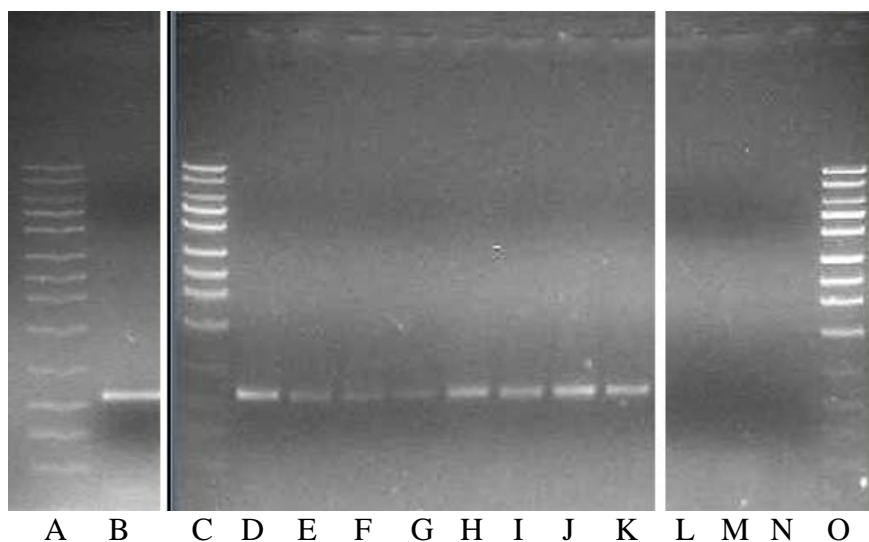
Figure 58: The presence of the lactonase transgene in the *A. bisporus'*
genome was confirmed by PCR. Highranger ladder (A, C, O);
transformed white (B, D-G); transformed brown (H-K); untransformed
white (L); untransformed brown (M); NTC (N).

5.3.2.3 Toxicity of *T. aggressivum* metabolites

A. bisporus transformed with lactonase were exposed to *T. aggressivum* secondary

metabolites to determine whether the *B. subtilis* lactonase gene can increase resistance to the

toxins (Figure 59). The toxicity test shows that all transformants are more sensitive or similar

sensitivity to *T. aggressivum* metabolites than the control. There are several reasons why the

lactonase transformants may not have increased *A. bisporus* resistance to *T. aggressivum*

metabolites. First, this experiment only tested for the presence of the transgene in the genome, it

did not look at transgene transcriptional expression. The transgene may have integrated into a

transcriptional inactive region of the genome and not been transcribed. This is unlikely, because

we generated multiple transformants and expect that some would integrate elsewhere in the
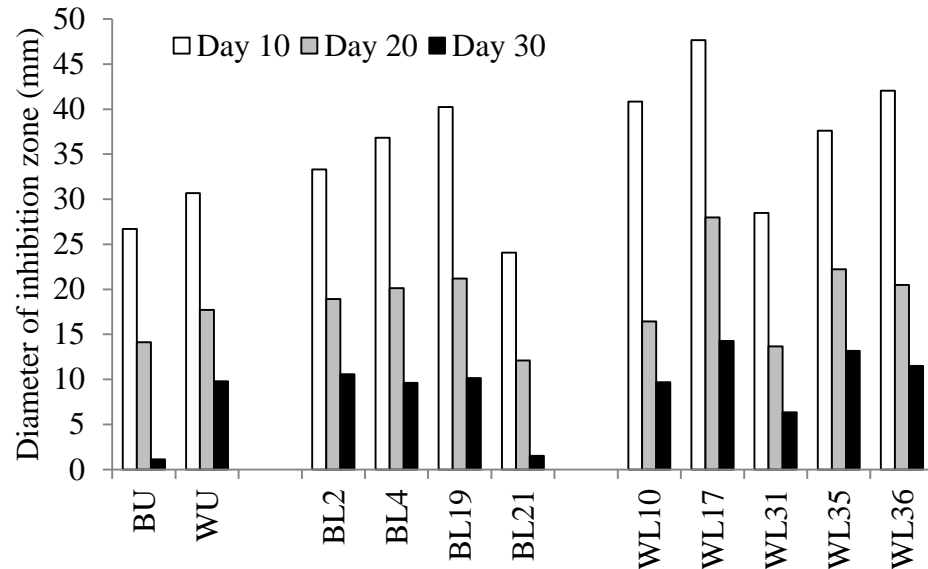
Figure 59: Inhibition zones from the presence of toxic secondary
metabolites from *T. aggressivum* in untransformed *A. bisporus* brown
and off-white strains (BU & WU) and those transformed with the
lactonase transgene (BL and WL).

genome. In addition, the gene is under the control of the strong GPDII promoter, and will likely

be transcribed. Secondly, we did not look at lactonase enzyme activity. Not every mRNA gets

translated into an active, functional protein, especially from a prokaryotic gene being expressed

in a eukaryotic cell. Chen et al. (2000) transformed a GFP gene into the *A. bisporus* genome,

confirmed its presence by DNA-DNA hybridization but were unable to detect GFP fluorescence

in any of their transformants. Burns et al. (2005) went on to show that efficient expression of

GFP in *A. bisporus* requires an intron. Our lactonase construct was amplified directly from *B.*

subtilis and does not have an intron, which may indicate that the lactonase mRNA is not being

translated. Lastly, the methylisocoumarin and other secondary metabolites may not be a

substrate for *B. subtilis* lactonase. Wang et al. (2004) looked at substrate spectrum of *Bacillus* lactonase and found they are highly specific to acyl homoserine lactones. Lactonase demonstrated over 83% activity against all the acyl homoserine lactones tested but among the non-acyl lactones, lactonase activity was only 1.85-6.72%. The authors suggested that the amide group and the ketone at C1 position in the acyl chain are important for enzyme-substrate interactions. The ability of lactonase to metabolize a methylisocoumarin has not been reported, but it is reasonable to expect activity to be low given the specificity of lactonase to the acyl homoserine structure.

The activity of *Bacillus* spp. lactonase against the lactonase moiety in the methylisocoumarin and the second unidentified toxic compound produced by *T. aggressivum* (Krupke et al. 2003) needs to be investigated. The ability to produce lactonase, combined with a general ability to inhibit the *T. aggressivum* (Savoie et al. 2001b) has led to the testing and approval of the Serenade ® fungicide on French mushroom farms (Largeteau & Savoie, 2010). The active ingredient in Serenade ® is *Bacillus subtilis* and its lipopeptides. Unless lactonase shows a unique ability (beyond that described by Wang et al. 2004) to degrade the toxins produced by *T. aggressivum;* the effectiveness of this fungicide likely has little to do with lactonase production.

5.4. Summary

This is one of only a few reports of using ATMT to deliver an RNAi cassette to study gene function in *A. bisporus*. *A. bisporus* was transformed with a cassette that would generate hpRNA, a substrate of the RNA silencing pathway. The effect of the cassette varied dramatically between the different transformants, but one conclusion can be obtained: increased laccase transcription correlates with increased resistance to *T. aggressivum* metabolites. These results provide further evidence that increased laccase transcription and activity is a defense mechanism used by both U1 and SB65 during exposure to *T. aggressivum* metabolites, and that

increased laccase production by the commercial brown strains may contribute to increase resistance to green mold disease.

Anticipating that the lactone ring in the methyl-isocoumarin would be a substrate of lactonase, *A. bisporus* was transformed with a lactonase gene from *B. subtilis*. However, all transformants had similar or increased sensitivity to *T. aggressivum* secondary metabolites. Further experiments are required to determine whether lactonase is transcribed, translated and able to metabolize the secondary metabolites.

General Conclusion

Trichoderma species inhibit the growth of other fungi using a combination of techniques including directed growth towards the host followed by coiling around and penetration of host hyphae, secretion of cell wall degrading enzymes and production of toxic secondary metabolites. Direct hyphae interactions, including penetration of host fungi, have not been observed in the *T. aggressivum - A. bisporus* interaction. Production of *T. aggressivum* hydrolytic enzymes and toxicity of extracellular components are greater towards the off-white strains of *A. bisporus* than the commercial brown strains, consistent with the brown strains increased resistance to green mold disease (Abubaker et al. 2013; Krupke et al. 2003).

This present work indicates that the U1 and SB65 strains of *A. bisporus* increase laccase production *in vitro* as a defense against toxic *T. aggressivum* metabolites. This defense mechanism is enhanced in the commercial brown strain, SB65, which may contribute to its increased resistance to green mold disease.

T. aggressivum organic extracellular components were significantly more toxic to the U1 off-white hybrid strain than the SB65 commercial brown strain of *A. bisporus*. Without the *T. aggressivum* mycelium and continual production of metabolites and hydrolytic enzymes, both *A. bisporus* strains were able to overcome the toxicity and grow in the inhibition zone. The formation of new colonies within the inhibition zone indicates that the toxins are fungistatic rather than fungicidal. The reduction of toxicity enabling *A. bisporus* to grow in the inhibition zone may result from toxin volatility or degradation through environmental or enzymatic mechanisms. Since volatility and environmental breakdown would occur at a similar rate in both strains of *A. bisporus*, the different growth rates in the inhibition zone implicates enzymatic breakdown of *T. aggressivum* secondary metabolites. Phenolic oxidizers including laccases and peroxidases are likely candidates for the enzymatic breakdown of methyl-isocoumarin

compounds, one of the secondary metabolites produced by *T. aggressivum* and toxic to the growth of *A. bisporus* (Krupke et al. 2003).

In solitary *A. bisporus* cultures, manganese peroxidase transcription and laccase transcription and activity increase minimally with time, likely proportional to an increase in fungal biomass and utilization of complex substrates. The addition of *T. aggressivum* toxic metabolites has significant transcription and post translational effects on specific *A. bisporus* hydrolytic enzymes. There was no effect on *MnP* or *lcc*1 transcription, indicating that these two genes likely do not play a direct role in resistance of the commercial brown strains to the *T. aggressivum* toxins. In contrast, laccase 2 behaves in a way consistent with what might be expected for an enzyme involved in a defense response to a known toxin of *Trichoderma* for several reasons. First, both *lcc*2 transcript abundance and LCC activity is higher in day 0 in the SB65 strain than U1 strain. In addition, *lcc*2 transcript abundance increases significantly in both *A. bisporus* strains, after 1 day in SB65 and 3 days in U1, and LCC activity increases in both strains by day 2. The resulting increase in *lcc*2 transcription and LCC activity is always to greater levels in the SB65 strain that the U1 strain. Furthermore, alteration of laccase transcription by RNAi resulted in transformants with variable levels of laccase transcript abundance. Those with a low number of laccase transcripts were very sensitive to *T. aggressivum* toxin, while those with a high number of laccase transcripts had increased resistance. The addition of the reducing agent glutathione, which likely reduces radical phenolic products generated by laccase back into the toxin phenolic metabolite, results in an increase in the toxicity of *T. aggressivum* metabolites to both U1 and SB65. Finally, when exogenous laccase with common laccase mediators, p-coumaric acid or acetosyringone, is incubated with the *T. aggressivum* metabolites the toxicity of the metabolites is reduced making the medium more hospitable to the growth of *A. bisporus*.

To explain the difference in laccase expression between the SB65 and U1 strains of *A. bisporus* in solitary culture and in the presence of *T. aggressivum* secondary metabolites, the sequences of *lcc*1 and *lcc*2 with 5`regulatory elements were determined from both strains. The sequences were very well conserved between the two strains. Due to lack of any apparent significant difference in the regulatory and coding sequences, the difference in laccase transcription and activity between the U1 and SB65 strains is unlikely a result of sequence differences. It may be a result of an undefined promoter element or transcription factor, from regulatory elements further upstream, or from factors that function earlier in the *lcc*2 regulatory pathway.

Increased laccase activity has been shown to be a general defense response of other basidiomycetes including *Lentinula edodes* (Savoie & Mata, 1999) and *Trametes versicolor* (Freitag & Morrell, 1992; Baldrian, 2004) resulting from interactions with different *Trichoderma* species. Increased laccase activity has also been shown to be a general response to stressful conditions including biotic and abiotic stresses (Baldrian, 2004). To determine if laccase expression is a general stress response to the presence of *Trichoderma* toxins, or an active defense enzyme produced by *A. bisporus* to reduce toxicity of the metabolites requires further investigation.

The timing of several processes described in this thesis occurs at similar periods in the production of commercial mushrooms. *A. bisporus* and *T. aggressivum* mycelium usually colonize compost together without any major effect on the competing fungus. However, shortly before *A. bisporus* initiates the development of fruiting bodies, *T. aggressivum* produces copious number of spores and the growth of *A. bisporus* is inhibited resulting in a significant loss in mushroom production. Production of *T. aggressivum* secondary metabolites often coincides with conidiation, and these metabolites are fungistatic to *A. bisporus*. Finally, *A. bisporus* laccase

production decreases rapidly following initiation of fruiting bodies. This project has shown that laccase is produced in response to *T. aggressivum* metabolites, and that a decrease in laccase activity results in increased sensitivity to these metabolites. If increased production of laccase confers resistance to toxic metabolites produced by *T. aggressivum*, the developmental down-regulation of laccase renders *A. bisporus* sensitive to the metabolites. A strategy of selecting strains, or using conditions that result in an increase, or at least maintenance, of laccase production and activity, especially during fruiting, may reduce symptoms of green mold disease.

This thesis is the first to describe a potential defence mechanism employed by *A. bisporus*, especially commercial brown strains, against green mold disease caused by *T. aggressivum*. While several studies have looked at interactions between the U1 strain of *A. bisporus* and their pathogens, none have included the commercial brown strains. This is astonishing considering that the commercial brown strains contain innate mechanisms for reducing symptoms of green mold disease and other pathogens. These mechanisms should be determined, described and utilized in the mushroom industry to reduce disease symptoms while alleviating the need for fungicides.

References

Abah, S.E., Abah, G. 2010. Antimicrobial and antioxidant potentials of *Agaricus bisporus*. Advances in Biological Research 4:277-282.

Abubaker, K.S. 2010. Cell wall degrading enzymes and interactions between *Trichoderma aggressivum* and *Agaricus bisporus*. Unpublished doctoral dissertations, Brock University – St. Catharines, ON.

Abubaker, K.S., Sjaarda, C., Castle, A.J. 2013. Regulation of genes encoding cell wall degrading enzymes of *Trichoderma aggressivum* during interaction with *Agaricus bisporus*. Canadian Journal of Microbiology 59: 417-424.

Anderson, M.G., Beyer, D.M., Wuest, P.J. 2001. Yield comparison of hybrid *Agaricus* mushroom strains as a measure of resistance to *Trichoderma* green mold. Plant Disease 85:731-734.

Arst, H.N., Penalva, M.A. 2003. pH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. Trends in Genetics 19:224-231.

Bailey, A.M., Collopy, P.D., Thomas, D.J., Sergeant, M.R., Costa, A.M.S.B., Barker, G.L.A., Mills, P.R., Challen, M.P., Foster, G.D. 2013. Transcriptomic analysis of the interactions between *Agaricus bisporus* and *Lecanicullium fungicola*. Fungal Genetics and Biology 55: 67-76.

Baldrain, P. 2004. Increase of laccase activity during interspecific interactions of white-rot fungi. FEMS Microbiology Ecology 50: 245-253.

Baldrian, P. 2006. Fungal laccases – occurrence and properties. FEMS Microbiology Reviews 30: 215-242.

Basehoar, A.D., Zanton, S.J., Pugh, B.F. 2004. Identification and distinct regulation of yeast TATA box-containing genes. Cell 116: 699-709.

Benhamou, N., Chet, I. 1993. Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: Ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology 83:1062-1071.

Benhamou, N., Rey, P., Picard, K., Tirilly, Y. 1999. Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soilborne plant pathogens. Phytopathology 89:506-517.

Benítez, T., Rincón, A.M., Limón, M.C., Codón, A.C. 2004. Biocontrol mechanisms of *Trichoderma* strains. International Microbiology 7:249-260.

Benoist, C., O'Hare, K., Breathnach, R., Chambon, P. 1980. The ovalbumin gene-sequence of putative control regions. Nucleic Acids Research 8: 127-142.

Berendsen, R.L. Baars, J.J.P. Kalkhave, S.I.E., Lugones, L.G., Wösten, H.A.B., Bakker, P.A.H.M. 2010. *Lecanicillium fungicola:* causal agent of dry bubble disease in white-button mushroom. Molecular Plant Pathology 11:585-595.

Birnbaumer, L. 1992. Receptor-to-effector signaling through G proteins: roles for beta gamma dimers as well as alpha subunits. Cell 71:1069–72.

Birnboim, H.C., Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7: 1513-1523.

Blakeman, J.P., 1978. Microbial competition for nutrients and germination of fungal spores. Annals of Applied Biology 89:151-155.

Bolker, M. 1998. Sex and crime: heterotrimeric G proteins in fungal mating and pathogenesis. Fungal Genetics and Biology 25:143–56.

Bonnen, A.M., Anton, L.H., Orth, A.B. 1994. Lignin-degrading enzymes of the commercial button mushroom, *Agaricus bisporus*. Applied and Environmental Microbiology 60: 960-965.

Borodynko, N., Hasiów-Jaroszewske, B., Rymelska, N., Psopeiszny, H. 2010. La France disease of the cultivated mushroom *Agaricus bisporus* in Poland. Acta Virologica 54:217-219.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Brodey, C.L., Rainey, P.B., Tester, M., Johnstone, K. 1991. Bacterial blotch disease of the cultivated mushroom is caused by an ion channel forming lipodepsipeptide toxin. Molecular Plant-Microbe Interactions 4:407-411.

Bucher, P. 1990. Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. Journal of Molecular Biology 212: 563-578.

Burns, C., Gregory, K.E., Kirby, M., Cheung, M.K., Riquelme, M., Elliott, T.J., Challen, M.P., Bailey, A., Foster, G.D. 2005. Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns. Fungal Genetics and Biology 42:191-199.

Calistru, C., McLean, M., Berjak, P. 1997. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. Mycopathologia 137:115-124.

Camarero, S., Ibarra, D., Martínez, M.J., Martínez, A.T. 2005. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Applied and Environmental Microbiology 71: 1775-1784.

Canessa, P., Alvarez, J.M., Polanco, R., Bull, P., Vicuna, R. 2008. The copper-dependent ACE1 transcription factor activates the transcription of the *mco*1 gene from the basidiomycete *Phanerochaete chrysosporium*. Microbiology 154: 491-499.

Carbajo, J.M., Junca, H., Terron, M.C. Gonzalez, T., Yague, S., Zapico, E., Gonzalez, A.E. 2002. Tannic acid induces transcription of laccase gene *cglcc*1 in the white-rot fungus *Coriolopsis gallica*. Canadian Journal of Microbiology 48: 1041-1047.

Carranco, R., Almoguera, C., Jordano, J. 1999. An imperfect heat shock element and different upstream sequences are required for the seed-specific expression of a small heat shock protein gene. Plant Physiology 121: 723-730.

Castle, A.J., Horgen, P.A., Anderson, J.B. 1987. Restriction fragment length polymorphisms in the mushrooms *Agaricus brunnescans* and *Agaricus bitorquis*. Applied and Environmental Microbiology 53:816-822.

Castle, A.J., Horgen, P.A., Anderson, J.B. 1988. Crosses among homokaryons from commercial and wild-collected strains of the mushroom *Agaricus brunnescens* (=*A. bisporus*). Applied and Environmental Microbiology 54:1643-1648.

Castle, A., Speranzini, D., Rghei, N., Alm, G., Rinker, D., Bissett, J. 1998. Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. Applied and Environmental Microbiology 64:133-137.

Celar, F. 2003. Competition for ammonium and nitrate forms of nitrogen between some phytopathogenic and antagonistic soil fungi. Biological Control 28:19-24.

Chakravarty, B. 2011. Trends in mushroom cultivation and breeding. Australian Journal of Agricultural Engineering 2 (4): 102-109.

Chang, S.T., Miles, P.G. 2004. Mushrooms. Cultivation, nutritional value, medicinal effect, and environmental impact. Second edition. CRC Press LLC. Boca Raton, FL.

Chang, S.S., Zhang, Z., Liu, Y. RNA interference pathways in fungi: mechanisms and functions. Annual Review of Microbiology 66: 305-323.

Chen, X., Ospina-Giraldo, M.D., Wilkinson, V., Royse, D.J., Romaine, C.P. 2003. Resistance of pre- and post-epidemic strains of *Agaricus bisporus* to *Trichoderma aggressivum* f. *aggressivum*. Plant Disease 87:1457-1461.

Chen, X., Romaine, C.P., Ospina-Giraldo, M.D., Royse, D.J. 1999. A polymerase chain reaction-based test for the identification of *Trichoderma harzianum* biotypes 2 and 4, responsible for the worldwide green mold epidemic in cultivated *Agaricus bisporus*. Applied Microbiology and Biotechnology 52:246-250.

Chen, X., Stone, M., Schlagnhaufer, C., Romaine, C.P. 2000. A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. Applied and Environmental Microbiology 66: 4510-4513.

Chet, I., Baker, R. 1981. Isolation and biocontrol potential of *Trichoderma hamatum* from soil naturally suppressive to *Rhizoctonia solani*. Phytopathology 71:286-290.

Chet, I., Harman, G.E., Baker, R. 1981. *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. Microbial Ecology 7:29-38.

Chowdhary, R., Bajic, V.B., Dong, D., Wong, L., Liu, J.S. 2010. Genome-wide analysis of regions similar to promoters of histone genes. IN: BMC Systems Biology. http://www.biomedcentral.com/1752-0509/4/S1/S4

Collopy, P.D., Largeteau-Mamoun, M.L., Romaine, C.P., Royse, D.J. 2001. Molecular phylogenetic analysis of *Verticillium fungicola* and related species causing dry bubble disease of the cultivated button mushroom, *Agaricus bisporus*. Phytopathology 91:905-912.

Cordoba Cañero, D., and Roncero, M. I. G. 2008. Functional analyses of laccase genes from *Fusarium oxysporum*. Phytopathology 98:509-518.

Costa, A.S.M.B, Thomas, D.J.I., Eastwood, D., Cutler, S.B., Baily, A.M., Foster, G.D., Mills, P.M., Challen, M.P. 2009. Quantifiable down regulation of endogenous genes in *Agaricus bisporus* mediated by expression of RNA hairpins. Journal of Microbiology and Biotechnology 19: 271-276.

Criquet, S., Tagger, S., Vogt, G., Iacazio, G., LePetit, J. 1999. Laccase activity of forest litter. Soil Biology and Biochemistry 31: 1239-1244.

Dana, M.M., Limón, M.C., Mejías ,R., Mach, R.L., Benítez, T., Pintor-Toro, J.A., Kubicek, C.P. 2001. Regulation of chitinase 33 (*chit33*) gene expression in *Trichoderma harzianum*. Current Genetics 38:335-342.

de Groot, M.J.A., Bundock, P., Hooykaas, P.J.J., Beijersbergen, A.G.M. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nature Biotechnology 16: 839-842.

Del Carmen, S.J., Largeteau-Mamoun, M.L., Rousseau, T., Regnault-Roger, C., Savoie, J.M. 2002. Genetic and physiological variation in isolated of *Verticillium fungicola* causing dry bubble disease of the cultivated button mushroom, *Agaricus bisporus*. Mycological Research 106:1163-1170.

Delgado-Jarana, J., Moreno-Mateos, A., Benítez, T. 2003. Glucose uptake in *Trichoderma harzianum*: Role of *gtt1*. Eukaryotic Cell 2:708-717.

Delgado-Jarana, J., Pintor-Toro, J.A. Benítez, T. 2000. Overproduction of β-1,6-glucanase in *Trichoderma harzianum* is controlled by extracellular acidic proteases and pH. Biochimica et Biophysica Acta 1481:289-296.

Delgado-Jarana, J., Rincón, A.M., Benítez, T. 2002. Aspartyl protease from *Trichoderma harzianum* CECT 2413: cloning and characterization. Microbiology 148:1305-1315.

Dodd, S.L., Crowhurst, R.N., Rodrigo, A.G., Samuels, G.J., Hill, R.A., Stewart, A. 2000. Examination of *Trichoderma* phylogenies derived from ribosomal DNA sequence data. Mycological Research 104:23-34.

Druzhinina, I.S., Kubicek, C.P., Komoń-Zelazowska, M. Mulaw, T.B., Bissett, J. 2010. The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. BMC Evolutionary Biology 10:94.

Dynan, W.S., Tijian, R. 1985. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature 316: 774-778.

Eastwood, D.C., Bains, N.K., Henderson, J., Burton, K.S. 2011. Genome organization and transcription response to harvest of two metallothionein-like genes in *Agaricus bisporus* fruiting bodies. Journal of Microbiology and Biotechnology 25: 455-463.

Eastwood, D.C. Challen, M.P., Zhang, C., Jenkins, H., Henderson, J., Burton, K.S. 2008. Hairpin-mediated down-regulation of the urea cycle enzyme argininosuccinate lyase in *Agaricus bisporus.* Mycological Research 112: 708-716.

Eisendle, M., Oberegger, H., Buttinger, R., Illmer, P., Haas, H. 2004. Biosynthesis and uptake of siderophores is controlled by the PacC-mediated ambient-pH regulatory system in *Aspergillus nidulans*. Eukaryotic Cell 3:561-563.

Elad, Y. 1996. Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. European Journal of Plant Pathology 102:719-732.

Elad, Y., Chet, I., Katan, J. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathology 70:119-121.

El-Katatny, M.H., Gudelj, M., Robra, K.H., Elnaghy, M.A., Gübitz, G.M. 2001. Characterization of a chitinase and an endo-β-1,3-glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*. Applied Microbiology and Biotechnology 56:137-143.

Everett, R.D., Baty, D., Chambon, P. 1983. The repeated GC-rich motifs upstream from the TATA box are important elements of the SV40 early promoter. Nucleic Acids Research 11: 2447-2464.

Ezzi, M.I., Lynch, J.M. 2002. Cyanide catabolizing enzymes in *Trichoderma* spp. Enzyme and Microbial Technology 31:1042-1047.

Fan, F., Zhuo, R., Sun, S., Wan, X., Jiang, M., Zhang, X., Yang, Y. 2011. Cloning and functional analysis of a new laccase gene from *Trametes* sp 48424 which had the highest yield for laccase and strong ability for decolorizing different dyes. Bioresource Technology 102: 3126-3137.

FAOSTAT 2012. Retrieved December 10, 2013 from http://faostat3.fao.org/home/index.html.

Faraco, V., Giardina, P., Sannia, G. 2003. Metal-responsive elements in *Pleurotus ostreatus* laccase gene promoters. Microbiology 149: 2155-2162.

Farkaš, V. 1979. Biosynthesis of cell walls of fungi. Microbiological Reviews 43:117-144.

Fermor, T.R. Wood, D.A. 1981. Degradation of bacteria by *Agaricus bisporus* and other fungi. Journal of General Microbiology 126: 377-387.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806-811.

Fletcher, J.T., Gaze, R.H., 2008. Mushroom Pest and Disease Control. Manson Publishing, London, UK, pp. 192

Fletcher, J.T., Jaffe, B., Muthumeenakshi, S., Brown, A.E., Wright, D.M. 1995. Variations in isolated of *Mycogone perniciosa* and in disease symptoms in *Agaricus bisporus*. Plant Pathology 44:130-140.

Flores, A., Chet, I., Herrera-Estrella, A., 1997. Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene *prb*1. Current Genetics 31, 30–37.

Foulongne-Oriol, M., Minvielle, N., Savoie, J.M. 2011. QTL for resistance to *Trichoderma* lytic enzymes and metabolites in *Agaricus bisporus*. In Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Production. France.

Fritsche, G. 1981. Some remarks on the breeding and maintenance of strains and spawn of *Agaricus bisporus* and *A. bitorquis*. In Proceedings of the Eleventh International Scientific Congress on the Cultivation of Edible Fungi (pp 367-385). Australia.

Fu. Y.H., Marzluf, G.A. 1990. *nit*-2, the major positive-acting nitrogen regulatory gene of *Neurospora crassa*, encodes a sequence-specific DNA-binding protein. PNAS. 87: 5331-5335.

Galano, A., Alvarez-Idaboy, J.R. 2011. Glutathione: mechanism and kinetics of its non-enzymatic defense action against free radicals. RSC Advances 1: 1763-1771.

Galhaup, C., Goller, S., Peterbauer, C.K., Strauss, J., Haltrich. 2002. Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. Microbiology 148: 2159-2169.

Gea, F.J., Tello, J.C., Navarro, M.J. 2010. Efficacy and effects on yield of different fungicides for control of wet bubble disease of mushroom caused by the mycoparasite *Mycogone perniciosa.* Crop Protection 29:1021-1025.

Geels, F.P. 1997. Rondetafel-bijeenkomst over *Trichoderma.* [Roundtable meeting over *Trichoderma.*]. Champignoncultuur 41:13.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., Sannia, G. 2010. Laccases: a never-ending story. Cellular and Molecular Life Sciences 67: 369-385.

Glenn, J.K., Gold, M.H. 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Archives of Biochemistry and Biophysics 242: 329-341.

Goodin, M.M., Schlagnhaufer, B., Romaine, C.P. 1992. Encapsidation of the La France Disease-specific double-stranded RNAs in 36-nm isometric viruslike particles. Phytopathology 82:285-290.

Guertin, M.J. Lis, J.T. 2010. Chromatin landscape dictates HSF binding to target DNA elements. PLOS Genetics 6(9): e1001114. doi:10.1371/journal.pgen.1001114.

Guthrie, J.L., Castle, A.J. 2006. Chitinase production during interaction of *Trichoderma aggressivum* and *Agaricus bisporus*. Canadian Journal of Microbiology 52:961-967.

Guthrie, J.L., Khalif, S., Castle, A.J. 2005. An improved method for detection and quantification of chitinase activities. Canadian Journal of Microbiology 51:491-495.

Gutkind, J.S. 1998. Cell growth control by G protein-coupled receptors: from signal transduction to signal integration. Oncogene 17:1331–42.

Graham, G. C., Mayers, P., Henry, R.J. 1994. A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. Biotechniques 16:48-50.

Gruber, S., Seidl-Seiboth, V. 2012. Self versus non-self: fungal cell wall degradation in *Trichoderma*. Microbiology 158:26-34.

Haran, S., Schickler, H., Oppenheim, A., Chet, I. 1996. Differential expression of *Trichoderma harzianum* chitinases during mycoparasitism. Molecular Plant Pathology 86:980-985.

Harkin, J.M. Larsen, M.J., Obst, J.R. 1974. Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi. Mycologia 66: 469-476.

Harkin, J.M., Obst, J.R. 1973. Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. Experientia 29: 381-387.

Harman GE, Hayes CK, Lorito M, Broadway RM, Di Pietro A, Peterbauer CK, Tronsmo A (1993) Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathology 83: 313–318.

Harman, G.E., Howell, C.R., Viterbo, A. Chet, I., Lorito, M. 2004. *Trichoderma* species – opportunistic, avirulent plant symbionts. Nature Review Microbiology 2:43-56.

Harmsen, M.C. Schuren, F.H.J., Moukha, S.M., van Zuilen, C.M., Punt, P.J., Wessels, J.G.H. 1992. Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium* and *Agaricus bisporus*. Current Genetics 22: 447-454.

Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiology Reviews 13: 125-135.

Hatvani, L., Antal, A., Manczinger, L., Szekeres, A., Druzhinina, I.S., Kubicek, C.P., Nagy, A., Nagy, E., Vágvölgyi, Kredics, L. 2007. Green mold diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* species. Ecology and Epidemiology 97:532-537.

Heid, C.A., Stevens, J., Livak, K.J. Williams, P.M. 1996. Real time quantitative PCR. Genome Methods 6: 986-994.

Heneghan, M.N. Costa, A.M.S.B., Challen, M.P., Mills, P.R., Bailey, A., Foster, G.D. 2007. A comparison of methods for successful triggering of gene silencing in *Coprinus cinerea*. Molecular Biotechnology 35: 283-297.

Hermosa, H.R., Grondona, I., Iturriaga, A., Diaz-Minguez, J.M., Castro, C., Monte, E., Garcia-Acha, I. 2000. Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. Applied and Environmental Microbiology 66:1890-1898.

Hilden, K., Makela, M.R., Lankinen, P., Lundell, T. 2013. *Agaricus bisporus* and related *Agaricus* species on lignocellulose: production of manganese peroxidase and multicopper oxidases. Fungal Genetics and Biology 55: 32-41.

Hofrichter, M. 2002. Review: lignin conversion by manganese peroxidase (MnP). Enzyme and Microbial Technology 30: 454-466.

Hou, Y., Zhang, H., Miranda, L., Lin, S. 2010. Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: microalgal *pcna* as the model gene. PLOS ONE 5: 1-7.

Howell, C. R., Hanson, L. E., Stipanovic, R. D., and Puckhaber, L. S. 2000. Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens.* Phytopathology 90:248-252.

Inbar, J., Chet, I. 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. Journal of Bacteriology 174:1055-1059.

Inbar, J., Chet, I. 1994. A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii*: purification, characterization and role in mycoparasitism. Microbiology 140(3), 651–657.

Inbar, J., Chet, I. 1995. The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum.* Microbiology 141(11), 2823–2829.

Janusz, G., Kucharzyk, K.H. Pawlik, A., Staszczak, M., Paszczynski, A.J. 2013. Fungal laccase, manganese peroxidase and lignin peroxidase: gene expression and regulation. Enzyme and Microbial technology 52: 1-12.

Juo, Z.S., Chiu, T.K., Leiberman, P.M., Baikalov, I., Berk, A.J., Dickerson, R.E. 1996. How proteins recognize the TATA box. Journal of Molecular Biology 261: 239-254.

Kaluskar, V.M., Kapadnis, B.P., Jaspers, C.H., Penninckx, M.J. 1999. Production of laccase by immobilized cells of *Agaricus* sp. Applied Biochemistry and Biotechnology 76: 161-170.

Kamzolkina, O.V., Volkova, V.N., Kozlova, M.V., Pancheva, E.V., Dyakov, Y.T., Callac, P. 2006. Karyological evidence for meiosis in the three different types of life cycles existing in *Agaricus bisporus*. Mycologia 5:763-770.

Karahanian, E., Corsini, G., Lobos, S., Vicuna, R. 1998. Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Biochimica et Biophyica Acta 1443: 65-74.

Karam, J., Nicell, J.A. 1997. Potential application of enzymes in waste treatment. Journal of Chemical Technology and Biotechnology 69:141-153.

Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., Satoh, T. 1991. Structure and function of signal-transducing GTP-binding proteins. Annual Review of Biochemistry 60:349–400.

Kerrigan, R.W., Challen, M.P., Burton, K.S. 2013. *Agaricus bisporus* genome sequence: a commentary. Fungal Genetics and Biology 55: 2-5.

Kredics, L., Antal, Z., Dóczi, I., Manczinger, L., Kevei, F., Nagy, E. 2003. Clinical importance of the genus *Trichoderma*. Acta Microbiologica et Immunologica Hungarica 50:105-117.

Krupke, O.A., Castle, A.J., Rinker, D.L. 2003. The North American mushroom competitor, *Trichoderma aggressivum* f. *aggressivum,* produces antifungal compounds in mushroom compost that inhibit mycelial growth of the commercial mushroom *Agaricus bisporus*. Mycological Research 107:1467-1475.

Kuwahara, M., Glenn, J.K., Morgan, M.A., Gold, M.H. 1984. Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS 169: 247-250.

Lankinen, P., Hilden, K., Aro, N., Salkinoja-Salonen, M., Hatakka, A. 2005. Manganese peroxidase of *Agaricus bisporus*: grain bran-promoted production and gene characterization. Applied Microbiology and Biotechnology 66: 401-407.

Largeteau, M.L., Savoie, J.M. 2010. Microbial induced diseases of *Agaricus bisporus*: biochemical mechanisms and impact on commercial mushroom production. Applied Microbiology and Biotechnology 86:63-73.

Largeteau-Mamoun, M.L., Mata, G., Savoie, J.M. 2002. Green mold disease: adaptation of *Trichoderma harzianum* Th2 to mushroom compost. Mushroom Biology and Mushroom Products 179-187.

Larrondo, L.F., Avila, M., Salas, L., Cullen, D., Vicuna, R. 2003. Heterologous expression of laccase cDNA from *Ceriporiopsis subvermispora* yields copper-activated apoprotein and complex isoform patterns. Microbiology 149: 1177-1182.

Lee, H.I., Jeong, K.S., Cha, J.S. 2002. PCR assays for specific and sensitive detection of *Pseudomonas tolaasii*, the cause of brown blotch disease of mushrooms. Letters in Applied Microbiology 35:276-280.

Leonowitcz, A., Cho, N, Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A. et al. 2001. Fungal laccase: properties and activity on lignin. Journal of Basic Microbiology 41: 185-227.

Lewis, J.A., Papavizas, G.C. 1991. Biocontrol of plant diseases: the approach for tomorrow. Crop Protection 10:95-105.

Limón, M.C., Pintor-Toro, J.A., Benítez, T. 1999. Increased antifungal activity of Trichoderma harzianum transformants that overexpress a 33-kDa chitinase. Phytopathology 89:254-261.

Litvintseva, A.P., Henson, J.M. 2002. Cloning, characterization, and transcription of three laccase genes from *Gaeumannomyces graminis* var. *tritici*, the take-all fungus. Applied and Environmental Microbiology 68: 1305-1311.

Lo, C.-T., Nelson, E. B., Hayes, C. K., Harman, G. E. 1998. Ecological studies of transformed *Trichoderma harzianum* strain 1295-22 in the rhizosphere and on the phylloplane of creeping bentgrass. Phytopathology 88: 129-136.

López-Mondéjar, R., Ros, M., Pascual, J.A. 2011. Mycoparasitism-related genes expression of *Trichoderma harzianum* isolates to evaluate their efficacy as biological control agent. Biological Control 56: 59-66.

Lora, J.M., DeLa Cruz, J., Benitez, T., & Pintor-Toro, J.A. 1995. A putative catabolite-repressed cell wall protein from the mycoparasitic fungus *Trichoderma harzianum* , Molecular and General Genetics 247: 639-645.

Lorito, M., Harmann, G.E., Hayes, C.K., Brodway, R.M., Woo, S.L., & Di Pietro, A. 1993. Chitinolitic enzymes produced by *T. harzianum*. Antifungal activity of purified endochitinase and chitobiosidase. Phytopathology 83: 302–307.

Lu, S.C., 2009. Regulation of glutathione synthesis. Molecular Aspects of Medicine 30: 42-59.

Lundell, T.K., Mäkelä, M.R., Hildén, K. 2010. Lignin-modifying enzymes in filamentous basidiomycetes – ecological, functional and phylogenetic review. Journal of Basic Microbiology 50: 5-20.

Mach, R.L., Peterbauer, C.K., Payer, K., Jaksits, S., Woo, S.I., Zeilinger, S., Kullnig, C.M., Lorito, M., Kubicek, C.P. 1999. Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. Applied and Environmental Microbiology 65: 1858-1863.

Mager, W.H. DeKruijff, A.J. 1995. Stress-induced transcriptional activation. Microbiological Reviews 59: 506-531.

Mamoun, M.L., Iapicco R., Savoie, J.M., Olivier, J.M. 2000a. Green mould disease in France: *Trichoderma harzianum* Th2 and other species causing damages on mushroom farms. Science and Cultivation of Edible Fungi 625-632.

Mamoun, M.L., Savoie, J.M., Olivier, J.M. 2000b. Interactions between the pathogen *Trichoderma harzianum* Th2 and *Agaricus bisporus* in mushroom compost. Mycologia 92:235-240.

Marzluf, G.A. 1997. Genetic regulation of nitrogen metabolism in the fungi. Microbiology and Molecular Biology Reviews 61: 17-32.

Matcham, S.E., Jordan, B.R., Wood, D.A. 1985. Estimation of fungal biomass in a solid substrate by three independent methods. Applied Microbiology and Biotechnology 21: 108-112.

Mattanovich, D., Rüker, F., Machado, A.C., Laimer, M., Regner, F., Steinkellnew, H., Himmler, G., Katinger, H. 1989. Efficient transformation of *Agrobacterium* spp. by electroporation. Nucleic Acids Research 17:6747.

Mayer, A.M., Staples, R.C. 2002. Laccase: new functions for an old enzyme. Phytochemistry 60: 551-565.

Medzihradszky, K.F. 2008. Characterization of site-specific N-glycosylation. Methods in Molecular Biology 446: 293-316.

Meister, A., Anderson, M.E. 1983. Glutathione. Annual Review of Biochemistry 52: 711-760.

Michielse, C.B., Hooykaas, P.J.J, vandenHondel, A.M.J.J, Ram, A.F.J. 2005. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Current Genetics 48: 1-17.

Migheli, Q., González-Candelas, L., Dealessi, L., Camponogara, A., Ramón-Vidal, D. 1998. Transformants of *Trichoderma longibrachiatum* overexpressing the β-1,4-endoglucanase gene *egl1* show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology 88:673-677.

Mikosch, T.S.P, Lavrijssen, B., Sonnenberg, A.S.M., van Griensven, L.J.L.D. 2001. Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from *Agrobacterium tumefaciens*. Current Genetics 39: 35-39.

Mills, P.R. Fermor, T., Muthumeenakshi, S., Lincoln, S. 2000. Cell wall degrading enzymes produced by *Verticillium* spp and their relationship to infection in *Agaricus bisporus*. Science and Cultivation of Edible Fungi: 601-609.

Morimoto, R.I. 1993. Cells in stress: transcriptional activation of heat shock genes. Science 259: 1409-1410.

Morin, E., Kohler, A., Baker, A.R., Foulongne-Oriol, M., Lombard, V., Nagy, L.G., et al. 2012. Genome sequence of the button mushroom *Agaricus bisporus* reveals mechanisms governing adaptation to a humic-rich ecological niche. PNAS. 109: 17501 – 17506.

Moquet, F. Mamoun, M., RamosGuedes-Lafargue, M., Loviier, J.M., Savoie, J.M. 1998. Differences in susceptibility of *Agaricus bisporus* strains to bacterial blotch and in natural cap colour related to compost composition. Plant Breeding 117: 385-388.

Mukherjee, P.K., Latha, J., Hadar, R., Horwitz, B.A. 2004. Role of two G-protein alpha subunits, TgaA and TgaB, in the antagonism of plant pathogens by *Trichoderma virens*. Applied and Environmental Microbiology 70:542–9.

Mumpuni, A., Sharma, H.S.S., Brown, A.E. 1998. Effect of metabolites produced by *Trichoderma harzianum* biotypes and *Agaricus bisporus* on their respective growth radii in culture. Applied and Environmental Microbiology 64:5053-5056.

Munsch P, Olivier J-M (1995) Biocontrol of bacterial blotch of the cultivated mushroom with lytic phages: some practical considerations. Mushroom Science 14:595–602

Muthumeenakshi, S., Brown, A.E., Mills, P.R. 1998. Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom compost in North America and the British Isles. Mycological Research 102:385-390.

Muthumeenakshi, S., Mills, P.R., Brown, A.E., Seaby, D.A. 1994. Intraspecific molecular variation among *Trichoderma harzianum* isolated colonizing mushroom compost in the British Isles. Microbiology 140:769-777.

Nagai, M., Kawata, M., Watanabe, H., Ogawa, M., Saito, K., Takesawa, T., Kanda, K., Sato, T. 2003. Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies. Microbiology 149: 2455-2462.

Napoli, C., Lemieux, C., Jorgensen, R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. The Plant Cell 2: 279-289.

Neer, E.J. 1995. Heterotrimeric G proteins: organizers of transmembrane signals. Cell 80: 249-257.

Nehlin, J.O., Carlberg, M., Ronne, H. 1991. Control of yeast *GAL* genes by MIG1 repressor: a transcriptional cascade in the glucose response. The EMBO Journal 10: 3373-3377.

Nikolov, D.B., Chen, H., Halay, E.D., Hoffmann, A., Roeder, R.G., Burley, S.K. 1996. Crystal structure of a human TATA box-binding protein/TATA element complex. PNAS 93: 4862-4867.

Oh, S.J., Kim, Han. K., Kim, Hee. K., Fermor, T.R. 2000. Effect of sodium hypochlorite for controlling bacterial blotch on *Pleurotus ostreatus*. Microbiology 28:123-126.

Ohga, S., Smith, M., Thurston, C.F., Wood, D.A. 1999. Transcriptional regulation of laccase and cellulose genes in the mycelium of *Agaricus bisporus* during fruit body development on a solid substrate. Mycological Research 103: 1557-1560.

Omero, C., Inbar, J., Rocha-Ramirez, V., Herrera-Estrella, A., Chet, I., Horwitz, B.A. 1999. G protein activators and cAMP promote mycoparasitic behaviour in *Trichoderma harzianum*. Mycological Research 103:1637-1642.

Ostling, J., Carlberg, M., Ronne, H. 1996. Functional domains in the Mig1 repressor. Molecular and Cellular Biology 16: 753-761.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., Sannia, G. 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus osteatus*. Applied and Environmental Microbiology 66: 920-924.

Papavizas, G.C. 1985. *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. Annual Review of Phytopathology 23:23-54.

Paszczyński, A., Huynh, V. Crawford, R. 1986. Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chryosporium*. Archives of Biochemistry and Biophysics 244: 750-765.

Paszczyński, A., Huynh, V. Crawford, R. 1985. Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiology Letters 29: 37-41.

Perry, C.R., Matcham, S.E., Wood, D.A., Thurston, C.F. 1993a. The structure of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus*. Journal of General Microbiology 139: 171-178.

Perry, C.R., Smith, M., Britnell, C.H., Wood, D.A., Thurston, C.F. 1993b. Identification of two laccase genes in the commercial mushroom *Agaricus bisporus*. Journal of General Microbiology 139: 1209-1218.

Piscitelli, A., Giardina, P., Lettera, V., Pezzella, C., Sannia, G., Faraco, V. 2011. Induction and transcriptional regulation of laccases in fungi. Current Genomics 12:104-112.

Pozo, M.J., Back, J.M., García, J.M., Kenerley, C.M. 2003. Functional analysis of *tvsp1*, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genetics and Biology 41:336-348.

Prusky, D., Yakoby, A.N. 2003. Pathogenic fungi: leading or led by ambient pH? Molecular Plant Pathology 4:509-516.

Rabindran, S.K., Wisniewski, J., Li, L., Li, G., Wu, C. 1994. Interaction between heat shock factor and hsp70 is insufficient to suppress induction of DNA-binding activity in vivo. Molecular and Cellular Biology 14: 6552-6560.

Raguz, S., Yague, E., Wood, D.A., Thurston, CF. 1992. Isolation and characterization of a cellulose-growth-specific gene from *Agaricus bisporus*. Gene 119: 183-190.

Raper, C.A., Raper, J.R., Miller, R.E. 1972. Genetic analysis of the life cycle of *Agaricus bisporus*. Mycologia 64: 1088-1117.

Rehman, A., Anjum, M.S. 2011. Multiple metal tolerance and biosorption of cadmium by *Candida tropicalis* isolated from industrial effluents: glutathione as detoxifying agent. Environmental Monitoring and Assessment 174: 585-595.

Reino, J.L., Guerrero, R.F., Hernández-Galán, R., Collado. I.G. 2008. Secondary metabolites from species of the biological agent *Trichoderma*. Phytochemical Reviews 7:89-123.

Reithner, B., Brunner, K., Schuhmacher, R., Peissl, I., Seidl, V., Krska, R., Zeilinger, S. 2005. The G protein alpha subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. Fungal Genetics and Biology 42:749–60.

Reithner, B., Ibarra-Laclette. E., Mach, R.L., Herrara-Estrella, A. 2011. Identification of mycoparasitism-related genes in *Trichoderma atroviride*. Applied and Environmental Microbiology 77:4361-4370.

Ride, J.P. 1980. The effect of induced lignification on the resistance of wheat cell walls to fungal degradation. Physiological Plant Pathology 16: 187-196.

Rinker, D. L. 1993. Disease management strategies for *Trichoderma* mould. Mushroom World 4: 3–5.

Rinker, D. L. and Alm, G. 1997. Investigations of factors influencing the expression of green mould. Mushroom World 8: 25–29.

Rinker, D.L., Alm, G., Castle, A., Nghei, N. 1997. Distribution of green mould on infected mushroom farms. Mushroom World: 71-75.

Rinker, D., Alm, G. 2002. Spawn and green mould disease. AMGA Journal: 19.

Rocha-Ramirez, V., Omero, C., Chet, I. Horwitz, B.A. Herrera-Estrella, A. 2002. *Trichoderma atroviride* G-protein alpha-subunit gene tga1 is involved in mycoparasitic coiling and conidiation. Eukaryotic Cell 1:594–605.

Romaine, C.P., Schlagnhaufer, B. 1995. PCR analysis of the viral complex associated with La France disease of *Agaricus bisporus*. Applied and Environmental Microbiology 61:2322-2325.

Romaine, C.P., Schlagnhaufer, B. 2006. Mushrooms (*Agaricus bisporus*). Methods in Molecular Biology, vol. 344: Agrobacterium Protocols, 2/e, volume 2. Humana Press Inc., Totowa, NJ.

Romaine, C.P., Schlagnhaufer, B., Goodin, M.M. 1994. Vesicle-associated double-stranded ribonucleic acid genetic elements in *Agaricus bisporus*. Current Genetics 25:128-134.

Romano, N., Macino, G. 1992. Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. Molecular Microbiology 6: 3343-3353.

Romero-Arenas, O., Lara, M.H., Huato, M.A.D., Hernández, F.D., Victoria, D.A.A. 2009. The characteristics of *Trichoderma harzianum* as a limiting agent in edible mushrooms. Revista Colombiana de Biotecnología 11:143-151.

Ross, R.C., Harris, P.J. 1983. The significance of thermophilic fungi in mushroom compost preparation. Scientia Horticulturae 20:61-70.

Royse, D.J. 2010. Effects of fragmentation, supplementation and the addition of phase II compost to 2nd break compost on mushroom (*Agaricus bisporus*) yield. Bioresource Technology 101:188-192.

Royse, D.J., Beelman, R.B., 2007. Six Steps to Mushroom Farming. Received from http://mushroomspawn.cas.psu.edu/SixSteps.shtml.

Royse, D.J., Sanchez, J.E., Beelman, R.B., Davidson, J. 2008. Re-supplementing and re-casing mushroom (*Agaricus bisporus*) compost for a second crop. World Journal of Microbiology and Biotechnology 24:319-325.

Rushmore, T.H., King, R.G., Paulson, K.E., Pickett, C.B. 1990. Regulation of glutathione S-transferase Ya subunit gene expression: identification of a unique xenobiotic-resistive element controlling inducible expression by planar aromatic compounds. PNAS 87: 3826-3820.

Rushmore, T.H., Morton, M.R., Pickett, C.B. 1991. The antioxidant responsive element. The Journal of Biological Chemistry 266: 11632-11639.

Rushmore, T.H., Pickett, C.B. 1990. Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. The Journal of Biological Chemistry 265: 14648-14653.

Sadler, M. 2003. Nutritional properties of edible fungi. Nutritional Bulletin 28: 305-308.

Sahai, A.S., Manocha M.S.1993. Chitinases of fungi and plants: their involvement in morphogenesis and host – parasite interaction. FEMS Microbiology Reviews 11: 317-338.

Salame, T.M., Ziv, C., Hadar, Y., Yarden, O. 2011. RNAi as a potential tool for biotechnological application in fungi. Applied Microbiology and Biotechnology 89: 501-512.

Sambrook, J., Russell, D.W. 2001. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Samuels, G.J. 2006. *Trichoderma*: Systematics, the sexual state, and ecology. Phytopathology 96:195-206.

Samuels, G.J., Dodd, S.L., Gams, W., Castlebury, L.A., Petrini, O. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia 94:146-170.

Sassoon, J., Mooibroek, H. 2001. A system of categorizing enzyme-cell wall associations in *Agaricus bisporus*, using operational criteria. Applied Microbiology and Biotechnology 56: 613-622.

Savoie, J.M., Iapicco, R., Largeteau-Mamoun, M. 2001a. Factors influencing the competitive saprophytic ability of *Trichoderma harzianum* Th2 in mushroom (*Agaricus bisporus*) compost. Mycological Research 105:1348-1356.

Savoie, J.M., Mata, G. 2003. *Trichoderma harzianum* metabolites pre-adapt mushrooms to *Trichoderma aggressivum* antagonism. Mycologia 95:191-199.

Savoie, J.M., Mata, G., Mamoun, M. 2001b. Variability in brown line formation and extracellular laccase production during interaction between white-rot basidiomycetes and *Trichoderma harzianum* biotype Th2. Mycologia 93:243-248.

Savioe, J.-M., Mata, G. and Billette, C. 1998. Extracellular laccase production during hyphal interactions between *Trichoderma* sp. and Shiitake, *Lentinula edodes*. Applied Microbiology and Biotechnology 49: 589-593.

Seaby, D.A. 1987. Infection of mushroom compost by *Trichoderma* species. The Mushroom Journal 179:355-360.

Seaby, D.A. 1989. Further observation on *Trichoderma*. The Mushroom Journal 197:147-151.

Seaby, D.A. 1996a. Differentiation of *Trichoderma* taxa associated with mushroom production. Plant Pathology 45:905-912.

Seaby, D.A. 1996b. Investigation of the epidemiology of green mould of mushroom (*Agaricus bisporus*) compost caused by *Trichoderma harzianum*. Plant Pathology 45:913-923.

Singh, A.K., Rana, M.K., Sing, S., Kumar, S., Kumar, R., Singh, R. 2013. CAAT box-derived polymorphism (CBDP): a novel promoter-targeted molecular marker for plants. Journal of Plant Biochemistry and Biotechnology DOI 10.1007/s13562-013-0199-5.

Sivan, A., Chet I. 1986. Biological control of *Fusarium* spp in cotton, wheat and muskmelon by *Trichoderma harzianum*. Journal of Phytopathology 116:39-47.

Smith, J.F., Claydon, N., Love, M.E., Allan, M., Wood, D.A. 1989. Effect of substrate depth on extracellular endocellulase and laccase production of *Agaricus bisporus*. Mycological Research 93: 292-296.

Smith, M., Shnyreva, A., Wood, D.A., Thurston, C.F. 1998. Tandem organization and highly disparate expression of the two laccase genes *lcc*1 and *lcc*2 in the cultivated mushroom *Agaricus bisporus*. Microbiology 144: 1063-1069.

Sobieralski, K., Siwulski, M., Frużyńska-Jóźwiak, D. 2009a. Growth of aggressive isolates of *Trichoderma aggressivum* f. *europaeum* in dependence on temperature and medium. Phytophathologia 53:11-18.

Sobieralski, K., Siwulski, M., Frużyńska-Jóźwiak, D. Górski, R. 2009b. Impact of *Trichoderma aggressivum* f. *europaeum* Th2 on the yielding of *Agaricus bisporus*. Phytophathologia 53:5-10.

Soden, D.M., Dobson, A.D.W. 2003. The use of amplified flanking region-PCR in the isolation of laccase promoter sequences form the edible fungus *Pleurotus sajor-caju*. Journal of Applied Microbiology 95: 553-562

Sorger, P.K. 1991. Heat shock factor and the heat shock response. Cell 65: 363-366.

Statistics Canada. Retrieved December 10, 2013 from http://www5.statcan.gc.ca.

Steyaert, J.M. Ridgway, H.J., Elad, I., Stewart, A. 2003. Genetic basis of mycoparasitism: a mechanism of biological control by species of *Trichoderma*. New Zealand Journal of Crop and Horticultural Science 31:281-291.

Steyaert, J.M., Weld, R.J., Steward, A. 2010. Ambient pH intrinsically influences *Trichoderma* conidiation and colony morphology. Fungal Biology 114:198-208.

Strauss, J., Horvath, H.K., Abdallah, B.M., Kindermann, J., Mach, R.L., Kubicek, C.P. 1999. The function of CreA, the carbon catabolite repressor of *Aspergillus nidulans,* is regulated at the transcriptional and post-transcriptional level. Molecular Microbiology 32: 169-178.

Suarez, B., Rey, M., Castillo, P., Monte, E., Llovell, A. 2004. Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. Applied Microbiology and Biotechnology 65:46-55.

Szczech, M., Staniaszek, M., Habdas, H., Uliński, Z., Szymański, J. 2008. *Trichoderma* spp. − the cause of green mould on polish mushroom farms. Vegetable Crops Research Bulletin 69:105-114.

Tanović, B. Poročnik, I., Delibašic, G., Ristić, M., Kostić, M. Marković, M. 2009. *In vitro* effect of essential oils from aromatic and medicinal plants on mushroom pathogens: *Verticullium fungicola* var. *fungicola*, *Mycogone perniciosa,* and *Cladobotryum* sp. Archives of Biological Sciences 61: 231-237.

Thurston, C.F. 1994. The structure and function of fungal laccases. Microbiology 140: 19-26.

Tora, L., Timmers, H.T.M. 2010. The TATA box regulates TATA-binding protein (TBP) dynamics in vivo. Trends in Biochemical Sciences 35: 309-314.

Treger, J.M., Magee, T.R., McEntee, K. 1998. Functional analysis of the stress response element and its role in the multistress response of *Saccharomyces cerevisiae*. Biochemical and Biophysical Research Communications 243: 13-19.

Tsukamoto, T., Murata, H., Shirata, A. 2002. Identification of non-Pseudomonad bacteria from fruit bodies of wild *Agaricales* fungi that detoxify tolaasin produced by *Pseudomonas tolaasii.* Bioscience, Biotechnology, and Biochemistry 66:2201-2208.

Turner, E.M. 1974. Phenoloxidase activity in relation to substrate and development stage in the mushroom, *Agaricus bisporus*. Transactions of the British Mycological Society. 63: 541-547.

Turner G.E. Borkovich K.A., 1993. Identification of a G protein alpha subunit from *Neurospora crassa* that is a member of the Gi family. The Journal of Biological Chemistry 268:14805–11.

Turner, E.M., Wright, M., Ward, T., Osborne, D.J., Self, R. 1975. Production of ethylene and other volatiles and changes in cellulase and laccase activities during the life cycled of the cultivated mushroom, *Agaricus bisporus*. Journal of General Microbiology 91: 167-176.

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y., Valéro, J.R. 2007. Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. Biochemical Engineering Journal 37:1-20.

Viterbo, A., Ramot, O., Chernin, L., Chet, I. 2002. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. Antonie van Leeuwenhoek 81:549-556.

Vuister, G.W., Kim, S.J., Orosz, A., Marquardt, J., Wu, C., Bax, A. 1994. Solution structure of the DNA-binding domain of *Drosophila* heat shock transcription factor. Structural Biology 1: 605-614.

Wach, M.P., Sriskantha, A., Romaine, C.P 1987. Double-stranded RNAs associated with La France disease of the commercial mushroom. Phytopathology 77:1321-1325.

Wang, L.H., Weng, L.X., Dong, T.H., Zhang, L.H. 2004. Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). The Journal of Biological Chemistry 279: 13645-13651.

Williams, J., Clarkson, J.M., Mills, P.R., Cooper, R.M. 2003. Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus*. Applied and Environmental Microbiology 69:4192-4199.

Weindling, R. 1932. *Trichoderma lignorum* as a parasite of other soil fungi. Phytopathology 22, 837-845.

Wells, H.D., Bell, D.K., Jaworski, C.A. 1972. Efficacy of *Trichoderma harzianum* as a biocontrol for *Sclerotium rolfsii*. Phytopathology 62:442-447.

Whitwam, R.E., Brown, K.R., Musick, M., Natan, M.J., Tien, M. 1997. Mutagenesis of the Mn^{2+}-binding site of manganese peroxidase affects oxidation of Mn^{2+} by both compound I and compound II. Biochemistry 36: 9766-9773.

Wood, D.A. 1980a. Production, purification and properties of extracellular laccase of *Agaricus bisporus*. Journal of General Microbiology 117: 327-338.

Wood, D.A. 1980b. Inactivation of extracellular laccase during fruiting of *Agaricus bisporus*. Journal of General Microbiology 117: 339-345.

Wood, D.A., Goodenough, P.W. 1977. Fruiting of *Agaricus bisporus*. Changes in extracellular enzyme activities during growth and fruiting. Archives of Microbiology 114:161-165.

Xiao, X., Marzluf, G.A. 1996. Identification of the native NIT2 major nitrogen regulatory protein in nuclear extracts of *Neurospora crassa*. Genetica 97: 153-163.

Xiao, Y.Z., Hong, Y.Z., Li, J.F., Hang, J., Tong, P.G., Fang, W., Zhou, C.Z. 2006. Cloning of novel laccase isozyme genes form *Trametes* sp. AH28-2 and analyses of their differential expression. Applied Microbiology and Biotechnology 71: 493-501.

Yang, H-H., Yang, S.L., Peng, K-C., Lo, C-T. and Liu, S-Y. 2009. Induced proteome of *Trichoderma harzianum* by *Botrytis cinerea*. Mycological Research 113: 924-932.

Yedidia, I., Benhamou, N., Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Applied and Environmental Microbiology 65:1061-1070.

Yoshida, H. 1883. Chemistry of lacquer (Urushi). Part 1. Journal of the Chemical Society 43:472–486.

Zeid, D.C., Pardo-Golzález, J.E., Minhoni, M.R.A., Pardo-Giméz, A. 2011. A reliable quality index for mushroom production. Journal of Agricultural Science 3:50-61.

Zeilinger, S., Galhaup, C., Payer, K., Woo, S.L., Mach, R.L., Fekete, C., Lorito, M., Kubicek, C.P. 1999. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genetics and Biology 26:131-140.

Zeilinger, S., Omann, M. 2007. *Trichoderma* biocontrol: signal transduction pathways involved in host sensing and mycoparasitism. Gene Regulation and Systems Biology 1:227-234.

Zeilinger, S., Reithner, B., Scala, V. Peissl, I., Lorito, M., Mach, R.L. 2005. Signal transduction by Tga3, a novel G protein alpha subunit of *Trichoderma atroviride*. Applied Environmental Microbiology 71(3):1591–7.

APPENDIX I

NORGEN ladders used through this project.

bp	ng/10µL
1000	78
900	70
800	62
700	55
600	47
500	78
400	31
300	24
200	31
100	24

10µL on 1.5% agarose
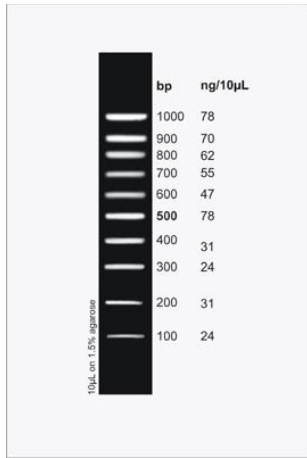
PCR Sizer 100 bp
DNA Ladder

bp	ng/10µL
5000	88
4000	78
3000	67
2500	58
2000	50
1500	43
1000	25
700	34
500	33
300	24

10µL on 1% agarose

MidRanger 1 kb
DNA Ladder

bp	ng/10µL
10000	42
8000	33
6000	25
5000	42
4000	35
3000	27
2500	22
2000	18
1500	13
1000	40
900	28
800	25
700	34
600	19
500	44
400	13
300	18
200	13
100	10

10µL on 1% agarose
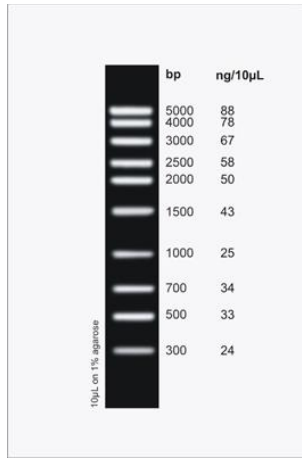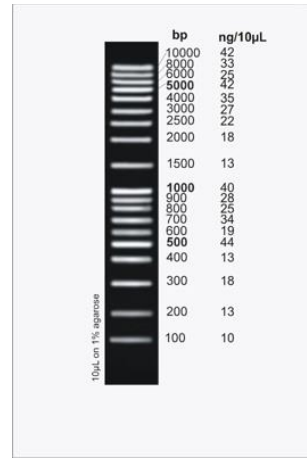
HighRanger Plus 100 bp
DNA Ladder

bp	ng/10µL
10000	58
8000	53
6000	46
5000	67
4000	56
3000	47
2500	41
2000	34
1500	29
1000	18
700	13
500	23
300	14

10µL on 1% agarose

HighRanger 1 kb
DNA Ladder

bp	ng/10µL
24000	30
14000	27
10000	60
8000	44
6000	36
5000	55
4000	49
3000	39
2500	35
2000	30
1500	33
1000	10
700	21
500	18
300	11

10µL on 0.9% agarose
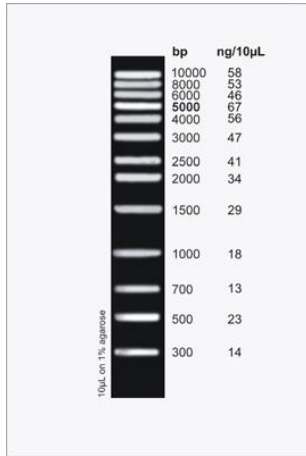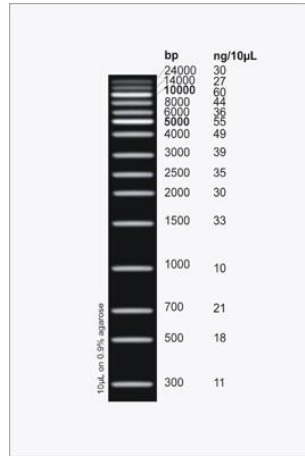
UltraRanger 1 kb
DNA Ladder

Toxicity of *T. aggressivum* metabolites

Table A1: The data from Figure 10 shows no statistically significant deviation from sphericity using Mauchly`s Test of Sphericity (p>0.05). There is a statistically significant difference between the white and brown strains sensitivity to the toxin, the effect of time on the inhibition zone, and a difference in the rate at which the two strains overcome the toxin with time.

	General Linear Model – Repeated Measures	
Within Subjects	F-value	Sig
Time	530.144	<0.001
*Time *treatment*	7.898	0.001
Between Subjects		
Treatment	30.356	<0.001

Response of *A. bisporus* to toxins from *T. aggressivum* – transcript abundance of *lcc*1, *lcc*2, *MnP*

Table A2: Laccase I mRNA levels (Figure 23) were tested using a repeated measures ANOVA to determine statistical significance. Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated (p<0.001) so the Huynh-Feldt correction was utilized. There is an effect of time, *lcc*1 is increasing through the experiment, but there is no difference in expression between the white and brown strains and the addition of *Trichoderma* metabolites also has no effect on transcript abundance.

General Linear Model – Repeated Measures		
White & Brown – sphericity (p<0.001); therefore, Huynh Feldt correction		
Within Subjects	F-value	Sig
Time	7.057	0.002
*Time *treatment*	1.834	0.117
Between Subjects		
Treatment	1.089	0.408
White – sphericity (p=0.008); therefore, Huynh Feldt correction		
Within Subjects	F-value	Sig
Time	6.494	0.012
*Time *treatment*	0.678	0.560
Between Subjects		
Treatment	0.242	0.649
Brown – sphericity (p=0.028); therefore, Huynh Feldt correction		
Within Subjects	F-value	Sig
Time	3.200	0.041
*Time *treatment*	0.418	0.793
Between Subjects		
Treatment	0.323	0.600

Table A3: A one-way ANOVA testing for difference in laccase 1 mRNA expressions (Figure 23) between the two strains of *A. bisporus* and the control and experimental conditions shows no statistically significant difference at all 5 time periods.

ANOVA			
		F	Sig.
Day 0	Between Groups	0.667	0.596
Day 1	Between Groups	0.976	0.451
Day 2	Between Groups	1.287	0.343
Day 3	Between Groups	3.806	0.058
Day 4	Between Groups	0.989	0.446

Table A4: Repeated measures ANOVA was used to test for statistically significant differences in laccase 2 mRNA levels (Figure 24). When Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, a Huynh-Feldt correction was used. Laccase levels are increasing as a result of time, the presence of *Trichoderma* metabolites increases the number of transcripts and the brown strain is producing significantly more than the white strain.

General Linear Model – Repeated Measures		
White & Brown – spericity (p=0.046); therefore, Huynh Feldt correction		
Within Subjects	F-value	Sig
Time	16.915	<0.001
*Time *treatment*	4.246	0.001
Between Subjects		
Treatment	110.660	<0.001
White – assume sphericity (p=0.163)		
Within Subjects	F-value	Sig
Time	20.474	<0.001
*Time *treatment*	8.857	0.001
Between Subjects		
Treatment	118.221	<0.001
Brown – assume sphericity (p=0.061)		
Within Subjects	F-value	Sig
Time	5.786	0.004
*Time *treatment*	5.327	0.006
Between Subjects		
Treatment	109.578	<0.001

Table A5: A one-way ANOVA testing for difference in laccase 2 mRNA expressions (Figure 24) between the two strains of *A. bisporus* and the control and experimental conditions is statistically significant at all 5 time periods. Bonferroni post-hoc test based on the ANOVA allows the determination which groups are significantly different (shown by letters on Figure #3)

ANOVA			
		F	Sig.
Day 0	Between Groups	18.721	.001
Day 1	Between Groups	10.368	.004
Day 2	Between Groups	8.241	.008
Day 3	Between Groups	18.987	.001
Day 4	Between Groups	92.491	<.001

Table A6: Repeated measures ANOVA was used to test for statistically significant differences in *MnP* mRNA levels (Figure 25). When Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, a Huynh-Feldt correction was used. *MnP* levels are increasing as a result of time, the presence of *Trichoderma* metabolites increases the number of transcripts in the brown strain, but not the white strain, and the white strain is producing significantly more than the brown strain.

General Linear Model – Repeated Measures		
White & Brown – spericity (p<0.001); therefore, Huynh Feldt correction		
Within Subjects	F-value	Sig
Time	3.328	0.069
*Time *treatment*	1.689	0.210
Between Subjects		
Treatment	0.05	0.952
White – assume sphericity (p=0.075)		
Within Subjects	F-value	Sig
Time	1.16	0.365
*Time *treatment*	1.954	0.229
Between Subjects		
Treatment	0.016	0.906
Brown – sphericity (p=0.013), therefore, Huynh-Feldt correction		
Within Subjects	F-value	Sig
Time	4.680	0.06
*Time *treatment*	0.405	0.642
Between Subjects		
Treatment	0.147	0.721

Table A7: A one-way ANOVA testing for difference in *MnP* mRNA expression between the two strains of *A. bisporus* and the control and experimental conditions is statistically significant at days 1 through 3 (p<0.05).

ANOVA			
		F	Sig.
Day 0	Between Groups	1.515	0.283
Day 1	Between Groups	7.414	0.011
Day 2	Between Groups	4.258	0.045
Day 3	Between Groups	4.403	0.042
Day 4	Between Groups	0.856	0.502

Response of *A. bisporus* to toxins from *T. aggressivum* – laccase enzymatic activity

Table A8: Repeated measures ANOVA was used to test for statistically significant differences in laccase enzyme activity (Figure 29). Over time, there is a statistically significant increase in the amounts of laccase produced by both the white and brown strain. There is also a statistically significant difference in laccase enzyme production between the off-white and brown control and experimental conditions.

General Linear Model – Repeated Measures		
White & Brown – assume spericity (p = 0.847)		
Within Subjects	F-value	Sig
Time	138.633	< 0.001
Time *treatment	24.239	< 0.001
Between Subjects		
Treatment	39.325	< 0.001
White – assume sphericity (p=0.202)		
Within Subjects	F-value	Sig
Time	134.011	<0.001
Time *treatment	41.267	< 0.001
Between Subjects		
Treatment	57.963	0.002
Brown – assume sphericity (p=0.845)		
Within Subjects	F-value	Sig
Time	46.910	<0.001
Time *treatment	17.812	0.001
Between Subjects		
Treatment	28.510	0.006

Table A9: A one-way ANOVA testing for difference in laccase enzyme activity between the two strains of *A. bisporus* and the control and experimental conditions showed statistically significant at all time periods.

ANOVA			
		F	Sig.
Day 0	Between Groups	31.368	<0.001
Day 2	Between Groups	27.943	<0.001
Day 4	Between Groups	45.242	<0.001

APPENDIX V

Effect of glutathione on *A. bisporus* sensitivity to *T. aggressivum* toxins.

Table A10: *A. bisporus* sensitivity to *T. aggressivum* toxins and the effect of glutathione was determined by the size of the inhibition zone surrounding the disc containing the toxin (Figure 33). A repeated measures ANOVA was used to determine significant difference in the size of the inhibition zone.

General Linear Model – Repeated Measures		
White and Brown – assume sphericity (p=0.086)		
Within Subjects	F-value	Sig
Time	530.144	<0.001
*Time *treatment*	7.898	0.001
Between Subjects		
Treatment	30.356	<0.001
White – assume sphericity (p=0.058)		
Within Subjects	F-value	Sig
Time	526.748	<0.001
*Time *treatment*	2.113	0.137
Between Subjects		
Treatment	17.454	0.001
Brown – assume sphericity (p=0.210)		
Within Subjects	F-value	Sig
Time	707.672	<0.001
*Time *treatment*	7.496	0.002
Between Subjects		
Treatment	18.239	<0.001

Sequencing and alignment of laccase genes

```
H97   :ATGAGGCTGTCCAACGCTTTTGTATTGGTTGCCGCATGCATTTCGAGCGTACTCGCCGATACCAAAACATTCGACTTCGACCTAGTCAATACCAGGCTCGCGCCTGACGGGGTTTGAAAGGGAgtgagtat: 130
D649  :ATGAGGCTGTCCAACGCTTTAGTATTGGTCGCCGCATGCATTTCGAGTGTGAGTTGCGAAAACCAGAACCTTCGACTTCGACCTAGTCAATACCAGGCTCGCGCCTGATGGGTTTGAAAGGGAgtgagtat: 130
U1a   :ATGAGGCTGTCCAACGCTTTTGTATTGGTTGCCGCATGCATTTCGAGCGTACTCGCCGATACCAAAACATTCGACTTCGACCTAGTCAATACCAGGCTCGCGCCTGACGGGTTTGAAAGGGAgtgagtat: 130
U1b   :ATGAGGCTGTCCAACGCTTTAGTATTGGTCGCCGCATGCATTTCGAGTGTGAGTTGCGAAAACCAGAACCTTCGACTTCGACCTAGTCAATACCAGGCTCGCGCCTGATGGGTTTGAAAGGGAgtgagtat: 130
SB65a :ATGAGGCTGTCCAACGCTTTTGTATTGGTTGCCGCATGCATTTCGAGCGTACTCGCCGATACCAAAACATTCGACTTCGACCTAGTCAATACCAGGCTCGCGCCTGACGGGTTTGAAAGGGAgtgagtat: 130
SB65b :ATGAGGCTGTCCAACGCTTTAGTATTGGTCGCCGCATGCATTTCGAGCGTACTCGCCGATACCAAAACATTCGACTTCGACCTAGTCAATACCAGGCTCGCGCCTGACGGGTTTGAAAGGGAgtgagtat: 130


H97   :atttctatacagattcatcgttagagtttactgaccttgattCaaactagCACCGTGGTCATCAATGGAGAATTCCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATA: 260
D649  :atttctatacagattcatcgttagagtttactgaccttgattTaaactagCACCGTGGTCATCAATGGAGAATTCCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATA: 260
U1a   :atttctatacagattcatcgttagagtttactgaccttgattCaaactagCACCGTGGTCATCAATGGAGAATTCCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATA: 260
U1b   :atttctatacagattcatcgttagagtttactgaccttgattTaaactagCACCGTGGTCATCAATGGAGAATTCCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATA: 260
SB65a :atttctatacagattcatcgttagagtttactgaccttgattCaaactagCACCGTGGTCATCAATGGAGAATTTCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATA: 260
SB65b :atttctatacagattcatcgttagagtttactgaccttgattCaaactagCACCGTGGTCATCAATGGAGAATTTCCTGGAACACTGATCCAAGTCAACAAGGGCGACAGTGTCCGCATCCCTCTCCATA: 260
          Intron I


H97   :ACAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATTgtgagttacttttttgagaccaaaattgacttgactatCtaacacaaattagCATTGGCATGGATTCTTCCAAGCGAGCACTTCTGGTCAG: 390
D649  :ACAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATTgtgagttacttttttgagcccagattgacttgactataaacacaaattagCATTGGCATGGATTTTTCCAAGCGAGAACTTCTGGTCAG: 390
U1a   :ACAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATTgtgagttacttttttgagaccaaaattgacttgactatCtaacacaaattagCATTGGCATGGATTCTTCCAAGCGAGCACTTCTGGTCAG: 390
U1b   :ACAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATTgtgagttacttttttgagcccagattgacttgactataaacacaaattagCATTGGCATGGATTTTTCCAAGCGAGAACTTCTGGTCAG: 390
SB65a :ACAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATTgtgagttacttttttgagaccaaaattgacttgactatCtaacacaaattagCATTGGCATGGATTTTTCCAAGCGAGCACTTCTGGTCAG: 390
SB65b :ACAAACTGACTAGCCCGACCATGAGGCGAAGTGTATCCATTgtgagttacttttttgagaccaaaattgacttgactatCtaacacaaattagCATTGGCATGGATTTTTCCAAGCGAGCACTTCTGGTCAG: 390
                                                                     Intron II


H97   :GACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTCACCTATGAATTCAGCGTCGCGGAGCAATCTGGAACGTTCgtcc----tgatccacaaaat-cattttcagcataaattg: 515
D649  :GACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTCACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAACGTTCgtcc----tgatcccttaaatacatcttcagcgtaaattg: 516
U1a   :GACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTCACCTATGAATTCAGCGTCGCGGAGCAATCTGGAACGTTCgtcc----tgatccacaaaat-cattttcagcataaattg: 515
U1b   :GACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTCACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAACGTTCgtcc----tgatcccttaaatacatcttcagcgtaaattg: 516
SB65a :GACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTCACCTATGAATTCAGCGTGGCGGAGCAATCTGGAACGTTCgtccgacctgatccacaaaat-cattttcagcataaattg: 519
SB65b :GACGGGCCTTCCTTCGTGAATCAGTGCCCTCAGCCACCCAACACAACCTTCACCTATGAATTCAGCGTGGCGGAGCAATCTGGAACGTTCgtccgacctgatccacaaaat-cattttcagcataaattg: 519
                                                                                         Intron III


H97   :acatttcttcaagttatTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGgtaattctttcctcttacatgcgaagatattttttgccgacttt-gttta: 644
D649  :acatttcttcaagttatTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGgtaattccttcctcttgcatgcgaagatattttttgccgactttcgttta: 646
U1a   :acatttcttcaagttatTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGgtaattctttcctcttacatgcgaagatattttttgccgacttt-gttta: 644
U1b   :acatttcttcaagttatTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGgtaattccttcctcttgcatgcgaagatattttttgccgactttcgttta: 646
SB65a :acatttcttcaagttatTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGgtaattctttcctcttacatgcgaagatattttttgccgacttt-gttta: 648
SB65b :acatttcttcaagttatTGGTACCATTCACACTTGTCCACCCAATACTGTGATGGTCTTCGCGGTGCATTCATCGTTTACGgtaattctttcctcttacatgcgaagatattttttgccgacttt-gttta: 648
                                                                                      Intron IV
```

```
H97   :gACCCACGTGATCCACTCAGACATCTCTATGATGTTGATGACGAAAGTACCGTCATCACTCTAGCTGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCgt: 774
D649  :gACCCACGTGATCCACTCAGACATCTCTATGATGTTGATGACGAAAGTACCGTCATCACTCTAGCTGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCgt: 776
U1a   :gACCCACGTGATCCACTCAGACATCTCTATGATGTTGATGACGAAAGTACCGTCATCACTCTAGCTGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCgt: 774
U1b   :gACCCACGTGATCCACTCAGACATCTCTATGATGTTGATGACGAAAGTACCGTCATCACTCTAGCTGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCgt: 776
SB65a :gACCCACGTGATCCACTCAGACATCTGTATGATGTCGATGACGAAAGTACCGTCATTACTTTAGCCGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCgt: 778
SB65b :gACCCACGTGATCCACTCAGACATCTGTATGATGTCGATGACGAAAGTACCGTCATTACTTTAGCCGAATGGTATCACATCCTCGCACCCGATGCCACTAACGAGTTTTTCAGCTCTGGCATTATACCgt: 778


H97   :aagcttcttaaaggaatatttattcatttt-cataatccatttcctgcagCGTACAAGATTCTGGCTTGATCAACGGGAAAGGTAGATTCAATGGAGGACCCCTCAgtaagtCgatcaaaacagaaaaggt: 903
D649  :aagcttcttaaaggaatatttattcattttcataacccaattcctgcagCGTGCAAGATTCTGGCTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCCTCAgtaagtagatcaaaacagaaaaggt: 906
U1a   :aagcttcttaaaggaatatttattcattt-cataatccatttcctgcagCGTACAAGATTCTGGCTTGATCAACGGGAAAGGTAGATTCAATGGAGGACCCCTCAgtaagtCgatcaaaacagaaaaggt: 903
U1b   :aagcttcttaaaggaatatttattcattttcataacccaattcctgcagCGTGCAAGATTCTGGCTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCCTCAgtaagtagatcaaaacagaaaaggt: 906
SB65a :aagcttcttaaaggaatatttattcattttcataacccaattcctgcagCGTACAAGATTCTGGCTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCCTCAgtaagtCgatcaaaacagaaaaggt: 908
SB65b :aagcttcttaaaggaatatttattcattttcataacccaattcctgcagCGTACAAGATTCTGGCTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCCTCAgtaagtCgatcaaaacagaaaaggt: 908
                  Intron V                                                                                                    Intron VI

H97   :gaCgatgaaaatttgacttttacttgcttagCACCATTCGCCGTCGTCAATGTTCGACGAGGCAAACGTTACAGGCTCCGTGTCATAGCTATTTCTTGCAGGCCGTTCTTCACCTTCTCCGTCGATAACCA:1033
D649  :gatgatgaaaatttgacttttacttgcttagCACCATTCGCCGTCGTCAATGTTCGCCGAGGCAAACGTTACAGGCTCCGTGTCATAGCTATTTCTTGCAGGCCGTTCTTCACCTTCTCCGTCGATAACCA:1036
U1a   :gaCgatgaaaatttgactttacttgcttagCACCATTCGCCGTCGTCAATGTTCGACGAGGCAAACGTTACAGGCTCCGTGTCATAGCTATTTCTTGCAGGCCGTTCTTCACCTTCTCCGTCGATAACCA:1033
U1b   :gatgatgaaaatttgactcttacttgcttagCACCATTCGCCGTCGTCAATGTTCGCCGAGGCAAACGTTACAGGCTCCGTGTCATAGCTATTTCTTGCAGGCCGTTCTTCACCTTCTCCGTCGATAACCA:1036
SB65a :gatgatgaaaatttgactctacttgcttagCGCCATTCGCCGTCGTCAATGTTCGACGAGGCAAACGTTACAGGCTCCGTGTCATAGCTATTTCTTGCAGGCCATTCTTCACCTTCTCCGTCGATAACCA:1038
SB65b :gatgatgaaaatttgactctacttgcttagCGCCATTCGCCGTCGTCAATGTTCGACGAGGCAAACGTTACAGGCTCCGTGTCATAGCTATTTCTTGCAGGCCATTCTTCACCTTCTCCGTCGATAACCA:1038


H97   :TAGCTTGGTCTTCATGGAAGCCGATGGTGTTGAACATGACCCTGTCGAAGTCCAAAACGTCGACATCTACGCGGCCCAGCGTGTCTCGGTTATTCTGCATGCCAATCAACCCATCGATAACTACTGGATC:1163
D649  :TAGCTTGGTCTTCATGGAAGCCGATGGTGTTGAACATGACCCTGTCGAAGTCCAAAACGTCGACATCTACGCGGCCCAGCGTGTCTCGGTTATTCTGCATGCCAATCAACCCATCGATAACTACTGGATC:1166
U1a   :TAGCTTGGTCTTCATGGAAGCCGATGGTGTTGAACATGACCCTGTCGAAGTCCAAAACGTCGACATCTACGCGGCCCAGCGTGTCTCGGTTATTCTGCATGCCAATCAACCCATCGATAACTACTGGATC:1163
U1b   :TAGCTTGGTCTTCATGGAAGCCGATGGTGTTGAACATGACCCTGTCGAAGTCCAAAACGTCGACATCTACGCGGCCCAGCGTGTCTCGGTTATTCTGCATGCCAATCAACCCATCGATAACTACTGGATC:1166
SB65a :TAGCTTGGTCTTCATGGAAGCCGATGGTGTTGAACATGACCCTGTCGAAGTCCAAAACGTCGACATCTACGCGGCCCAGCGTGTCTCGGTTATTCTGCATGCCAATCAACCCATCGATAACTACTGGATC:1168
SB65b :TAGCTTGGTCTTCATGGAAGCCGATGGTGTTGAACATGACCCTGTCGAAGTCCAAAACGTCGACATCTACGCGGCCCAGCGTGTCTCGGTTATTCTGCATGCCAATCAACCCATCGATAACTACTGGATC:1168


H97   :AGAGCTCCTATGACTGGCGGCAACCCAGACAGGAACCCCAATCgtaagcgtcattttgccacctgctagcagctgtaatgaaacgtgtttccaaagTAAACATCAGCCTCACTCTCGCCATCCTGCGCTA:1293
D649  :AGAGCTCCTATGACTGGCGGCAACCCAGACAGGAACCCCAATCgtaagcgtcattttgccacctgctagcagctgtaatgaaacgtgtttccaaagTAAACATCAGCCTCACTCTCGCCATCCTGCGCTA:1296
U1a   :AGAGCTCCTATGACTGGCGGCAACCCAGACAGGAACCCCAATCgtaagcgtcattttgccacctgctagcagctgtaatgaaacgtgtttccaaagTAAACATCAGCCTCACTCTCGCCATCCTGCGCTA:1293
U1b   :AGAGCTCCTATGACTGGCGGCAACCCAGACAGGAACCCCAATCgtaagcgtcattttgccacctgctagcagctgtaatgaaacgtgtttccaaagTAAACATCAGCCTCACTCTCGCCATCCTGCGCTA:1296
SB65a :AGAGCTCCTATGACTGGCGGCAACCCAGACAGGAACCCCAATCgtaagcgtcattttgccacctgctttgtagctgtaatgaaacgtgtttccaaagTAAACATCAGCCTCACTCTCGCCATCCTGCGCTA:1298
SB65b :AGAGCTCCTATGACTGGCGGCAACCCAGACAGGAACCCCAATCgtaagcgtcattttgccacctgctttgtagctgtaatgaaacgtgtttccaaagTAAACATCAGCCTCACTCTCGCCATCCTGCGCTA:1298
                                                                            Intron VII
```

```
H97   :CCATGGTGCACGTCACGTCGAACCGACTACGGTCAACGTTCCCGGACACAAACTCCTTGATCAAGAAATGCATgtaagaataacttgcgcaaaggcattccgaaatgcttactaacgcacaccgatctag:1423
D649  :CCATGGTGCACGTCACGTCGAACCGACTACGGTCAACGTTCCCGGACACAAACTCCTTGATCAAGAAATGCATgtaagaataacttgcgtaaaggcattccgaaatacttactaacgcgcaccaatctag:1426
U1a   :CCATGGTGCACGTCACGTCGAACCGACTACGGTCAACGTTCCCGGACACAAACTCCTTGATCAAGAAATGCATgtaagaataacttgcgcaaaggcattccgaaatgcttactaacgcacacgatctag:1423
U1b   :CCATGGTGCACGTCACGTCGAACCGACTACGGTCAACGTTCCCGGACACAAACTCCTTGATCAAGAAATGCATgtaagaataacttgcgtaaaggcattccgaaatacttactaacgcgcaccaatctag:1426
SB65a :CCATGGTGCACGTCACGTCGAACCGACTACGGTCAACGTTCCCGGACACAAACTCCTTGATCAAGAAATGCATgtaagaataacttgcgcaaaggcattccgaaatgcttactaacgcgcaccaatctag:1428
SB65b :CCATGGTGCACGTCACGTCGAACCGACTACGGTCAACGTTCCCGGACACAAACTCCTTGATCAAGAAATGCATgtaagaataacttgcgcaaaggcattccgaaatgcttactaacgcgcaccaatctag:1428
                                                                                                       Intron VIII

H97   :CCGATCCGCCAGGAAGGACCAGGCAAAgtgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcg:1553
D649  :CCGATCCGCCAGGAAGGACCAGGCAAAgtgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcg:1556
U1a   :CCGATCCGCCAGGAAGGACCAGGCAAAgtgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcg:1553
U1b   :CCGATCCGCCAGGAAGGACCAGGCAAAgtgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcg:1556
SB65a :CCGATCCGCCAGGAAGGACCAGGCAAAgtgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcg:1558
SB65b :CCGATCCGCCAGGAAGGACCAGGCAAAgtgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcg:1558
                            Intron IX

H97   :tctcattattcaaatcaaactcgacttctgacatgagcacagCCCAACGCTCCCTTCTTCGATATCAACGGAATCTCTTACATTTCCCCGACTGTCCCTGTTCTTCTCCAAATCCTCAGCGGTGCCAAGA:1683
D649  :tctcattattcaaatcaaactcgacttctgacatgagcacagCCCAACGCTCCCTTCTTCGATATCAACGGAATCTCTTACATCTCCCCGACTGTCCCTGTTCTTCTCCAAATCCTCAGCGGAGCCAAGA:1686
U1a   :tctcattattcaaatcaaactcgacttctgacatgagcacagCCCAACGCTCCCTTCTTCGATATCAACGGAATCTCTTACATCTCCCCGACTGTCCCTGTTCTTCTCCAAATCCTCAGCGGAGCCAAGA:1683
U1b   :tctcattattcaaatcaaactcgacttctgacatgagcacagCCCAACGCTCCCTTCTTCGATATCAACGGAATCTCTTACATCTCCCCGACTGTCCCTGTTCTTCTCCAAATCCTCAGCGGAGCCAAGA:1686
SB65a :actcattattcaaatcaaactcgacttctgacatgagcacagCCCAACGCTCCTTTCTTCGATATCAACGGAATCTCTTACATTTCCCCGACTGTCCCTGTTCTTCTCCAAATCCTCAGCGGTGCCAAGA:1688
SB65b :actcattattcaaatcaaactcgacttctgacatgagcacagCCCAACGCTCCTTTCTTCGATATCAACGGAATCTCTTACATTTCCCCGACTGTCCCTGTTCTTCTCCAAATCCTCAGCGGTGCCAAGA:1688
              Intron X

H97   :GGCCCGAAGATGTTCTTCCATCCGAGCAAATCTTCTTTGTCCCCAAGAACTCGTTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatggtaacctcattttctcctcaattcct:1813
D649  :GGCCCGAAGATGTTCTTCCATCCGAACAAATCTTCTTTGTCCCCAAGAATTCATTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatggtaacctcattttctcctcaattcct:1816
U1a   :GGCCCGAAGATGTTCTTCCATCCGAGCAAATCTTCTTTGTCCCCAAGAACTCATTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatggtaacctcattttctcctcaattcct:1813
U1b   :GGCCCGAAGATGTTCTTCCATCCGAACAAATCTTCTTTGTCCCCAAGAATTCATTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatggtaacctcattttctcctcaattcct:1816
SB65a :GGCCCGAAGATGTTCTTCCATCCGAACAAATCTTCTTTGTCCCCAAGAACTCGTTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatgataacctcattttctcctcaattcct:1817
SB65b :GGCCCGAAGATGTTCTTCCATCCGAACAAATCTTCTTTGTCCCCAAGAACTCGTTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatgataacctcattttctcctcaattcct:1817
                                                                                                   Intron XI

H97   :ctacttacgtccctgtttcagTGCACGGTCATAACTTTGATGTCGTTCTGGCTTCTAACGACGACACTTTCAACTTCAAGAAgtgagttaattgaaaaaggaagttacctgctgaacactaatctcaaat:1943
D649  :ctacttacgtccctgtttcagTGCACGGTCATAACTTTGATGTCGTTCTGGCTTCTAACGACGACACTTTCAACTTCAAGAAgtgagttaattgaaaaaggaagttacctgctgaacactaatctcaaat:1946
U1a   :ctacttacgtccctgtttcagTGCACGGTCATAACTTTGATGTCGTTCTGGCTTCTAACGACGACACTTTCAACTTCAAGAAgtgagttaattgaaaaaggaagttacctgctgaacactaatctcaaat:1943
U1b   :ctacttacgtccctgtttcagTGCACGGTCATAACTTTGATGTCGTTCTGGCTTCTAACGACGACACTTTCAACTTCAAGAAgtgagttaattgaaaaaggaagttacctgctgaacactaatctcaaat:1946
SB65a :ctacttacgtccctgtttcagTGCACGGTCATAACTTTGATGTCGTTCTGGCTTCTAACGACGATACTTTCAACTTCAAGAAgtgagttaattgataaaggaagttacctgctgaacactaatctcaaat:1947
SB65b :ctacttacgtccctgtttcagTGCACGGTCATAACTTTGATGTCGTTCTGGCTTCTAACGACGATACTTTCAACTTCAAGAAgtgagttaattgataaaggaagttacctgctgaacactaatctcaaat:1947
                                                                                                        Intron XII
```

```
H97   :gaaagCCCGCCTCGTCGTGACGTATACCCCATCAATGGTGGCAACACCACTTTCCGCTTCTgtaagattccacgttgctttctgaagaataactctttctgaacgacccctcagTCACCGATAACCCAGG:2073
D649  :gaaagCCCGCCTCGTCGTGACGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCTgtaagattccacgtttctttctgaagaataactctttctgaacgacccctcagTCACCGATAACCCAGG:2076
U1a   :gaaagCCCGCCTCGTCGTGACGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCTgtaagattccacgtttctttctgaagaataactctttctgaacgacccctcagTCACCGATAACCCAGG:2073
U1b   :gaaagCCCGCCTCGTCGTGACGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCTgtaagattccacgtttctttctgaagaataactctttctgaacgacccctcagTCACCGATAACCCAGG:2076
SB65a :gaaagCCCGCCTCGTCGTGACGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCTgtaagattccacgttgctttctgaagaataactctttctgaacgacccctcagTCACCGATAACCCAGG:2077
SB65b :gaaagCCCGCCTCGTCGTGACGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCTgtaagattccacgttgctttctgaagaataactctttctgaacgacccctcagTCACCGATAACCCAGG:2077
                                                                                                                 Intron XIII

H97   :CGCATGGTTCTTGCATTGgtgaggaacatttctatccatttctctcgaggcgaatactgattgaatcttgtgacatagCCATATCGATTGGCATCTTGAAGCTGGTCTCGCTATTGTGTTTGCTGAGGCT:2203
D649  :CGCATGGTTCTTGCATTGgtgaggaacatttctatccatttctctcgaggcgaatactgattgaatcttgtgacatagCCATATCGATTGGCATCTTGAAGCTGGTCTCGCTATTGTGTTTGCTGAGGCT:2206
U1a   :CGCATGGTTCTTGCATTGgtgaggaacatttctatccatttctctcgaggcgaatactgattgaatcttgtgacatagCCATATCGATTGGCATCTTGAAGCTGGTCTCGCTATTGTGTTTGCTGAGGCT:2203
U1b   :CGCATGGTTCTTGCATTGgtgaggaacatttctatccatttctctcgaggcgaatactgattgaatcttgtgacatagCCATATCGATTGGCATCTTGAAGCTGGTCTCGCTATTGTGTTTGCTGAGGCT:2206
SB65a :CGCATGGTTTTTGCATTGgtgaggaacatttctatccatttctctcgaggcgaatactgattgaatcttgcgacatagCCATATCGATTGGCATCTTGAAGCTGGTCTCGCTATTGTATTTGCTGAGGCT:2207
SB65b :CGCATGGTTTTTGCATTGgtgaggaacatttctatccatttctctcgaggcgaatactgattgaatcttgcgacatagCCATATCGATTGGCATCTTGAAGCTGGTCTCGCTATTGTATTTGCTGAGGCT:2207
                                    Intron XIV

H97   :CCGGAGGATAACGTTTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTGGTTGGATCTCTGTCCAGAATACAACGCAATCGAGCCTGAATTCCAGTAG------- : 2302
D649  :CCGGAGGATAACGTTTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTGGTTGGATCTCTGTCCAGAATACAACGCAATCGAGCCTGAATTCCAGTAG------- : 2305
U1a   :CCGGAGGATAACGTTTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTGGTTGGATCTCTGTCCAGAATACAACGCAATCGAGCCTGAATTCCAGTAG------- : 2302
U1b   :CCGGAGGATAACGTTTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTGGTTGGATCTCTGTCCAGAATACAACGCAATCGAGCCTGAATTCCAGTAG------- : 2305
SB65a :CCGGAGGATAACGTCTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTGGTTGGATCTCTGTCCAGAATACAACGCAATCGAGCCTGAATTCCAGTAG------- : 2306
SB65b :CCGGAGGATAACGTCTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTGGTTGGATCTCTGTCCAGAATACAACGCAATCGAGCCTGAATTCCAGTAG------- : 2306
```

Figure A1 Alignment of *lcc*1 alleles from the database (H97 and D649) and the U1 and SB65 strains of *A. bisporus*. Arrows indicate the forward and reverse primers used to generate the RNAi silencing cassette.

```
H97  :ATGAGGCTTTTCCAACGCTTTTGTACTGGTCGCCGCCTGCATTTCGAGCGTACTCGCCGATACCAAAACCTTCAACTTCGACCTAGTCAATACTAGGCTTGCACCTGATGGGTTTGAAAGGGAgtgagtac:130
D649 :ATGAGGTTTTTCCAACGCTTTTGTACTGGTCGCCGCCTGCATTTCGAGCGTACTCGCCGATACCAAAACCTTCAACTTCGACCTAGTCAATACTAGGCTTGCACCTGATGGGTTTGAAAGGGA-------:122
U1   :ATGAGGCTTTTCCAACGCTTTTGTACTGGTCGCCGCCTGCATTTCGAGCGTACTCGCCGATACCAAAACCTTCAACTTCGACCTAGTCAATACTAGGCTTGCACCTGATGGGTTTGAAAGGGAgtgagtac:130
SB65 :ATGAGGCTTTTCCAACGCTTTTGTACTGGTCGCCGCCTGCATTTCGAGCGTACTCGCCGATACCAAAACCTTCAACTTCGACCTAGTCAATACTAGGCTTGCACCTGATGGGTTTGAAAGGGAgtgagtac:130


H97  :atctttatacagaatcatcgttagagtttattgaccttgatccaaatcagCACCGTGGTTATCAATGGAGAATTCCCTGGAACACTGGTCCAAGTCAACAAGGGCGATAGTGTCCGTATCCCGGTCAACA:260
D649 :--------------------------------------------------CACCGTGGTTATCAATGGAGAATTCCCTGGAACACTGGTCCAAGTCAACAAGGGCGATAGTGTCCGTATCCCGGTCAACA:202
U1   :atctttatacagaatcatcgttagagtttattgaccttgatccaaatcagCACCGTGGTTATCAATGGAGAATTCCCTGGAACACTGGTCCAAGTCAACAAGGGCGATAGTGTCCGTATCCCGGTCAACA:260
SB65 :atctttatacagaatcatcgttagagtttattgaccttgatccaaatcagCACCGTGGTTATCAATGGAGAATTCCCTGGAACACTGGTCCAAGTCAACAAGGGCGATAGTGTCCGTATCCCGGTCAACA:260
                 Intron I

H97  :ACAAACTGACCAGCTCAACCATGAGGCGAAGTGTATCCATTgtgagttgtctttcgagacccaattcaatctactaaaaccaaactagCACTGGCATGGATTCTTCCAAGCGAGGACTTCTGGTCAGGAC:390
D649 :ACAAACTGACCAGCTCAACCATGAGGCGAAGTGTATCCATT----------------------------------------------CACTGGCATGGATTCTTCCAAGCGAGGACTTCTGGTCAGGAC:285
U1   :ACAAACTGACCAGCTCAACCATGAGGCGAAGTGTATCCATTgtgagttgtctttcgagacccaattcaatctactaaaaccaaactagCACTGGCATGGATTCTTCCAAGCGAGGACTTCTGGTCAGGAC:390
SB65 :ACAAACTGACCAGCTCAACCATGAGGCGAAGTGTATCCATTgtgagttgtctttcgagacccaattcaatctactaaaaccaaactagCACTGGCATGGATTCTTCCAAGCGAGGACTTCTGGTCAGGAC:390
                                                  Intron II

H97  :GGGCCTGCATTCGTGAATCAGTGCCCCCAACCACCCAACACAACCTTCACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAACgttagtcctgatccatgaaatacatattcagcgtaaacttatgttct:520
D649 :GGGCCTGCATTCGTGAATCAGTGCCCCCAACCACCCAACACAACCTTCACCTATGAATTCAGCGTGGCGGACGAGTCTGGAAC--------------------------------------------:368
U1   :GGGCCTGCATTCGTGAATCAGTGCCCCCAACCACCCAACACAACCTTCACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAACgttagtcctgatccatgaaatacatattcagcgtaaacttatgttct:520
SB65 :GGGCCTGCATTCGTGAATCAGTGCCCCCAACCACCCAACACAACCTTCACCTATGAATTCAGCGTGGCGGAGCAGTCTGGAACgttagtcctgatccatgaaatacatattcagcgtaaacttatgttct:520
                                                                                    Intron III

H97  :ctcttcaagTTTCTGGTACCATTCACACTTGTCCACCCAATATTGTGATGGCCTTCGTGGTGCCTTCGTCGTTTATGgtaattctttactcttgcatgcgaagatatttttgctgatctccgcctagACC:650
D649 :---------TTTCTGGTACCATTCACACTTGTCCACCCAATATTGTGATGGCCTTCGTGGTGCCTTCGTCGTTTATG--------------------------------------------------ACC:439
U1   :ctcttcaagTTTCTGGTACCATTCACACTTGTCCACCCAATATTGTGATGGCCTTCGTGGTGCCTTCGTCGTTTATGgtaattctttactcttgcatgcgaagatatttttgctgatctccgcctagACC:650
SB65 :ctcttcaagTTTCTGGTACCATTCACACTTGTCCACCCAATATTGTGATGGCCTTCGTGGTGCCTTCGTCGTTTATGgtaattctttactcttgcatgcgaagatatttttgctgatctccgcctagACC:650
                                                                                   Intron IV

H97  :CTGAGGATCCACTCGGGCATCTTTACGATGTTGATGATGAAACTACTGTCATCACCCTAGCTGAATGGTATCACGTCCTCGCACCCGATATCAACAACGAATTCTTCAGCTCTGGCATTATACCgtgagt:780
D649 :CTGAGGATCCACTCGGGCATCTTTACGATGTTGATGATGAAACTACTGTCATCACCCTAGCTGAATGGTATCACGTCCTCGCACCCGATATCAACAACGAATTCTTCAGCTCTGGCATTATACC------:563
U1   :CTGAGGATCCACTCGGGCATCTTTACGATGTTGATGATGAAACTACTGTCATCACCCTAGCTGAATGGTATCACGTCCTCGCACCCGATATCAACAACGAATTCTTCAGCTCTGGCATTATACCgtgagt:780
SB65 :CTGAGGATCCACTCGGGCATCTTTACGATGTTGATGATGAAACTACTGTCATCACCCTAGCTGAATGGTATCACGTCCTCGCACCCGATATCAACAACGAATTCTTCAGCTCTGGCATTATACCgtgagt:780


H97  :ttcccaaggaatacttattcattttcataacccaattcctgcagCGTGCAAGATTCTGGTTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCGAGAgtaagtagaccaaaacagaaaaggtgatgg:910
D649 :---------------------------------------------CGTGCAAGATTCTGGTTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCGAGA----------------------------:619
U1   :ttcccaaggaatacttattcattttcataacccaattcctgcagCGTGCAAGATTCTGGTTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCGAGAgtaagtagaccaaaacagaaaaggtgatgg:910
SB65 :ttcccaaggaatacttattcattttcataacccaattcctgcagCGTGCAAGATTCTGGTTTGATCAACGGTAAAGGTAGATTCAATGGAGGACCCGAGAgtaagtagaccaaaacagaaaaggtgatgg:910
           Intron V                                                                                                          Intron VI
```

184

```
H97  :aaatttgactttacttgcttagCGCCATTCGCCGTCGTCAACGTCGAGCAAGGAAAACGCTATAGGTTCCGTGTCATCGCCATCTCCTGCAGGCCATTCTTCACCTTCTCCGTCGATAACCACAACTTGA:1040
D649 :---------------------CGCCATTCGCCGTCGTCAACGTCGAGCAAGGAAAACGCTATAGGTTCCGTGTCATCGCCATCTCCTGCAGGCCATTCTTCACCTTCTCCGTCGATAACCACAACTTGA: 727
U1   :aaatttgactttacttgcttagCGCCATTCGCCGTCGTCAACGTCGAGCAAGGAAAACGCTATAGGTTCCGTGTCATCGCCATCTCCTGCAGGCCATTCTTCACCTTCTCCGTCGATAACCACAACTTGA:1040
SB65 :aaatttgactttacttgcttagCGCCATTCGCCGTCGTCAACGTCGAGCAAGGAAAACGCTATAGGTTCCGTGTCATCGCCATCTCCTGCAGGCCATTCTTCACCTTCTCCGTCGATAACCACAACTTGA:1040


H97  :CCTTCATGGAAGCCGATAGTGTTGAACACGATCCCGTCGAAATCCAGAACGTCGACATTTATGCAGCCCAGCGTGTCTCGGTTATCCTGAATGCCAATCAACCCGTCGATAACTACTGGATGAGAGCTCC:1170
D649 :CCTTCATGGAAGCCGATAGTGTTGAACACGATCCCGTCGAAATCCAGAACGTCGACATTTATGCAGCCCAGCGTGTCTCAGTTATCCTGAATGCCAATCAACCCGTCGATAACTACTGGATGAGAGCTCC: 857
U1   :CCTTCATGGAAGCCGATAGTGTTGAACACGATCCCGTCGAAATCCAGAACGTCGACATTTATGCAGCCCAGCGTGTCTCGGTTATCCTGAATGCCAATCAACCCGTCGATAACTACTGGATGAGAGCTCC:1170
SB65 :CCTTCATGGAAGCCGATAGTGTTGAACACGATCCCGTCGAAATCCAGAACGTCGACATTTATGCAGCCCAGCGTGTCTCGGTTATCCTGAATGCCAATCAACCCGTCGATAACTACTGGATGAGAGCTCC:1170


H97  :TATGACTGGCGGCAACCCAGCTAGGAACCCCAATCgtaagcgccattttgtcgcctgctagcaactgtaatgtaacgtgttcccaaagTCAACATCAGTCTCACTCTCGCCATCCTGCGCTACAAGGGTG:1300
D649 :TATGACTGGCGGCAACCCAGATAGGAACCCCAATC--------------------------------------------------------TCAACATCAGTCTCACTCTCGCCATCCTGCGCTACAAGGGTG: 934
U1   :TATGACTGGCGGCAACCCAGCTAGGAACCCCAATCgtaagcgccattttgtcgcctgctagcaactgtaatgtaacgtgttcccaaagTCAACATCAGTCTCACTCTCGCCATCCTGCGCTACAAGGGTG:1300
SB65 :TATGACTGGCGGCAACCCAGCTAGGAACCCCAATCgtaagcgccattttgtcgcctgctagcaactgtaatgtaacgtgttcccaaagTCAACATCAGTCTCACTCTCGCCATCCTGCGCTACAAGGGTG:1300
                                                        Intron VII


H97  :CACCTGAAGTCGAGCCAACCACTGTCAACGTCCCCGGACACAAGCTCCTTGATCAAGAGATGCATgtaagattaacccgcgcaaaaatgtcccgaaacgctcactaacgcacaccaatctacCCGATTGC:1430
D649 :CACCCGAAGTCGAGCCGACCACGGTTAATGTTCCCGGACACAAACTCCTTGATCAAGAAATGCAT-------------------------------------------------------CCGATTGC:1007
U1   :CACCTGAAGTCGAGCCAACCACTGTCAACGTCCCCGGACACAAGCTCCTTGATCAAGAGATGCATgtaagattaacccgcgcaaaaatgtcccgaaacgctcactaacgcacaccaatctacCCGATTGC:1430
SB65 :CACCTGAAGTCGAGCCAACCACTGTCAACGTCCCCGGACACAAGCTCCTTGATCAAGAGATGCATgtaagattaacccgcgcaaaaatgtcccgaaacgctcactaacgcgcaccaatctacCCGATTGC:1430
                                                         Intron VIII


                                                              - - - - - - - - - - - →
H97  :CCAGGAAGGACCAGGCAAAgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcgtctcatta:1560
D649 :CCAGGAAGGCCCAGGCAAA-----------------------------------------------------CTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAA---------------:1074
U1   :CCAGGAAGGACCAGGCAAAgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcgtctcatta:1560
SB65 :CCAGGAAGGACCAGGCAAAgtgtgtatatatttacatgaacataatcatcaactcaacgttctctagCTCGGTGACGGTCCCCCCGATAAGCACATCACCCTCAACATTGCTCAAgtaagcgtctcatta:1560
            Intron IX                                                        - - - - - - - - - - - →


H97  :ttcaaatcaaacttgacttctgacatgagcacagCCCAACGCTCCCTTCTTCGATATCAACGGAATCTCTTACATCTCCCCGACTGTCCCTGTTCTTCTCCAAATTCTCAGCGGAGCCAAGAGGCCCGAA:1690
D649 :----------------------------------CCCAACGCTCCTTTCTTCGATATCAACGGAATTTCTTACATTTCCCCCACTGTCCCTGTTCTTCTCCAAATCCTCAGCGGAGCCAAGAGGCCCGAA:1170
U1   :ttcaaatcaaacttgacttctgacatgagcacagCCCAACGCTCCCTTCTTCGATATCAACGGAATCTCTTACATCTCCCCGACTGTCCCTGTTCTTCTCCAAATTCTCAGCGGAGCCAAGAGGCCCGAA:1690
SB65 :ttcaaatcaaacttgacttctgacatgagcacagCCCAACGCTCCCTTCTTCGATATCAACGGAATCTCTTACATCTCCCCGACTGTCCCTGTTCTTCTCCAAATTCTCAGCGGAGCCAAGAGGCCCGAA:1690
          Intron X


H97  :GATGTTCTTCCATCCGAGCAAATCTTCTTTGTCCCCAAGAATTCATTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatgatcatcccattttctcctcatttcctctacttac:1820
D649 :GATGTTCTTCCATCCGAGCAAATCTTCTTTGTCCCCAAGAACTCATTGATCGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATT-------------------------------------:1261
U1   :GATGTTCTTCCATCCGAGCAAATCTTCTTTGTCCCCAAGAATTCATTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatgatcatcccattttctcctcatttcctctacttac:1820
SB65 :GATGTTCTTCCATCCGAGCAAATCTTCTTTGTCCCCAAGAATTCATTGATTGAAGTCAACATTCCAGGAGAAGGAGCTCATCCATTCCATTgtatgatcatcccattttctcctcatttcctctacttac:1820
                                                                                                   Intron XI
```

```
H97   :atccccgtttcagTGCACGGTCACAACTTTGATGTCGTTCTCGCCTCTAACGATGACACCTTCAACTTCGTGAAgtgagtcacctgaaagaaaaatatctagctgaatactaataccacatgaaagCCCT:1950
D649  :-------------TGCACGGTCACAACTTTGATGTCGTTCTCGCCTCTAACGATGACACCTTCAACTTCGTGAA-------------------------------------------------------CCCG:1326
U1    :atccccgtttcagTGCACGGTCACAACTTTGATGTCGTTCTCGCCTCTAACGATGACACCTTCAACTTCGTGAAgtgagtcacctgaaagaaaaatatctagctgaatactaataccacatgaaagCCCT:1950
SB65  :atccccgtttcagTGCACGGTCACAACTTTGATGTCGTTCTCGCCTCTAACGATGACACCTTCAACTTCGTGAAgtgagtcacctgaaggaaaaatatctagctgaatactaataccacatgaaagCCCG:1950
           Intron XII

H97   :CCTCGTCGTGATGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCTgtgagatttatatttacttttcggaaatgtttgtttctcaacgatacctcagTCACCGATAACCCAGGAGCATGGTTC:2080
D649  :CCTCGTCGTGATGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCT----------------------------------------------------TCACCGATAACCCAGGAGCATGGTTC:1404
U1    :CCTCGTCGTGATGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCTgtgagatttatatttacttttcggaaatgtttgtttctcaacgatacctcagTCACCGATAACCCAGGAGCATGGTTC:2080
SB65  :CCTCGTCGTGATGTATACCCCATCAACGGTGGCAACACCACTTTCCGCTTCTgtgagatttatatttacttttcggaaatgtttgtttctcaacgatacttcagTCACCGATAACCCAGGAGCATGGTTC 2080
                                                                                    Intron XIII

H97   :TTGCATTGgtgaggactttttttatccattcctctcgaagcgtatactgatttaacattgttacatagTCACATTGACTGGCATCTTGAAGCTGGTCTCGCTATCGTGTTCGCTGAGGCTCCGGAGGACAA:2210
D649  :TTGCATTG---------------------------------------------------------------TCACATTGACTGGCATCTTGAAGCTGGTCTCGCTATCGTGTTCGCTGAGGCTCCGGAGGACAA:1475
U1    :TTGCATTGgtgaggactttttttatccattcctctcgaagcgtatactgatttaacattgttacatagTCACATTGACTGGCATCTTGAAGCTGGTCTCGCTATCGTGTTCGCTGAGGCTCCGGAGGACAA:2210
SB65  :TTGCATTGgtgaggactttttttatccattcctctcgaagcgtatactgatttaacattgttacatagTCACATTGACTGGCATCTTGAAGCTGGTCTCGCTATCGTGTTTGCTGAGGCTCCGGAGGACAA:2210
                        Intron XIV

H97   :CGTCTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTGGTTGGATCTCTGCCCAGAATACAACGCAATCGAGCCTGAATTCCAGTAGTTCAGTTAAATATTTATCTTCCTCTCATAAGGAATCTATC--:2338
D649  :CGTATCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTGGTTGGATCTCTGCCCAGAATACAACGCAATCGAGCCTGAATTCCAGTAGTTCAGTTAAATATTTATCTTCCTCTCATAAGGAATCTATCTT:1605
U1    :CGTCTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATT----------------------------------------------------------------------------------------------:2248
SB65  :CGTCTCAGGTCCCCAGTCTCAGATCACTCCTCAAGATTG---------------------------------------------------------------------------------------------:2249
```

Figure A2 Alignment of *lcc*2 gene from the database (H97 and D649) and the U1 and SB65 strains of *A. bisporus*. Arrows indicate the forward and reverse primers used to generate the RNAi silencing cassette.