Genome-wide analysis of fitness data in *Ceratocystis albifundus*

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree MSc in Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for any degree at this or any other university.

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PREFACE

The genus *Ceratocystis* includes important tree pathogens that pose a threat to commercial forestry and plantation forestry. One of these, *Ceratocystis albifundus*, is an important pathogen causing Ceratocystis wilt on black wattle (*Acacia mearnsii*, indigenous to Australia), particularly of plantation trees. The disease is characterized by the presence of wilting, gummosis and in extreme cases dieback, which contributes to substantial economic losses, and considerable negative impacts in the forestry industry. In the last decade the fungus has also emerged as devastating pathogen of certain cultivars of orchard-grown *Protea cynaroides*, which is posing a significant risk to the cut flower industry in the Western Cape region of South Africa. Also, the fungus is commonly found on various indigenous tree species occurring in natural stands, where the plants display no apparent symptoms of infection or disease. It is unclear why *C. albifundus* does not cause disease on these plants, while being pathogenic on plantation or orchard-grown species. Therefore, the main goal of this study was to increase current knowledge for the pathogen's pathogenicity and fitness, by focusing specifically on populations collected in South Africa (including the Western Province, Gauteng, KwaZulu-Natal, Limpopo and Mpumalanga) from native tree species, as well as cultivated *P. cynaroides* in the Western Cape and Australian *Acacia* species, including *A. mearnsii*.

This is because these enzymes play crucial roles in the oxidation of phenolic compounds, which could assist with the breakdown of plant-produced compounds, either to access nutrients or to detoxify harmful substances. Therefore, the first chapter is presented as a review article focussing on plant-fungus interactions and the role of fungal laccases in their biology. The second chapter describes a genome-wide association study (GWAS) for identifying the genes and pathways associated with oxidation of the phenolic compound α-naphthol (i.e., used as an indication of laccase). In the third chapter, correlations between mycelial growth and pathogenicity to *Acacia mearnsii* plants are investigated, together with other traits (i.e., isolates' laccase production, reproductive potential, host plant and origin). Results of this study will thus complement the existing body of knowledge on the the ecology of *C. albifundus*.

The research conducted during this study were performed at the Forestry and Agricultural Biotechnology Institute (FABI), Department of Biochemistry, Genetics and Microbiology at the University of Pretoria, as well as at the Agricultural Research Council- Biotechnology Platform (BTP). It was performed under the supervision of Drs. Magriet van der Nest, Lieschen

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This thesis consists of three independent units and, therefore, contain some duplicated information and references. Chapter 1 is written as a review article based on the available literature, whereas Chapter 2 and Chapter 3 are written in the format required for submission to scientific journals.

Chapter One

Literature study: Plant-fungus interactions and the role of fungal laccases

1.1. INTRODUCTION

Many fungi obtain their energy from woody plant biomass (Goodell et al., 2020), which consists of carbohydrates, lignin, extraneous organic molecules, and minerals (Cragg et al., 2015; Kameshwar et al., 2019). Most of these wood degraders form part of the phyla Ascomycota or Basidiomycota, which are sister clades that together represent the Dikarya (Hibbett et al., 2007). Members of this subkingdom is characterized by the ability to establish a dikaryon (i.e., cells containing two sets of distinct nuclei without karyogamy) due to the cytoplasmic fusion of two haploid or monokaryotic hyphae (Hibbett et al., 2018). The main diagnostic differences between the two phyla pertains to the extent of their dikaryotic phase, and how their reproductive spores (meiospores) are formed and borne on meiosporangia (i.e., the fruiting structures that produce spores resulting from sexual reproduction) (Hibbett et al., 2018). Ascomycota produce meiospores (known as ascospores) in sac-like meiosporangia (known as asci), and their dikaryotic mycelium is typically short-lived and restricted to the zone of interaction between different individuals. Basidiomycota bear their meiospores (known as basidiospores) exogenously on basidia, and these fungi usually have prolonged, free-living dikaryotic phases (McLaughlin et al., 2009; Peraza Reyes and Berteaux-Lecellier, 2013; Hibbett et al., 2018; Ogidi et al., 2020).

Ascomycota and Basidiomycota also differ in how they decay woody plant biomass, because evolution afforded each group with distinct strategies, although there may be overlaps. Overall, however, the two phyla differ in their mechanism and ability to utilize lignocellulosic substrates (Janusz et al., 2013; Cragg et al., 2015; Janusz et al., 2017). This ability is conveyed by the networks and cocktails of enzymes they produce and which facilitate breakdown of the lignocellulosic substrate matrix of the plant cell wall (Filler, 2001; Levasseur et al., 2008, 2013; Kameshwar and Qin, 2017). These enzymes are classified as CAZymes (carbohydrate-active enzymes (CAZymes) that catalyse, degrade, synthesize or modify carbohydrates and glycoconjugates (Levasseur et al., 2013; Lombard et al., 2014; Ohm et al., 2014). CAZymes are separated into different classes based on amino acid sequence similarities, protein folds, and enzymatic mechanisms (Levasseur et al., 2008, 2013). Classes involved in the breakdown of carbohydrates include glycoside hydrolases (GH) that catalyse the hydrolytic cleavage of

the glycosidic linkage to form carbohydrate hemiacetal, carbohydrate esterases (CE) that catalyse the de-*O* or de-*N*-acylation of substituted saccharides, as well as polysaccharide lyases (PL) that cleave uronic acid-containing polysaccharides via a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new reducing end at the point of cleavage (Levasseur et al., 2013). Glycosyltransferases (GT) catalyse the formation of the glycosidic linkage to form a glycoside, which is the only class involved in the assembly of carbohydrates (Levasseur et al., 2013).

In terms of substrates involved in lignocellulose conversion, the CAZyme class named "auxiliary activity" (AA) is important and includes many families of ligninolytic enzymes (Levasseur et al., 2008). For example, a group of fungal AA enzymes known as lignin oxidases belong to the multicopper oxidase family, the members of which degrades phenolic compounds such as lignin (Baldrian, 2006). Notable members of the AA class include the multicopper oxidase family (CAZyme AA1), lignin-active class-II peroxidases (CAZyme AA2), as well as chloroperoxidase, manganese peroxidase, lignin peroxidase, versatile peroxidase, and cellobiose dehydrogenase (Levasseur et al., 2008). The AA1 multicopper oxidases are composed of subfamilies AA1_1 (laccases), AA1_ 2 (ferroxidases), and AA1_3 (laccase-like multicopper oxidases), of which the subfamily accounting for the majority is the AA1_1 oxidoreductase that can oxidize both phenolic and non-phenolic substances (Levasseur et al., 2008). In their genome-based analyses, Levasseur et al. (2013) showed that many Basidiomycota encode for lignin-active class-II peroxidases (AA2), which was absent from the Ascomycota genomes examined. The Ascomycota genomes also lacked genes encoding for ligninolytic laccase (AA1_1) genes, but the authors did find genes encoding for laccase-like multicopper oxidases (AA1_3) in these fungi.

In fungi, laccases are assumed to perform various physiological functions. These enzymes play a vital role in the development and production of spores and pigments such as melanin (Sbaghi, 1996; Lu et al., 2017), as well as pathogenicity to plants (Mayer, 2002). Laccases also influence virulence through various mechanisms, including lignin degradation to mediate the pathogen's infection of the plant and to utilize the plant tissue for nutrients (Thurston, 1994; Arora and Sharma, 2010). Laccase can also afford the fungus protection from host defence responses by increasing its cell wall integrity or supporting the cell wall through melanin production (Giardina et al., 2010). This is because melanin acts as a protective barrier against oxidative stress caused by the plant during the invasion by the pathogen (Litvintseva and Henson, 2002).

During melanin formation, laccase catalyses the conversion of diphenolic compounds such as l-3,4-dihydroxyphenylalanine, norepinephrine, epinephrine, and other related aromatic compounds to quinones, which can then rapidly autopolymerize to form melanin. However, despite their importance in diverse biological processes, most previous studies on laccases focused on Basidiomycota, especially the white-rot fungi, as they are the only fungi capable of fully degrading lignin (Ohm et al., 2014; Cragg et al., 2015; Andlar et al., 2018).

Fungal pathogens represent an important threat to native and commercial plants (Wingfield et al., 2008; Macpherson et al., 2017). As a result, plants have evolved several mechanisms to prevent attacks from these pathogenic organisms that can either be pre-existing or induced during pathogen infection (Maor and Shirasu, 2005; van der Does and Rep, 2007). For example, *Fusarium* species tend to produce plant hormones to promote fungal growth and reproduction by manipulation of the plants' hormones (Maor and Shirasu, 2005). In turn, pathogens evolve due to a need for overcoming the defence mechanisms of its host to allow infection and disease development, while the host needs to overcome the pathogen's onslaughts (Anderson et al., 2010). This constant selective pressure on the host to defend itself against the pathogen and vice versa is widely referred to as the biological arms race (Shittu et al., 2021a).

Understanding the interaction of plants and fungal pathogens is vital (van der Does and Rep, 2007). This also extends to a thorough understanding of the co-evolution of these organisms to outcompete each other (Shittu & Obiazikwor, 2021b). In this review, the overall focus is on the mechanisms (particularly those involving biochemical compounds) fungi use to overcome the structural and chemical barriers plants use to defend themselves. For this reason, the role of the plant cell wall during plant-fungal interactions is discussed, and the differences are highlighted in how Basidiomycota and Ascomycota utilize the plant cell. An overview of the important biochemical defences used by fungi for overcoming the stresses and hurdles presented by the plant's defence mechanisms is given. Because of their central role in these processes, the next section of the review considers multicopper oxidases, with special reference to laccases. The review is concluded with a section on the fungus *Ceratocystis albifundus*, which forms the focal species for the other chapters in this dissertation.

1.2. PLANT CELL WALL AND PLANT-FUNGUS INTERACTIONS

Plants have evolved several structural defence mechanisms against pathogens to prevent infection (Shittu et al., 2021a). For example, plant stomata and lenticles have evolved to prevent entry of pathogens (Cao et al., 2001; Shittu et al., 2021a). Water-repellent plant surfaces (known as wax) prevent the formation of water films that is essential for fungi to germinate (Marcell and Beattie, 2002), while a thick cuticle prevents the pathogen penetrating the plant surface (Horsfall and Cowling, 1978; Marcell and Beattie, 2002). Plants also produce callose (a sugar polymer that consists of β-1,3 linked D-glucose units) that reinforce the cell wall to prevent the penetration of pathogens (Maor and Shirasu, 2005). However, if the pathogen has penetrated the plant surface, the epidermal cell wall is the next layer of defence against the invading pathogen (Doughari, 2015). It is difficult for pathogens to penetrate the thick and tough outer epidermal layer that consists of polymers such as cellulose, hemicelluloses, lignin and polymerized organic compounds (Vance et al., 1980).

Plants have developed several histological defence structures that prevent the spread of pathogens. For example, the formation of an abscission layer creates a gap between healthy and diseased tissues, which prevents the spread of the pathogen (Odjakova and Hadjiivanova, 2001). The formation of a cork layer prevents the spread of infection, as well as the spread of toxic substances produced by the pathogen (Hutcheson, 1998). Gum deposition creates a barrier that prevents the spread of infection from diseased to healthy tissues (Shittu et al., 2021a), while tylose formation deprives water to the point of infection (Jibril et al., 2016). The last line of defence includes morphological and/or chemical changes, for example cell wall inflammation that limit hyphal penetration (Jibril et al., 2016). Reorganization of the cell cytoplasm also prevents the development of the hyphae by disintegrating the hyphae into smaller granular bodies (Horsfall and Cowling, 1978; Jibril et al., 2016; Shittu et al., 2021a).

The plant cell wall represents the first line of defence against pathogen infection (Miedes et al., 2014; Underwood, 2012). It physically separates the plant from the outside environment and organisms seeking access to water and the nutrient rich protoplasts protected by the cell wall (Hückelhoven 2007). Access can only be gained via small wounds created by physical damage or enzymatic disruption of the plant cell wall (Cantu et al., 2008; Underwood, 2012). In the absence of physical damage, fungal infection of plants is thus dependent on the make-up and integrity of the plant's cell wall.

1.2.1. Composition of the plant cell wall

The plant cell wall represents a complex structure that is composed of three layers (Goodell et al., 2020). The three layers includes a middle lamella, and a primary wall that are both composed of cellulose (polymers consisting of β-1,4 linked D-glucose units), pectin (heteropolysaccharides rich in galacturonic acid), and hemicellulose (polymers consisting of linked units of sugars such as xylose, mannose and arabinose), as well as a secondary wall that consists of three compartments (S1, S2, and S3) composed of cellulose, hemicellulose, and lignin (Figures 1.1 and 1.2).

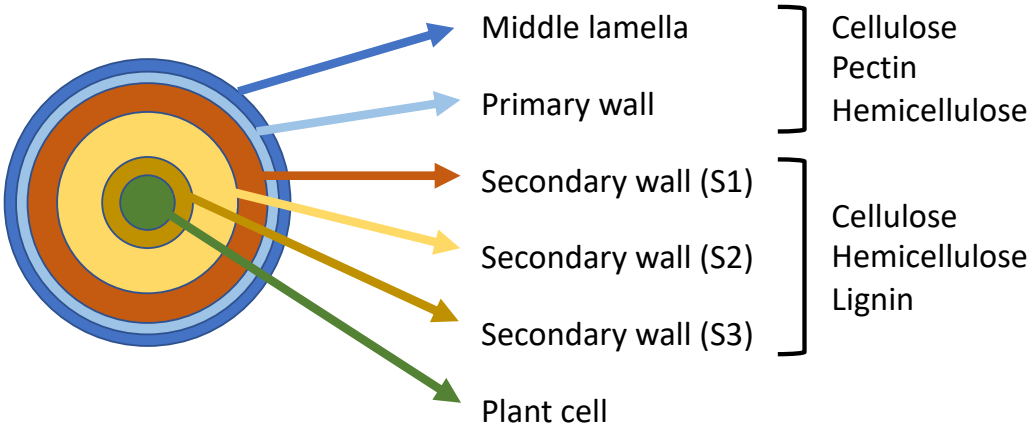

Figure 1.10. Diagrammatic representations of the layers of the plant cell wall (adapted from Online Science Notes available at https://onlinesciencenotes.com/differences-between-primary-and-secondary-cell-wall-in-plants/). The various layers are as follows:

1. *Middle lamella.* This layer represents the "glue" that attaches the primary cell walls of neighbouring cells to one another. It consists of calcium and magnesium pectates.

2. *Primary cell wall.* This layer is present in all plant cells and is characterized by a relatively low cellulose content and high hemicellulose, protein and lipid contents. It accounts for 40-44% of the cell wall and grows in thickness by the deposition of more cellulose. Cellulose polymers, held together by hydrogen-bonding among them, form microfibrils with high tensile strength. In this layer, the cellulose microfibrils are short, wavy and loosely arranged, affording the growing cell elasticity and expandability.

3. *Secondary cell wall.* This layer is present in only certain plant cells and contains additional compounds such as lignin and suberin. The layer is characterized by a relatively high cellulose content and low hemicellulose, protein and lipid contents. The secondary cell wall accounts for 15-32% of the cell wall and grows in thickness by the gradual accumulation of additional layers. Its cellulose microfibrils are long, straight and compactly arranged, thus making it rigid. The secondary cell wall may have distinct layers (S1, S2 and S3) due to the unique direction of the cellulose microfibrils in each.

The plant cell wall is highly dynamic, consisting of a specialized network compounds and polymers (Figure 1.2; Ochoa-Villarreal et al., 2012). However, their structure and composition largely depend on the plant's taxonomic identity. Also, separation between the composition and make-up of primary and secondary cell walls are not straightforward (Ochoa-Villarreal et al., 2012). Overall, the cell wall represents a heterogeneous mixture of cellulose, hemicellulose and pectin, some proteins and lignin, where the pectin provides an amorphous, gelatinous matrix within which the cellulose and hemicellulose are embedded, with everything being stabilized by the proteins and phenolic compounds. This means that all components occur together, and it is only their quantities that might differ between the primary and secondary cell walls. For example, lignin occurs together with cellulose, hemicellulose and pectin, but it is mostly associated with secondary cell walls. Lignin can also cross-link different cell wall polysaccharides as it covalently binds hemicellulose, thus conveying mechanical strength to the cell (Ochoa-Villarreal et al., 2012).

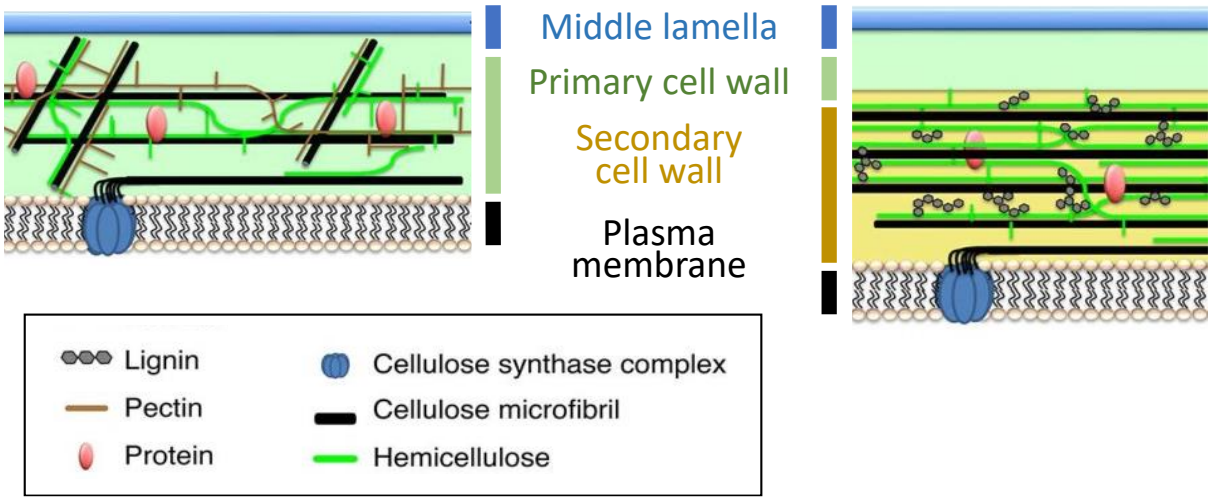

Figure 1.2. Schematic depictions of the primary (left panel) and secondary (right panel) cell walls of plants, (adapted from Nakano et al., 2015). The inset provides a key to the various symbols used in the two panels.

1.2.2. Utilization of the plant cell wall by Basidiomycota and Ascomycota

The breakdown of all components of the plant cell wall is required for a fungal pathogen to gain access. This includes disruption and breaking down of the three-dimensional phenolic network lignin that had been gradually deposited upon maturation of the cell wall. Lignin consists of an aromatic backbone composed of dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (p-hydroxyphenyl, H)

phenylpropanoid units that are linked by C-C and aryl-ether linkages (Levasseur et al., 2013). Fungi differ widely in their capability to degrade lignin, hemicellulose, and cellulose (Goodell et al., 2008; Kameshwar and Qin, 2017; Lundell et al., 2010; Broda, 2020).

Based on their ability to degrade lignin, fungi can cause soft, brown, or white rot. Ascomycota generally cause soft rot as they digest wood polysaccharides (mainly cellulose and hemicellulose) and not lignin, while Basidiomycota can cause either white or brown rot involving the decomposition of lignin (Baldrian, 2006; Arora and Sharma, 2010; Janusz et al., 2017; Kameshwar and Qin, 2017). Only white-rot fungi can fully degrade lignin, which gives the wood a white appearance (Ohm et al., 2014; Cragg et al., 2015; Andlar et al., 2018). Brown-rot fungi cannot fully degrade lignin, and lignin is left in the wood in an oxidated state, causing the decayed tissue to have a brown appearance (Cragg et al., 2015; Andlar et al., 2018). During brown or white rot, the decay process may in some instances also involve digestion of cellulose and hemicellulose (Kuës, 2015). Studies have reported that brown rot fungi have evolved from the saprotrophic white-rot fungi by losing several essential genes encoding for lignocellulose-degrading enzymes (Arantes and Goodell, 2014).

Compared to Basidiomycota, few studies have investigated lignin degradation by fungi in the Ascomycota. This is because very few fungi in this phylum are known to degrade this cell wall component. Their most common mode of degradation of moist wood results in the loss of texture (powdery dark wood), as well as dark discoloration of the wood (Daniel, 2014; Kameshwar and Qin, 2017; Andlar et al., 2018). Soft-rot fungi form cavities in the secondary cell wall on the cellulose microfibrils (Daniel, 2014; Kameshwar and Qin, 2017). Despite these differences, both Basidiomycota and Ascomycota are essential for nutrient recycling associated with woody plant biomass (Kuës, 2015).

1.3. BIOCHEMICAL DEFENCES DURING PLANT-FUNGUS INTERACTIONS

1.3.1. Plant defences

Plants have evolved biochemical defence mechanisms against pathogens to prevent infection (Shittu et al., 2021a). An important defence mechanism by the plant includes the accumulation of organic substances that act as inhibitors or antagonist against pathogens (Dangl and Jones, 2001; Leong et al., 2002). This includes proteins associated with plant development (e.g.,

chitinases and ß- glucanases) (Castroverde et al., 2010), as well as small proteins rich in the amino acid cysteine (e.g., lectins, defensins, proteinases and amylases) (Facchini and St-Pierre, 2005; Doughari, 2015). It also includes fatty acids such as phenolics (compounds that contain one or more benzene rings and hydroxyl groups) that are produced close to the site of infection to inhibit the pathogen from spreading to uninfected tissues/sites (Fritig et al., 1998; Hutcheson, 1998). Due to the energy required, these biochemical defenses are only made in significant quantities after an attack (Odjakova and Hadjiivanova, 2001). For example, plants only produce toxic phenolics (mainly phytoalexins) after an attack to interrupt metabolism or cellular structures of pathogens (Hammerschmidt, 1999; Agrios, 2005).

As part of the defence mechanisms, plants use metabolic processes to detoxify toxins produced by the plant (as well as those produced by the pathogen) (Shittu et al., 2021a). This is because, as part of the hypersensitive response, free radicals (e.g., superoxide, and hydrogen peroxide) are released, which is harmful for pathogens and the plant (Freeman and Beattie, 2008). When an avirulent gene product is released into the host plant to trigger the hypersensitive response, the enzymes and toxins produced as part of the plant defence are localized to the infected cells (Dangl and Jones, 2001; Harvey et al., 2008). The free radicals produced by the plant limit the spread of the pathogen due to the increased redox and oxidative stresses imposed upon it (Williamson, 1997a; Iakovlev and Stenlid, 2000; ÎAkovlev, 2001; Glass and Dementhon, 2006).

1.3.2. Defence mechanism of fungi against the plant host's defence response

Fungi have evolved several biochemical defence mechanisms against the host's defence response. For example, fungal pathogens can use such defenses to protect themselves against oxidative attack from the host (Williamson, 1997; ÎAkovlev, 2001). Fungi also secrete multicopper oxidases such as laccases for protection against the harsh biochemical environment created by the host (Karkowska-Kuleta et al., 2009; Prakash et al., 2019; Grigoriev et al., 2011). Fungal laccases are involved in wood degradation, degradation of phenolic compounds, as well as in protection of the fungus against oxidative attack from the host (Baldrian, 2006; Feng et al., 2015). Research has shown that the laccase genes *lcc1* and *lcc3* in the tomato pathogen *Fusarium oxysporum*, the *lac3* in the cereal root pathogen *Gaeumannomyces graminis var. tritici* as well as *lac1* in the mango decolourizer *Colletotrichum gloeosporioides* influence pathogenicity (Litvintseva and Henson, 2002; Canero and Roncero, 2008; Wei et al., 2017). This is because the rate at which these fungi are

capable of degrading plant material is believed to be a direct consequence of the genes encoding the enzymes responsible for lignin degradation (Kameshwar and Qin, 2017).

It is well-documented that the mycelia of fungi in the phyla Basidiomycota and Ascomycota secrete laccases, which aid them in overcoming the host defence response (McLaughlin et al., 2009). However, the function of laccases differs depending on the fungal species (Laufer et al., 2006). Below, I summarize the available information on multicopper oxidases, especially those occurring in fungi. I also briefly discuss the three recognized subfamilies of multicopper oxidases (Levasseur et al., 2008; Levasseur et al., 2013).

1.3.3. Multicopper oxidases

This family of enzymes (CAZyme class AA1) belong to a group of copper-containing proteins that participate in oxygen transport and activation and electron(s) transfer in redox processes (Crichton and Pierre, 2001; Baldrian, 2006). It's members all catalyse oxido-reductions, where the substrate being oxidized (i.e., aromatic phenols and amines such as hydroquinone and p-phenylenediamine) represents the electron donor, with molecular oxygen (O_2) as the final acceptor (Figure 1.3). In other words, these enzymes reduce O_2 to H_2O without the concomitant production of reactive oxygen species (ROS), which are biologically harmful molecules (Janusz et al., 2020). The only other enzyme capable of this four-electron reduction reaction is cytochrome c oxidase (Solomon et al., 2008).

$$4H^+ + 4\text{substrate} + O_2 \xrightarrow{\text{MCO}} 2H_2O + 4\text{substrate}^+$$

Figure 1.3. The chemical reaction catalysed by enzymes in the multicopper oxidase (MCO) family (CAZyme class AA1). Substrates are diverse, but all represent diphenolic molecules or similar compounds (Hakulinen et al., 2002; Solomon et al., 2008; Levasseur et al., 2013).

During multicopper oxidase-catalysed reactions, electrons are accepted at the enzyme's mononuclear copper centre and transferred to its trinuclear copper centre, from where four electrons are transferred to an O_2 (bound to the trinuclear copper centre) to mediate its reduction to two molecules of water. The resulting radicalised phenolic compound is highly reactive and can undergo homo- and hetero-coupling to mediate the synthesis of dimeric, oligomeric, polymeric, or cross-coupling products (Solomon et al., 1996). However, they can also mediate depolymerization of complex organic molecules by non-specifically cleaving of the inter-unit

C-C or ether bonds in such molecules, which is typically associated with the formation of ROS due to partial reduction of O_2 (Solomon et al., 1996).

The multicopper oxidases of fungi are generally made up of three cupredoxin domains (Gräff et al., 2020). The only known exceptions are the Fet3 and Fet5 multicopper oxidases in yeast with six cupredoxin domains (Nakamura and Go, 2005). Cupredoxin is a small polypeptide that binds copper in its +1 oxidative state (Cu I) via a particular protein motif (referred to as the T1 binding site) (Roberts et al., 2002). The T1 copper binding site contains two histidine (His) residues, a cysteine (Cys) residue, and a methionine (Met) residue in the sequence His-Cys-His-Met. In this motif, the first three residues form a tight, triagonal coordination with the copper molecule, and this constrained environment stabilizes the Cu I molecule (Roberts et al., 2002). A cupredoxin domain usually consists of a β-sandwich with 7 strands in 2 β-sheets that are arranged as Greek-key β-barrel fold (di Patti et al., 1999; Hakulinen et al., 2002, 2006; Stoj et al., 2006; Mayer and Staples, 2002; Valderrama et al., 2003; Hakulinen and Rouvinen, 2015; Soni et al., 2020). Interestingly, these enzymes are often referred to as the blue-copper family of multicopper oxidases due to properties of their T1 copper binding site (Messerschmidt and Huber, 1990; Alcalde, 2007; Hakulinen and Rouvinen, 2015). With copper bound in this site, it has a maximum absorbance of about 600 nm, thus conferring a characteristic blue colour to the protein (Kuës and Ruhl, 2011; Reiss et al., 2013).

In multicopper oxidase, individual cupredoxin domains attaches to one another via copper ions located at copper binding sites referred to as T2 and T3 (di Patti et al., 1999; Hakulinen et al., 2002, 2006; Stoj et al., 2006; Hakulinen and Rouvinen, 2015). T2 and T3 copper binding sites occurring between domains consist only of His residues, where four residues from each site coordinates binding of copper and clustering of the sites. This leads to the formation of a trinuclear copper centre, with T2 binding one and T3 binding two copper (T3 and T3') molecules (as opposed to the mononuclear centre of T1) (McCaig et al., 2005; Kosman, 2010; Kuës and Ruhl, 2011; Hakulinen and Rouvinen, 2015).

The different copper binding sites are responsible for the multicopper oxidase reaction (Figure 1.4). In the fully reduced enzyme, the T1 copper removes one electron from one molecule of the substrate and transports it via the His-Cys-His motif to the coppers at the T2-T3 centre (Gräff et al., 2020). This changes the oxidation state of the coppers to +2 (Cu II) and mediates the cleavage of molecular oxygen, while the appearance of water allows the enzyme to return

to the reduced state. Therefore, the oxidase mechanism requires the protein to temporarily "store" four electrons before the reduction of oxygen (di Patti et al., 1999; Hakulinen et al., 2002, 2006; Stoj et al., 2006; Hakulinen and Rouvinen, 2015), and it is the geometric and electronic structure of the trinuclear copper centre that ultimately facilitate the reductive cleavage of the O-O bond (Solomon et al., 2008).

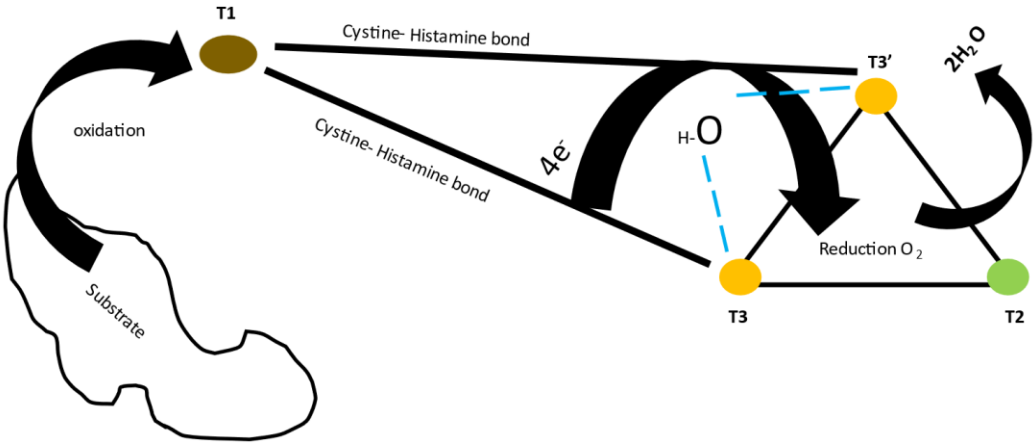

Figure 1.4. Schematic representation of the reaction that takes place in multicopper oxidases. At T1 various substrates bind and are oxidized. Four electrons are transferred to the trinuclear centre composed of T2 and T3 coppers, where oxygen is reduced to a water.

Common substrates used in the multicopper oxidase reaction are aromatic phenols and amines such as hydroquinone and p-phenylenediamine (Hakulinen et al., 2002; Levasseur et al., 2013). Multicopper oxidases may be further categorized based on their structure, function, and biochemistry. For example, the copper-containing motifs vary in terms of their spectroscopic traits, their electron paramagnetic resonance spectra, and the reactions they catalyse (Copete et al., 2015). According to the InterPro system (Paysan-Lafosse et al., 2022), multicopper oxidases (IPR045087) include amongst others the copper-type of nitrite reductase, (IPR001287), the L-ascorbate oxidase (IPR017762), laccase (IPR017761), division protein FtsP (IPR026589) and Copper-resistance protein CopA (IPR006376), of which the latter two are not found in fungi. According to the CAZyme system (Drula et al., 2022), multicopper oxidases have been separated into the subfamilies AA1_1 (laccases), subfamily AA1_2 (ferroxidases), and subfamily AA1_3 (laccase-like copper oxidase) (Levasseur et al., 2013). Among these CAZymes however, ferroxidases are different in that they utilize metals such as ferric iron as a substrate (Stoj et al., 2006).

1.3.3.1 Laccases (EC 1.10.3.2)

Laccase (benzenediol: oxygen oxidoreductase) is one of the oldest characterized enzymes (Yoshida, 1883; Thurston, 1994; Mayer and Staples, 2002; Giardina et al., 2010; Rodríguez-Couto, 2012; Giardina and Sannia, 2015). The enzyme was initially extracted and identified towards the end of the 19th century by Yoshida (1883) from the Japanese lacquer tree *Rhus vernicifera*. It was later characterized as a multicopper oxidase by Bertrand (1985). The basic reaction catalysed by laccases is indicated below in Figure 1.5.

$$4\ \text{benzenediol} + O_2 = 4\ \text{benzosemiquinone} + 2\ H_2O$$

Figure 1.5.

Figure 11.5. An overview of the basic chemical reactions catalysed by laccases (Revised from Kuës and Ruhl, 2011).

Laccases are copper metalloenzymes that contain four copper sites that are responsible for catalysing the oxidation of various substrates. These include *o*-phenols, *p*-phenols, aminophenols and phenylenediamines (Kuës and Ruhl, 2011). As with other multicopper oxidases (Figure 1.6), these compounds are oxidized with the concomitant four-electron reduction of oxygen to water (Thurston, 1994; Mayer and Staples, 2002; Giardina et al., 2010; Vrsanska et al., 2016). For the latter the proton transfer is assisted with the C-terminal carboxylate group in Ascomycota or an aspartic acid (Asp) residue in Basidiomycota (Kallio et al., 2011). Together with two serine (Ser) residues, the latter forms part of a Ser-Asp-Ser gate (or SDS-gate) that enhances the flow of protons from the phenolic substrate to the T1 centre, which is later transferred to the trinuclear copper cluster (Kallio et al., 2011; Moreno et al., 2017).

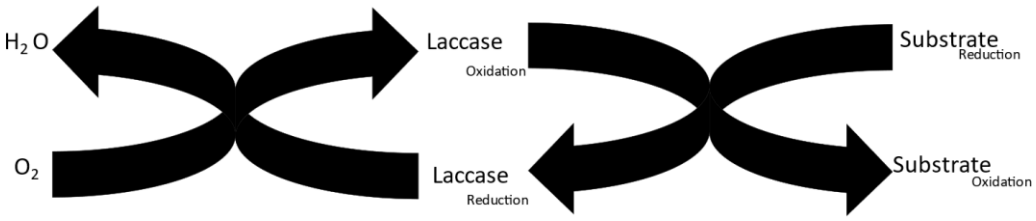

Figure 1.6. Schematic representation of the redox reaction that takes place in laccases, oxidation of the substrate, and the reduction of oxygen reduction to a water molecule.

1.3.3.2. Laccase-like copper oxidase (EC 1.10.3.-)

Laccase-like multicopper oxidases have a broader substrate specificity against urushiol (an unsaturated alkyl catechol) (Reiss et al., 2013; Berni et al., 2019; Zerva et al., 2021). The name for this group of enzymes were proposed by Reiss et al. (2013). Their function(s) is not yet fully understood, but it is assumed to be similar to those of laccase (Levasseur et al., 2008; Reiss et al. 2013). However, they are distinguished from bona fide laccases by the presence of conserved amino acid sequences (Reiss et al., 2013; Berni et al., 2019; Zerva et al., 2021). This group of multicopper oxidases also have extensive industrial applications (i.e., food processing, biofuel production, textile) and in plants they are responsible for postharvest plant physiology (Berni et al., 2019).

1.3.3.3 Other multicopper oxidases in fungi

Ferroxidases (Fe II: oxygen oxidoreductase; EC 1.16.3.1) are metalloxidase enzymes that are widely distributed in fungi, algae and mammals, where they catalyse the oxidation of ferrous iron II (Fe^{2+}) that is soluble in water, to ferric iron III (Fe^{3+}) that is insoluble in water (Lang et al., 2012). The basic reaction catalysed by these enzymes is indicated in Figure 1.7.

$$4Fe^{2+} + 4H^{+} + O_2 \longrightarrow 4Fe^{3+} + 2H_2O$$

Figure 1.7. Schematic representation of the basic chemical reactions catalysed by ferroxidase (Lang et al., 2012).

In these enzymes, the T1 site harbours the iron-binding site (Fe2+) and have conserved residues (i.e., three acidic residues, 1 glutamate, and 2 aspartates near the T1) (Copete et al., 2015). Iron is a critical metal but the concentration/levels of iron should be balanced, thus ferroxidase activity has been observed to play an important role in iron uptake and homeostasis in various organisms (Stoj et al., 2006, Lang et al., 2012, Copete et al., 2015). Fet3p is an iron protein alongside ferric permease (Ftr1p) responsible for the redox reaction that reduces the soluble ferrous iron to ferric iron to control iron homeostasis and uptake in yeast (Saccharomyces cerevisiae), whilst in mammals, ceruloplasmin and hephaestin are responsible for the homeostasis of iron in the brain and bloodstream respectively (Stoj et al., 2006). Iron has also been observed in contributing to the virulence of certain pathogens by employing ferroxidase activity to suppress iron cytotoxicity (Copete et al., 2015).

Nitrite reductase (EC 1.7.2.1; nitric-oxide: ferricytochrome-c oxidoreductase) is involved in nitrate assimilation in the denitrification pathway. This reaction is mostly associated with bacteria, but some protozoa, algae and fungi (mainly in the Sordariomycetes class of the Ascomycota) can also perform it (Mothapo et al., 2015). Phylogenetic studies pointed to a proto-mitochondrial origin for this enzyme in the denitrifying system in eukaryotes (Nakanishi et al., 2010; Chen et al., 2016). Similar to what happens in bacteria, fungal nitrite reductase catalyses the reduction of NO_2^- (nitrite) to NO (nitric oxide) to produce adenosine triphosphate (ATP) (Nakanishi et al., 2010). The enzyme localizes the mitochondrion, where the single electron needed for the reduction reaction is obtained from cytochrome *c* in the respiratory electron transport system (Suzuki et al., 1999; Nakanishi et al., 2010).

Ascorbate oxidase (L-ascorbate: O_2 oxidoreductase; EC 1.10.3.3) is most commonly encountered in plants, but it is also produced by certain animals and a few fungi (Pöggeler 2011; Braunschmid et al., 2022). It reduces molecular oxygen to water while oxidizing ascorbate to dehydroascorbate (Messerschmidt and Huber, 1990). Although not much is known about the diversity and function of these enzymes, their substrates are likely not strictly limited to ascorbate. For example, the enzyme can oxidise substrates with a lactone ring containing an enediol group next to a carbonyl group (Messerschmidt and Huber, 1990). Also, the enzyme from *Aspergillus flavus* can also oxidize ABTS (2, 2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid]), which is a common substrate for laccases (Braunschmid et al., 2022).

1.4. STRUCTURE OF FUNGAL LACCASES

Even though the vast majority of the available information on laccases is from Basidiomycota (i.e., due to the importance of laccases in lignin degradation), the available literature has broadened our understanding of the broad structure of laccases in fungi. Several authors studied the three-dimensional crystal structure of laccases to understand the contrast between and among these enzymes in Ascomycota and Basidiomycota. For example, an overview of the structure and function of Ascomycota laccases were obtained by comparing crystal structures for the TaLcc1laccase from *Thielavia arenaria* (Figure 1.8) and the MaL laccase from *Melanocarpus albomyces* (Kallio et al., 2011; Hakulinen et al., 2002). Among the Basidiomycota, crystal structures for laccases have been determined for *Trametes versicolor*

(Piontek et al., 2002), *Coprinus cinereus* (Ducros et al., 1998), *Lentinus tigrinus* (Ferraroni et al., 2007), and *Coriolopsis caperata* (Glazunova et al., 2015).

Laccase of both Ascomycota and Basidiomycota have three domains, namely I, II, and III, which are cupredoxin-like domain monomers accompanied by a β-barrels fold that attaches to the functional unit (Type I, II, III copper site) (Figure 1.8) (Kallio et al., 2011). However, laccases from these fungi differ in structure, pH, redox reaction potential, substrate specificity, and other properties (Mayer and Staples, 2002). Several factors, such as SDS gate amino acid, and C-terminus, accounts for the structural differences (Kallio et al., 2011). Mutant studies have been widely used in understanding the functions of laccases. For example, (Hakulinen et al., 2006; Hakulinen and Rouvinen, 2015) performed a study on laccase from *M. albomyces* to determine the function of C-terminals of laccase proteins. They demonstrated that the trinuclear site of the gene encoding the laccase protein present at the C-terminal provides stability to the structure, which is necessary for the function of the protein (Hakulinen et al., 2006).

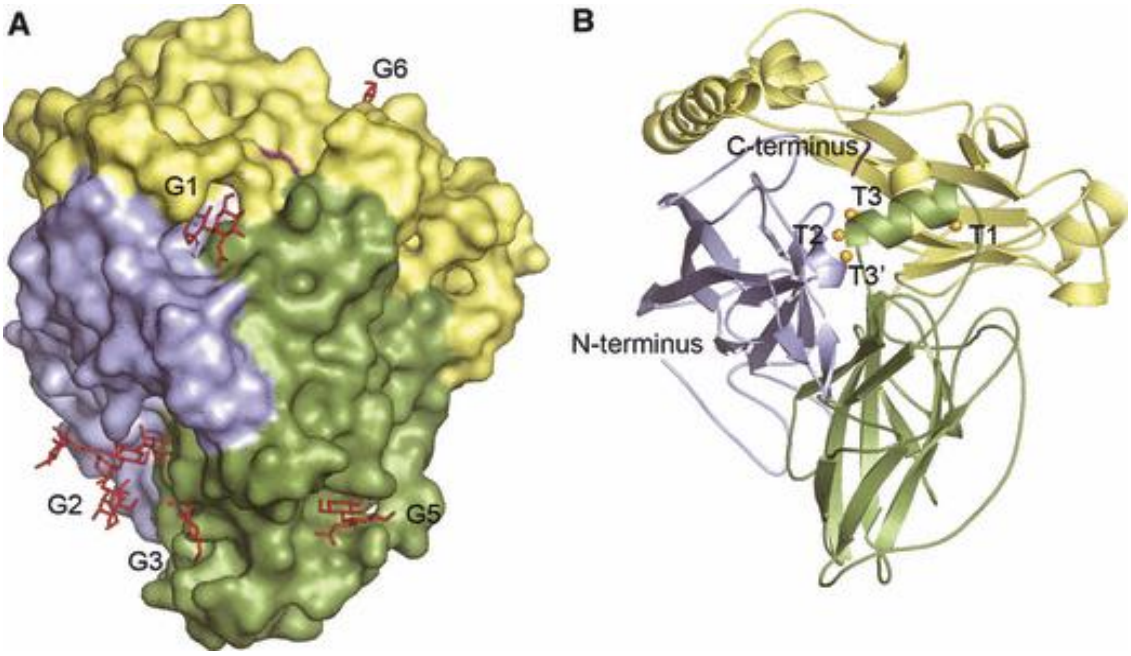

Figure 1.8. Overview details of the crystal structure of laccase TaLcc1 from *Thielavia arenaria*. (A) Surface model showing the associated *N*-glycans (oligosaccharides that are covalently attached to the protein) as red sticks, and the three cupredoxin domains in purple, green and yellow. (B) Representation of the laccase in cartoon format, where the catalytic coppers are shown in orange.

1.5. DISTRIBUTION OF FUNGAL LACCASE GENES IN BASIDIOMYCOTA AND ASCOMYCOTA

Laccases are encoded by a multigene family (Feng et al., 2015), but the number of laccase genes in different fungi is not conserved (Table 1.1). For example, laccase genes identified in the fungal phylum Ascomycota include *lac1* found in *Phoma sp*. (Junghanns et al., 2009), *lcc1-5* and *lcc9* found in *Fusarium oxysporum* (Canero and Roncero, 2008), *lacc1* and *lacc2* found in *Hortaea acidophila* (Tetsch et al., 2005), and *lacc* found in *Neurospora crassa* (Galagan et al., 2003). Laccase genes identified in the fungal phylum Basidiomycota include *pfaL* found in *Phanerochaete flavido-alba* (Rodríguez-Rincón et al., 2010), *Pr-lac2* found in *Phlebia radiata* (Mäkelä et al., 2006), *pel3* found in *Pleurotus eryngii* (Rodríguez et al., 2008), as well as *TvLac1* and *TvLac3-5* found in *Trametes versicolor* (National Center for Biotechnology Information, NCBI) (Janbon et al., 2014) (Table 1.1). In contrast to Ascomycota where laccase genes are generally clustered together in the genome, those of Basidiomycota are usually randomly distributed across the genome (Moreno et al., 2017). Overall, the genomes of Basidiomycota contain a greater number of laccase genes compared to Ascomycota (Moreno et al., 2017).

Table 1.1. Examples of fungal laccase genes showing that their numbers and lengths differ markedly, irrespective of taxonomic affilitations.

Organism name (strain number)	Gene name	Length (bp)	Protein (aa)	References
Ascomycota				
Neurospora crassa (OR74A)	*lacc*	2415	619	Galagan et al. (2003)
*Fusarium oxysporum f.*sp. *lycopersici* (4287)	*lcc1*	1755	585	Canero and Roncero (2008)
	lcc2	1920	640	
	lcc3	1869	623	
	lcc4	1698	566	
	lcc5	1890	630	
	lcc9	1638	546	
Hortaea acidophila (CBS 113389)	*lacc1*	1998	594	Tetsch et al. (2005)
	lacc2	1696	577	
Basidiomycota				
Phanerochaete flavido-alba (FPL 106507)	*pfaL*	4589	567	Rodríguez-Rincón et al. (2010)
Phlebia radiata 79 (ATCC 64658)	*Pr-lac2*	2889	521	Mäkelä et al. (2006)
Pleurotus eryngii (CBS 613.91)	*pel3*	3460	532	Rodríguez et al. (2008)
Trametes versicolor (FP-101664)	*TvLac4*	1563	521	*
	TvLac3	2074	520	
	TvLac1	2222	519	
	TvLac5	2266	527	

*Unpublished

The four copper site motifs encoded by laccase genes, along with the motif with His and Cys residues that hold the copper sites together, are highly conserved (Thurston, 1994; Pezzella et al., 2009). However, laccase genes differ widely in terms of the amino acid composition and multimeric quaternary structure (Pelley, 2011), as well as functions (Vrsanska et al., 2016; Moreno et al., 2017) of the proteins they encode. The expression of the specific laccase genes further varies based on environmental conditions (Bollag and Leonowicz, 1984; Rogalski and Leonowicz, 1992; Moreno et al., 2017). The kinetic parameters and regulatory properties of the laccase are also impacted by the availability of copper ions (Baldrian and Gabriel, 2002), as well as the substrates they interact with (Galhaup et al., 2002). For example, this may influence the ability of the fungus to perform/survive in different environmental conditions (Zhang et al., 2018).

1.6. CERATOCYSTIS ALBIFUNDUS

The Ceratocystidaceae (Phylum Ascomycota, Class Sordariomycetes, and Order Microascales) represent an important group of fungi, containing members with diverse ecological properties (de Beer et al., 2014). It includes various genera that are morphological similar, but that have wide geographic distribution and are associated with range of hosts and substrates (De Beer et al., 2014). These fungi further exhibit a range of ecological strategies, ranging from wound-inhabiting saprobes to serious plant pathogens (e.g., Roux et al. 2000; van Wyk et al. 2007; Heath et al. 2009; de Beer et al., 2014).

The economically important plant pathogens in Ceratocystidaceae infect various plant hosts. For example, *Thielaviopsis* species are pathogens of monocots, while *Ceratocystis* species are pathogens of dicots (de Beer et al., 2014). Among the latter, *C. albifundus* represent an important threat to commercial wattle-based plantation forestry in Southern and Eastern Africa (de Beer et al., 2014, Lee et al., 2015). In addition to wattles (i.e., *Acacia* species introduced into the region from Australia), the fungus infects various unrelated dicotyledonous tree species (Roux et al. 2007). The latter includes species native to the regions such as *Protea gigantea*, *P. grandiceps*, *Senegalia caffra*, *Burkea africana*, *Combretum molle*, *C. zeyheri*, *Faurea saligna* and *Ochna pulchra* (Roux et al. 2007).

1.6.1 Biological and genetic background

The ability of *C. albifundus* to cause disease on different plant species varies. While the fungus represents a serious threat to non-native wattle trees, it does not cause any disease symptoms on native tree species (Roux et al. 2007). However, it has also been reported as the causal agent of a disease outbreak in the Western Cape Province (South Africa) on orchard-grown *P. cynaroides* that is native to the region (Lee et al. 2016). It was subsequently theorized that the disease on commercially cultivated protea cultivars represent a host jump and subsequent invasion. This is because the fungus has not been found in this region prior to 2008, as well as the low overall genetic diversity of the fungus in the Western Cape Province (Lee et al. 2016). Furthermore, it was previously suggested that *C. albifundus* is a native African fungus that has undergone host shift to a non-native host (Roux et al. 2001; Barnes et al. 2005). These changes in host association might have been associated with the emergence of isolates that are more pathogenetic on the new hosts (i.e., wattle and *P. cynaroides*). This is consistent with the notion that after host jumps, selection favours fungal genotypes with high levels of aggressiveness to new hosts (Desprez Loustau et al. 2007; Pariaud et al. 2009).

To manage *C. albifundus* and reduce the economic losses caused by the pathogen, it is important to increase our understanding of the molecular mechanisms that the pathogen utilises to infect and cause disease. Such information will help to identify the genes and pathways that determine the outcome of the plant/pathogen interactions, as well as to why the pathogen cause disease native and not on non-native host.

1.6.2 Genomic resources

Because of their economic and ecological importance, the genome sequences for numerous members of the *Ceratocystidacea*e have been determined (Wilken et al., 2014; van der Nest et al. 2014; Wingfield et al. 2015). MycoCosm (Grigoriev et al., 2014) includes the genome sequences for only two members this family. However, as of 1 January 2023, the genome database of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/data-hub/genome/) included the genome sequences for 50 Ceratocystidaceae isolates, of which 24 were for species or isolates of *Ceratocystis*. For *C. albifundus* specifically, the NCBI database include the sequences for 7 isolates. Additionally, the sequence data for a population of South Africa *C. albifundus* isolates were available from a previous study. These unpublished data originated from an ongoing study launched

immediately prior to the COVID-19 pandemic, and were available for the research described in this MSc dissertation.

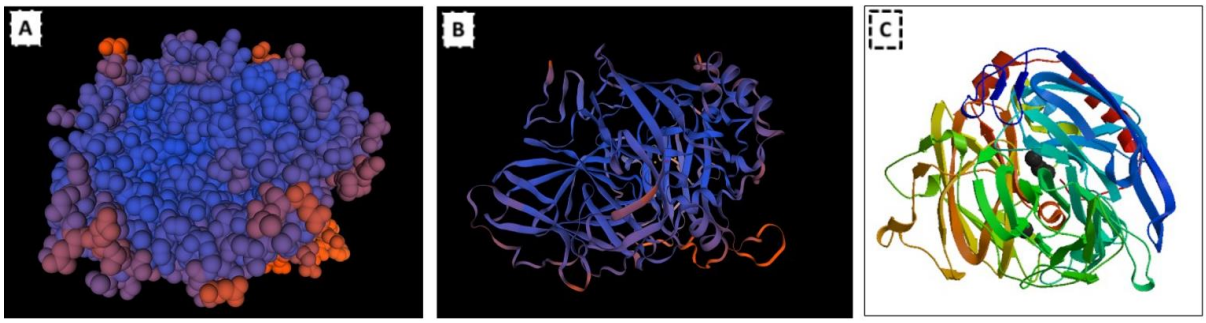

Figure 1.9. SWISS MODEL protein folding structures for a putative laccase from *C. albifundus* based on the best template match using SWISS Model Tools (https://swissmodel.expasy.org/). The surface (A) and ribbon (B) models for the *C. albifundus* laccase, and (C) ribbon model of the laccase from the *Myceliophthora thermophila* reference (Ernst et al., 2018).

Very little is known regarding the multicopper oxidases of *C. albifundus*. A routine BLAST search using a multicopper oxidase sequence from *F. oxysporum* against the available *Ceratocystis* genomic resources confirmed the presence of at least one laccase gene in *C. albifundus*. Its inferred amino acid sequence also conformed to that of the three-dimensional structure of other fungal laccases (Figure 1.9).

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CHAPTER 2

GWAS to identify genes and pathways associated with laccase activity in *Ceratocystis albifundus*

Abstract

Ceratocystis albifundus is an emerging pathogen of non-native *Acacia mearnsii* in Southern Africa. Although the fungus generally does not cause disease symptoms on native hosts, disease symptoms have been recently observed on commercial *Protea cynaroides*. Since laccases, an oxidase enzyme, have been implicated to influence plant-pathogen interactions, this study aimed to use a genome-wide association study (GWAS) approach to identify genes and pathways associated with oxidase activity across a South African *C. albifundus* population originating from a wide geographic and host range. These individuals were genotyped using low-coverage genome sequencing technologies and laccase activity was determined for each isolate. This study demonstrates that oxidative response represents a quantitative trait in this species and is associated with multiple genomic regions. This implies that oxidative activity may influence plant-pathogen interactions. Correlation between the single nucleotide polymorphism data for the isolates and their corresponding phenotype information allowed for the identification of a collection of genomic regions that were significantly associated with oxidase activity. For example, one of the regions contained a gene that codes for a protein in the multicopper oxidase superfamily, of which laccase is a member. However, further research experiments are required to validate computational study, with the ultimate goal, of improving our knowledge regarding pathogenesis in this economically important fungus.

2.1. INTRODUCTION

Ceratocystis albifundus (*Ceratocystidaceae, Microascales*, Ascomycota) and most other members of this genus are important plant pathogens (de Beer et al., 2014). In addition to *C. albifundus (*Heath et al., 2010; Lee et al., 2016; Roux et al., 2001), these include *C. fimbriata* (Engelbrecht and Harrington, 2005; Marincowitz et al., 2020; Oliveira et al., 2015; Suwandi et al., 2021; Webster and Butler, 1967), *C. eucalypti* and *C. fagacearum* (de Beer et al., 2017). *Ceratocystis* species are morphologically very similar and all have ascospores that are produced in sticky droplets with a strong aroma, which facilitate attraction and adhesion to insect vectors for dispersal (Kile, 1993; Wingfield et al., 2017). Despite their similar morphology, *C. albifundus* can be easily distinguished from other species based on the light colour of the base of the ascomata from which it derives its name (de Beer et al., 2014; Wingfield et al., 1996). This morphological characteristic is supported by DNA sequence comparisons of various housekeeping genes (de Beer et al., 2014; Marincowitz et al., 2020; Suwandi et al., 2021; Valdetaro et al., 2015; Wingfield et al., 1996).

Ceratocystis albifundus is widely distributed in Southern and Eastern Africa, having been reported from South Africa, Kenya, Malawi, Zambia, Tanzania, and Uganda (Roux et al., 2001, 2005). It is thought to have emerged in Africa, initial speculations pointing to an origin in South Africa (Roux et al., 2001), but populations from Uganda and South Africa both have high genetic diversity (Barnes et al., 2005) and this has led to a lack of consensus as to the origin. Nevertheless, in its natural habitat, *C. albifundus* needs wounds potentially caused by harsh weather, insect damage or pruning to infect its host plant (Heath et al., 2009, 2010; Lee et al., 2016; Nasution et al., 2019). Therefore, the widespread distribution of the fungus could be facilitated by movement due to wind and air currents, the activity of its insect vectors (e.g., Nitidulid beetles) (Heath et al., 2009), or as a result of human activity and movement (Lee et al., 2016).

The notion that *C. albifundus* is native to Africa, particularly South Africa, emerged from its apparent wide host range and propensity to only induce disease symptoms on plants that are grown commercially and/or that occur outside their natural ranges (Barnes et al., 2005). Hosts on which the fungus does not cause disease include native plant species such as *Combretum zeyheri, Burkea africana* and *Faurea saligna* (Roux et al., 2007). However, in the case of *Acacia mearnsii* and *Protea cynaroides*, *C. albifundus* is a significant pathogen associated with

substantial annual losses to the South African economy (Roux et al., 1999). On *A. mearnsii* (indigenous to Australia and extensively used in plantation forestry in South Africa), the fungus causes *Ceratocystis* wilt, a disease characterized by severe gummosis and die-back (de Beer et al., 2014; Morris et al., 1993). On *P. cynaroides* (indigenous to the South African Cape Floristic region and cultivated for the cut-flower market), infection by the fungus causes stem cankers (Lee et al., 2016). However, it is not well understood why *C. albifundus* has such a wide host range, nor is it known why the fungus differs in its ability to cause disease on only some of these plants. One possibility is that *C. albifundus* is not pathogenic to the plants with which it has co-evolved (Anderson et al., 2010), while an introduced species such as *A. mearnsii* would not have developed the requisite antifungal response for resisting disease (Roux et al., 2007). However, this does not explain why the fungus is also capable of causing disease in *P. cynaroides*, which has a native range overlaping with that of *C. albifundus*.

Fungal pathogens have developed mechanisms to by-pass plant host defense responses, especially the range of chemical compounds produced by the plant (Lattanzio et al., 2006, 2009; Westrick et al., 2021). To cope with such chemical onslaughts the pathogen produces an array of enzymes (Karlsson et al., 2008; Xu et al., 2015). For example, the pathogenic ability of fungi on various host species has been linked to the production of oxidases, especially multicopper oxidases such as laccase (benzenediol: oxygen oxidoreductase; E.C 1.10.3.2) (Mayer and Staples, 2002). Laccases influence the outcome of host-pathogen interactions because these enzymes oxidise the plant's phenolic defense compounds (McCaig et al., 2005). Previous studies have also demonstrated that laccases influence pathogenicity and host interaction in *Magnaporthiopsis maydis* (Degani & Goldblat, 2020), *Fusarium graminearum* (Soni et al., 2020), and *Colletotrichum gloeosporioides* (Wei et al., 2017). However, nothing is known regarding the oxidases or laccases in *C. albifundus*, nor their potential role during the interaction of the fungus with its host plants.

An efficient approach for investigating the molecular basis of a polygenic phenotype is to conduct a genome-wide association study (GWAS). Previously, GWAS was used to identify genomic regions associated with important traits in humans (Visscher et al., 2012), *Drosophila* (Burke et al., 2014), and many plant crop species (Sbaraini et al., 2017). However, to date, only a few studies have employed this approach for studying traits in fungi. These include ascomycetous model species such as *Saccharomyces cerevisiae* (Connelly and Akey, 2012) and *Neurospora crassa* (Palma-Guerrero et al., 2013), as well as the plant pathogens *F.*

graminearum (e.g., identified genes associated with aggressiveness and pathogenicity) (Talas et al., 2016) and *Parastagonospora nodorum* (e.g., to identify virulence factors) (Gao et al., 2016). GWAS was also used in the basidiomycetous fungus *Heterobasidion annosum* to identify possible virulence-associated genomic regions and their correlation to known virulence gene regions previously found using quantitative trait loci (QTLs) (Dalman et al., 2013).

As a consequence of the likely important role of oxidases such as laccases during the interaction of *C. albifundus* with its various plant hosts, the overall goal of this study was to use a GWAS approach for exploring the potential of this fungus to produce these enzymes. For this purpose, *C. albifundus* isolates from a wide range of hosts and geographic regions were genotyped using genome-wide single nucleotide polymorphism (SNP) analysis. The isolates were also phenotyped for laccase production by using the phenolic compound α-naphthol which undergoes a colour change when oxidized (Kulys et al., 2022). The latter data were then combined with the genotype data in a GWAS to identify genes and genomic regions underlying this phenotype. These analyses accounted for population stratification, which is well-known to affect the results of GWAS (Price et al., 2010). The findings of this study would thus provide a strong foundation from which to investigate if and how oxidase-related genes and their products influence the outcome of *C. albifundus* pathogen-host interactions.

2.2. MATERIALS AND METHODS

2.2.1 Fungal isolates

A total of 67 isolates of *C. albifundus* from South Africa were used for this study (Table 2.1). These isolates were collected from various indigenous hosts, non-native *Acacia* species occurring in the region, as well as known insect vectors of the fungus. All cultures were obtained from the CMW Culture Collection at the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria and preserved on MEA (20 gL^{-1} agar, 20 gL^{-1} malt extract, Biolab, Midrand, South Africa) media at 25°C and subcultured every 2 to 3 weeks.

2.2.2 Oxidase activity

The extent to which the respective isolates were capable of oxidizing α-naphthol (Sigma-Aldrich, USA) was determined using Petri plate-based assays. For this purpose, *C. albifundus*

isolates were grown for two weeks on 1% potato dextrose agar (PDA) (Biolab, Midrand, South Africa) medium, after which the plates were flooded with a solution of 5mM α-naphthol (made up using 96% ethanol) (Iakovlev & Stenlid, 2000). After incubation at 25°C for 2 hours, cultures were visually monitored for the development of a deep purple colouration, indicative of oxidation of α-naphthol by enzymes that the fungus produced (More et al., 2011; Shrestha et al., 2016). The latter was scored as 1, while isolates producing no colour change were scored as 0 (Table 2.1).

2.2.3 Genomes

Genome sequence sequences for all 67 isolates included in this study were used. Among these, the genomes of CMW17620, and CMW17274 were previously sequenced using the Illumina® platform (van der Nest et al., 2014, 2019). For isolate CMW4068, a high-quality hybrid assembly based on PacBio® RS II and Illumina HiSeq™ technologies is publicly available (van der Nest et al., 2019). For the remaining isolates, DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Möller et al., 1992; Murray and Thompson, 1980). The concentration of DNA was measured with a Thermo Scientific NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). These high-quality DNAs were then used to obtain single reads of 400 bp using the Ion-Torrent™ Ion S5™ system and Ion 530 Chip Kit at the Central Analytical Facility (University of Stellenbosch, Stellenbosch, South Africa). The sequence reads generated were then quality filtered with CLC Genomics Workbench v.10 (CLC bio, Aarhus, Denmark) (using P error limit = 0.05, max number of ambiguous nucleotides = 2) and Trimmomatic v.0.39 (Bolger et al., 2014; Kanzi et al., 2020) (using default settings (http://www.usadellab.org/cms/?page=trimmomatic).

2.2.4 SNP discovery

Quality-filtered reads were mapped to the largest 16 scaffolds of the publicly available genome of isolate CMW4068 as described above. This was done using the "bwa mem" command in Burrows-Wheeler Aligner (BWA) v.0.7.17 to align the filtered reads to the reference genome (Li, 2013; Li & Durbin, 2009). Default settings were implemented to align the filtered reads to the reference genome using the "ploidy" function, as *C. albifundus* is a haploid fungus. SAMtools v.1.9/0.1 was used to convert the SAM file into a BAM file, as well as to sort the BAM files (Li & Durbin, 2009). bcftools v.1.6.33 was used for genotype likelihood calculation and variant calling (Li & Durbin, 2009). SNP & Variation Suite (SVS) v.8.8.3 (Golden Helix

Inc., Bozeman, MT, USA; www.goldenhelix.com) was used for quality control and filtering of the SNPs. This was done using default settings for call rate (CR) <0.95, minor allele frequency (MAF), and pairwise linkage disequilibrium (LD) pruning, thus removing SNPs with MAF < 0, 05 and pairwise LD ≥ 0, 5. To remove duplicate samples between pairs of individuals, identity-by-state (IBS) values were estimated using SVS.

2.2.5 Population stratification, GWAS, and functional annotation of genes

To evaluate the possible impact of population stratification in GWAS (McCarthy et al., 2008; Power et al., 2017), the SNP data were subjected to Principal Components Analysis (PCA) (Patterson et al., 2006; Price et al., 2006) implemented using SNP & Variation Suite (SVS) v.8.8.3. Since oxidase/laccase is a complex trait controlled by several large-effect loci, a single-locus test would not be appropriate, especially in the presence of population structure. For this reason, we employed a multi-locus, mixed linear regression model (MLMM) (Miles et al. 2021) in SVS to test for association between the SNP and oxidase/laccase data by employing a P-value threshold of -log10 (Pawlowski et al., 2020). In this way the analysis corrected for possible cryptic relatedness among individuals (Kang et al., 2010; Korte et al., 2012; Segura et al., 2012). To account for biases associated with population stratification, kinship based on the PCA data was included as a random effect in the MLMM analysis.

The MLMM model uses a forward and backward stepwise approach to select markers as fixed effect covariates. This model performs an EMMAX (Efficient Mixed-Model Association eXpedited) scan through all markers and selects the most significant marker and adds it to the model as a new fixed-effect covariate ("cofactor"), creating a new model. This is repeated (forward inclusion) until a pre-specified maximum number of forward steps is reached. For each selected marker in the current model, this is temporarily removed from the fixed effects and an EMMAX scan is performed over only that marker. After a new smaller model is created, backward elimination is repeated until only one selected marker is left. The variance components are re-estimated between each forward and backward step, while the same kinship matrix is used throughout the calculations. Finally, to determine whether the P-values observed were inflated and whether they followed the null distribution, a quantile-quantile plot of expected versus observed p-values was generated (Rashid et al., 2018).

Putative genes associated with oxidase/laccase production in *C. albifundus* (i.e., genes found within 1 kb upstream and downstream of SNPs significantly associated with this phenotype)

were subjected to *in silico* functional annotation. To accomplish this, the Blast2GO plugin of CLC Genomics Workbench v.20 (Conesa et al., 2005) and InterProScan v.5 (Jones et al., 2014) were used to identify Gene Ontology (GO) terms and to characterize protein domains. Revigo was used to summarize the GO terms (http://revigo.irb.hr/) (Supek et al., 2011).

2.3. RESULTS

2.3.1 Oxidase activity

Results of the α-naphthol assay showed that the fungi examined varied in their ability to oxidize this phenolic compound (Figure 2.1). Out of the 67 *C. albifundus* individuals, 27 showed no or very little colour reaction when exposed to α-naphthol (scored as 0). The remaining 40 isolates reacted strongly (scored as 1) to this compound, the oxidation of which caused a deep purple coloration in the medium.

2.3.2 Genomes and SNP discovery

Quality filtering of the Illumina and Ion Torrent reads resulted in 9587601 to 10032768 reads per isolate, with mean read lengths ranging from 164 to 220 base pairs (bp) (Supplementary Table S2.1). Mapping of these reads to the reference genome yielded a total of 521828 polymorphic SNPs. Removal of SNPs with CR<0.95, MAF<0.05 and LD>0,5 yielded a dataset containing 112 558 SNPs. Additionally, all of the isolates were retained in the dataset as none had more than 5% missing data, nor did any of them represent duplicates of one another (i.e., all IBS-values were below 1) (Supplementary Table S2.2). Thus, the final dataset contained 68 individuals and 112 558 SNPs that were spread across the 16 scaffolds of the reference genome at a density of one SNP per 2,949 Kilobase pair (Kbp).

2.3.3 Population stratification and GWAS

The first two principal components of the PCA explained 22,76% of the total genetic variation in the data (Figure 2.2). Clustering based on geographic origin and/or plant host was detected among the isolates. The isolates associated with commercial or non-native hosts formed two separate clusters, with those from *P. cynaroides* sampled in the Stellenboshch region (Western Cape Province) grouping separately from the group of isolates obtained from *Acacia melanoxylon* in the Garden Route region (Western Cape Province) and *Acacia mearnsii* in KwaZulu-Natal Province. The isolates obtained from native trees in the Gauteng, Mpumalanga and Limpopo Provinces, as well as those originating from Nitidulid beetles (Gauteng Province) formed a separate cluster.

Analysis with the MMLM model allowed for the identification of 6 SNPs (P< 0,05) in the *C. albifundus* population. Six of the SNPs were significantly (P< 0,05; FDR< 0,05) associated with oxidase/laccase activity (Table 2.2) in the *C. albifundus* population. The P-values observed did not seem inflated (Rashid et al., 2018), and followed the null distribution (Figure S2.1). The genomic regions of the identified 6 SNPs were positioned based on the reference assembly of genome CMW 4068. These SNPs were located on scaffolds 2,4,8,10,15, linked to different plant-pathogen interactions (Figure 2.3). Gene sequence position, length, and GO terms within 10 kbp up- and downstream associated with the significant SNP included genes associated with oxidases activity and pathogenicity functions (Supplementary Table S2.3).

2.3.4 Functional annotation of genes

The genes located in the genomic regions identified using the GWAS approach encode proteins associated with oxidase activity and pathogenicity (Figure 2.4, Table 2.3). Genes associated with oxidoreductase activity included a putative NmrA-like family protein (g5407), a putative NAD(P)H dependent FMN reductase LOT6 (g5411) and a putative cytochrome P450 mono-oxygenase (g5385) on scaffold on 8 (MAOA02000031.1:957970), as well as a putative peroxidase (g7006) on scaffold 15 (MAOA02000024.1:365346) (Koch et al., 2017; Karlsson et al., 2008; Kong et al., 2020; Xu et al., 2015). Genes that encoded proteins that may be associated with pathogenicity included a gene (g2870) that encodes a WD40 repeat-like protein, a gene (g2867) that encoded a putative protein sey1, as well as a gene (g2862) that encodes a putative heat shock 70kDa protein (Dai et al., 2007; Sørensen et al., 2003; Xu et al., 2015) which were on scaffold 4 (MAOA02000035.1:301201). Interestingly, several genes detected across various scaffolds are the genes responsible for oxidase activity.

Revigo analyses of Molecular Function identified enriched GO terms associated with dioxygenase activity, enzyme regulator activity, FAD binding, 3-oxoadipate enol-lactonase activity, G protein-coupled activity, hydrolase activity lyase activity, lysine-acetylated histone binding, carbohydrate binding, iron sulphur cluster binding, etc. (Figure 2.5A). Revigo analyses of Biological Process identified enriched GO terms associated with double-stranded break repair involved in meiotic recombination, cellular potassium ion homeostasis, carbohydrate metabolic process, cell cycle, cell division, methylation, sporulation resulting in formation of a cellular spore, protein folding, electron transport chain, etc. (Figure 2.5B). Revigo analyses of Cell Component identified enriched GO terms associated with host cell

cytoplasm, extracellular region, membrane raft, membrane, cellular anatomical entity, etc. (Figure 2.5C).

2.3 DISCUSSION

This study showed that isolates of *C. albifundus* differed in their ability to oxidize the bicyclic phenolic compound α-naphthol. This is because an insoluble, violet or blue coloured precipate is formed during the oxidative oligomerization of α-naphthol (Kulys et al., 2003). The latter process involved radicalization of α-naphthol molecules, after which they are covalently linked to one another, while at the same time reducing molecular oxygen to water (Islam et al., 2014; Bollag et al., 1977). Although numerous environmental bioremediation and detoxification strategies involve the oxidative oligomerization of harmful polyphenolics, this reaction is also exploited in studies aiming to assay a microorganism's capacity for degrading particular polyphenolic compounds (Karigar and Rao, 2011). In fungi, for example, assays based on α-naphthol and other structural analogs of lignin, a complex polyphenol common plant tissue, have revealed the ability of certain species to transform and degrade this biopolymer (Harms et al., 2011). In such cases, however, α-naphthol oxidation is most often used as a presumptive indicator of laccase activity (Okino et al., 2000; Gramss et al., 1998; Burke and Cairney, 2002). Therefore, the blue colour observed in the α-naphthol assays was interpreted as likely reflecting the ability of *C. albifunudus* to produce laccase.

Laccase production by members of the *Ceratocystidaceae*, including *C. albifundus* is not surprising. This enzyme is produced by many fungi, where it is implicated in a range of biological activities, including lignin degradation (Baldrian, 2006; Burke and Cairney, 2002). Additionally, laccases have been demonstrated to play major roles during both the vegetative and sexual growth and development of various fungi. Laccases are also involved in pathogen-plant interactions during which these enzymes are thought to facilitate detoxification of plant-produced phenolics, as well as protection against the plant's oxidative responses to the pathogen's presence and activities (reviewed by Burke and Cairney, 2002). Laccase activity could thus be expected to be an important tool in the enzyme armory of a pathogenic, tree tissue-inhabiting fungus like *C. albifundus*.

Despite clear differences among the *C. albifundus* isolates that were capable and incapable of oxidizing α-naphthol, none of the SNPs significantly associated with this phenotype were

located in or near genes encoding laccase. This could reflect the fact that α-naphthol is not only oxidised by laccases, but also other phenol oxidases (Gramss et al., 1998), potentially leading to the blue coloration observed during assays (Kimura et al., 2012). For example, the genes located in the vicinity of α-naphthol oxidation-linked SNPs included those encoding for oxidoreductase, oxidases and peroxidase. However, the findings presented here probably also reflect the polygenic nature of laccase production as a phenotypic trait. Despite being the product of a single gene, expression and activity of laccase are dependent on a range of factors (e.g., nitrogen metabolism, carbon metabolism, stress responses, aspects of the fungal cell cycle), most of which are themselves subject to complex regulation (Rivera-Hoyos et al., 2013). Therefore, how laccase is produced in the presence of an inducer such as α-naphthol would be expected to vary among isolates. This is even more so for the collection of *C. albifundus* isolates that were specifically included to represent the diversity known for this fungus.

Several genes that were located in the genomic regions associated with laccase activity may influence the ability of *C. albifundus* to overcome the plant host defences. This included putative peroxidases, NAD(P) H-dependent FMN reductase LOT6, and Cytochrome P450 mono-oxygenase which are known to be associated with pathogenicity and virulence in microorganisms (Koch et al., 2017; Kong et al., 2020). The identified enriched GO terms associated with double-stranded break repair, cellular potassium ion homeostasis, protein folding and electron transport chain will help *C. albifundus* to overcome the plant host defences. The enriched GO terms associated with dioxygenase activity, hydrolase activity, lyase activity and others that are involved in the oxidative stress response may also influence the host-pathogen interactions by modifying plant chemicals used in the host defense response (Karlsson et al., 2008; Xu et al., 2015). However, it has also been demonstrated that plant pathogens use oxidoreductase activity to cause diseases in their host such as *Rhizoctonia solani* (Kant et al., 2019), *Glomerella cingulate* (Sygmund et al., 2011), *Candida albicans, Botrytis cinerea, Pseudomonas aeruginosa* (Koch et al., 2017), and *Magnaporthe oryzae* (Kong et al., 2020).

Several of the genes present in the genomic regions associated with laccase activity may encode proteins that are involved in pathogencity in *C. albifundus*. This included WD40 repeat-like protein that is involved in non-self- /self-recognition and Protein sey1 that is involved in GTP-dependent signaling (Chong et al., 2020; Praefcke and McMahon, 2004). Also included in the associated genes is a Heat shock 70 kDa protein which has previously been shown to influence

pathogenicity (Dai et al., 2007; Sørensen et al., 2003; Xu et al., 2015), as well as cellulases (related to endo-beta-1,4-glucanases) that have been reported to be involved in cell wall degradation important for host penetration and infection (Kikuchi et al., 2004; Walton, 1994; Xu et al., 2015). The presence of these genes is expected, since previous studies have underlined the role of plant-pathogen laccases in various hosts and attributed these to pathogenicity and virulence (Degani and Goldblat, 2020). *Magnaporthiopsis maydi* causing maize wilt produces plant-pathogen laccases in various conditions (Degani and Goldblat, 2020), while *Cryphonectria parasitica* laccase assist in neutralizing the tannic-acid rich environment associated with plant defense mechanism upon infection in chestnut (Mayer and Staples, 2002). *Heterobasidion annosum s.s* on scot pine was suggested to contain "a true virulence factor" annotated as the laccase gene *HaLCC15* involved in host plant interactions (Kuo et al., 2015). It is therefore possible that the genes identified in this study may be factors helping *C. albifundus* to attach/attack/invade the plant.

The current study adds to the growing body of work that demonstrates the value of GWAS for unravelling the molecular mechanisms and procesess underpinning biological traits in fungi (Sanglard, 2019; Sánchez-Vallet et al., 2018). As in previous studies (e.g., Broberg et al., 2018; Singh et al., 2022; Iqbal et al., 2020), it was possible to identify genetic regions containing SNPs that are significantly associated with the phenotype of interest. This was despite the relatively small number of isolates (n=68) included in the GWAS panel. Various previous studies could also make highly significant SNP-phenotype associations using such smaller GWAS panels (n<100; Sánchez-Vallet et al., 2018), with the number of isolates ranging from 23 or 24 (Dalman et al., 2013; Palma-Guerrero et al., 2013), 36 or 45 isolates (Mehmood et al., 2011, Guys et al., 2013), through to 63 (Connelly and Akey, 2012). This is because most fungi, including *C. albifundus*, are haploid (or have haploid stages) and experience mixed reproductive modes where sexual reproduction reduces genome-wide association (Bertucci et al., 2014; Dalman et al., 2013; Muller et al., 2011; Plissonneau et al., 2017).

In terms of population biology, PCA of the genome data for *C. albifundus* suggested that the nature of fungus's interaction with its plant host impacts the structuring of its populations. For example, isolates originating from apparently healthy native trees grouped clearly distinct from those obtained from diseased *Acacia* and *Protea* trees. Also, isolates obtained from diseased *Acacia* trees grouped separately from those obtained from commercially grown *P. cynaroides.* The separation between populations originating from diseased *Acacia* and *P. cynaroides*

tissues, as well as the groupings observed within the three bigger clusters are consistent with previous microsatellite-based studies (Lee et al., 2016). Although extensive population genetic analyses were not performed, the genome-wide SNP data did appear to have more resolving power than the microsatellites typically employed in population's studies of *C. albifundus*. It has also been shown in numerous other studies that genome-wide SNPs outperformed microsatellites in this regard (Plissonneau et al., 2017). The use of genome-wide SNPs would undoubtedly improve our understanding of the population biology of *C. albifundus* (especially regarding the drivers of it population dynamics), while also potentially shedding light on its native host and geographic range(s).

2.4. CONCLUSIONS

The findings presented demonstrated the power of GWAS for studying the molecular basis of important phenotypic traits in *C. albifundus* and fungal pathogens in general. The function of laccase has not yet been exploited in *C. albifundus,* especially one concerning virulence and pathogenicity. Future research should move toward validating the associations by characterizing the genes through gene knockouts and fungal transformations, as has been reported in previous studies (Dalman et al., 2013). Analysis of other fitness traits, such as that of laccase activity or pathogenicity would be of interest. Ultimately, the genomic regions associated with laccase activity in the population of *C. albifundus* isolates were determined, with a bright and challenging future ahead for fungal GWAS.

2.6. REFERENCES

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Table 2.1. Geographic origin and host of *C. albifundus* isolates screened for laccase activity.

Isolate number [a]	Geographic region	Substrate or host	Laccase activity [b]	Collector and year[c]
CMW4068*	KwaZulu-Natal	*Acacia mearnsii*	1	Roux J, 1997
CMW4065	KwaZulu-Natal	*A. mearnsii*	1	Roux J, 1997
CMW4075	KwaZulu-Natal	*A. mearnsii*	1	Roux J, 1997
CMW4090	KwaZulu-Natal	*A. mearnsii*	1	Roux J, 1997
CMW23823	Mpumalanga	*A. mearnsii*	1	Heath RN, 2006
CMW42100	Western Cape	*Acacia melanoxylon*	1	Misse A, 2014
CMW42101	Western Cape	*A. melanoxylon*	1	Misse A, 2014
CMW42102	Western Cape	*A. melanoxylon*	1	Misse A, 2014
CMW42103	Western Cape	*A. melanoxylon*	1	Misse A, 2014
CMW42104	Western Cape	*A. melanoxylon*	1	Misse A, 2014
CMW42108	Western Cape	*A. melanoxylon*	0	Misse A, 2014
CMW42117	Western Cape	*A. melanoxylon*	0	Misse A, 2014
CMW21150	Limpopo	*Acacia nigrescens*	1	Heath RN, 2005
CMW21146	Limpopo	*Acacia grandicornula*	0	Heath RN, 2005
CMW17274*	Gauteng	*Faurea saligna*	0	Roux J, 2005
CMW17628	Gauteng	*F. saligna*	0	Heath RN, 2005
CMW17620[#]	Limpopo	*Terminalia serecia*	0	Roux J, 2005
CMW23839	Limpopo	*T. serecia*	0	Heath RN, 2006
CMW37312	Mpumalanga	*T. serecia*	0	Mbenoun M, 2010
CMW42119	Mpumalanga	*T. serecia*	0	Lee DH, 2014
CMW42120	Mpumalanga	*T. serecia*	1	Lee DH, 2014
CMW42123	Mpumalanga	*T. serecia*	0	Lee DH, 2014
CMW41580	Mpumalanga	*T. serecia*	1	Lee DH, 2014
CMW41601	Mpumalanga	*T. serecia*	1	Misse A, 2014
CMW37313	Mpumalanga	*Combretum zeyheri*	0	Mbenoun M, 2010
CMW37949	Mpumalanga	*Combretum apiculatum*	0	Mbenoun M, 2010
CMW21473	Gauteng	Nitidulid beetle	1	Heath RN, 2006
CMW22302	Gauteng	*Brachypephus* beetle	1	Heath RN, 2006
CMW41550	Mpumalanga	*Lannea schweinfurthii*	1	Lee DH, 2014
CMW41551	Mpumalanga	*L. schweinfurthii*	0	Lee DH, 2014
CMW41552	Mpumalanga	*L. schweinfurthii*	1	Lee DH, 2014
CMW41421	Mpumalanga	*Peltophorum africanum*	0	Lee DH, 2014
CMW40625	Western Cape	*Protea gaguedi*	1	Roux J, 2013
CMW42408	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42409	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42410	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42413	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42414	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42415	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42416	Western Cape	*Protea cynaroides*	1	Roux J, 2013

CMW42417	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42419	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42420	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42421	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42423	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42425	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42428	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42429	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42432	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42434	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42436	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42437	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42438	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42441	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42442	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42444	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42445	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42446	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42450	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42474	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42476	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW42477	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42478	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW42479	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW43681	Western Cape	*Protea cynaroides*	0	Roux J, 2013
CMW43682	Western Cape	*Protea cynaroides*	1	Roux J, 2013
CMW43683	Western Cape	*Protea cynaroides*	1	Roux J, 2013

[a] Those isolates for which genomes were sequenced in previous studies are indicated as follows: * for genomes from the study by van der Nest et al (2019), and # for the genome sequenced by van der Nest et al. (2014). The genome (CMW4068) used as a reference during genotyping is indicated in bold.

[b] Results of laccase/oxidase activity in the presence of α-naphthol. The presence of the activity of this enzyme(s) was evident with the colour change to purple (1), while no colour change indicated limited or no oxidation of this phenolic compound (0).

[c] The isolates used emerged from the studies by Lee et al., 2016; Barnes et al., 2005; Sayari et al., 2019; van der Nest et al., 2014, 2019. All isolates are available from the CMW culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria.

Table 2.2. Significant SNPs associated with laccase production in the GWAS of *Ceratocystis albifundus*.

Scaffold[a]	**Allele**[b]	**P-Value**	**FDR**[c]
2:1710324	A/G	5,49E-05	0,386115218
2:1656046	G/C	5,49E-05	0,411856232
4:301201	C/T	5,49E-05	0,441274535
8:1543018	A/G	9,09E-13	1,02E-07
10:790562	C/T	7,73E-08	0,002901352
15:365346	C/G	6,05E-09	0,000340505

[a] Scaffold: The scaffolds are known as, scaffold 2 (MAOA02000037.10), 4 (MAOA02000035.1), 8 (MAOA02000031.1), 10 (MAOA02000029.1), and 15 (MAOA02000024.1). The scaffold name consists of the position (number: position). For example, Scaffold2:1710324 was located on scaffold 2 at position 1 710 324bp.

[b] Allele indicates biallelic SNP.

[c] FDR indicates a false discovery rate, FDR<0,05 is considered significant.

Table 2.3. Significant SNPs associated with oxidases production across the *C. albifundus* population determined by Blast2GO terms.

Scaffold [a]	Sequence name [b]	Description	GO terms [c]	Function
2	g1478	iron copper transporter	F:GO:0046872	Metal ion binding
	g1483	FAD dependent oxidoreductase-like protein	F:GO:0003884	D-amino-acid oxidase activity
				Oxidoreductase activity
4	g2858	steroid alpha reductase family protein	F:GO:0016627	Oxidoreductase activity
	g2862	heat shock 70kDa	F:GO:0005524	ATP binding
	g2867	protein sey1	F:GO:0003924	GTPase activity and binding
	g2870	WD40 repeat-like protein	F:GO:0005515	Protein binding
8	g5411	NAD(P)H-dependent FMN reductase LOT6	F:GO:0016491	Oxidoreductase activity
	g5407	NmrA-like family protein	F:GO:0016491	Oxidoreductase activity
	g5385	cytochrome P450 monooxygenase	F:GO:0016705	Oxidoreductase activity
10	g5915	Pisatin demethylase	F:GO:0016705	Oxidoreductase activity
15	g7006	Peroxidase	P:GO:0034599	Oxidative stress response

[a] Scaffold: The scaffolds are known as, scaffold 2 (MAOA02000037.10), 4 (MAOA02000035.1), 8 (MAOA02000031.1), 10 (MAOA02000029.1), and 15 (MAOA02000024.1).

[b] Sequence name was determined by Blast2GO.

[c] GO terms protein (P) and/ function (F) number.

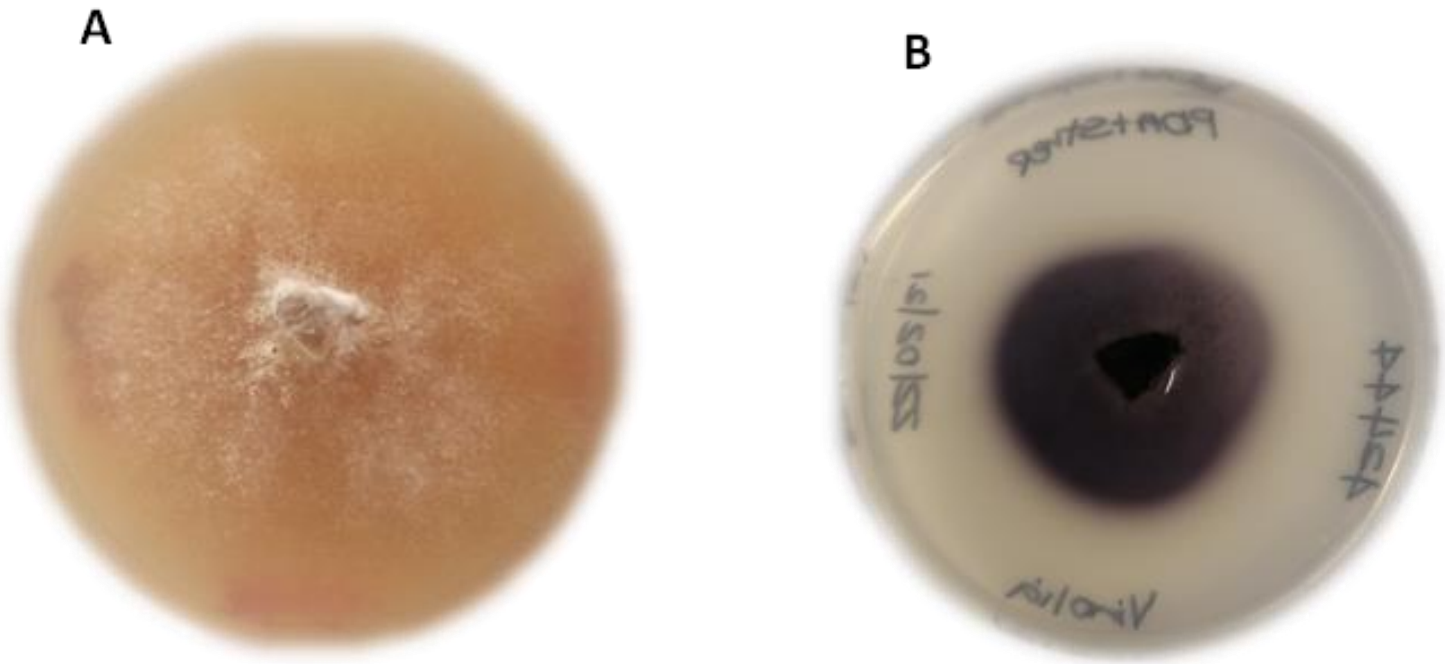


Figure 2.1. Laccase/oxidase activity differed greatly amongst individuals of *C. albifundus* that originated from different host species and across many geographic regions. (**A**) Some individuals did not produce much laccase or other oxidative enzymes, as indicated by an absence of colour change when flooded with α-naphthol. (**B**) Other isolates produced higher levels of these enzymes, as seen by the strong colour change (turning dark purple), which is due to the oxidation of α-naphthol.

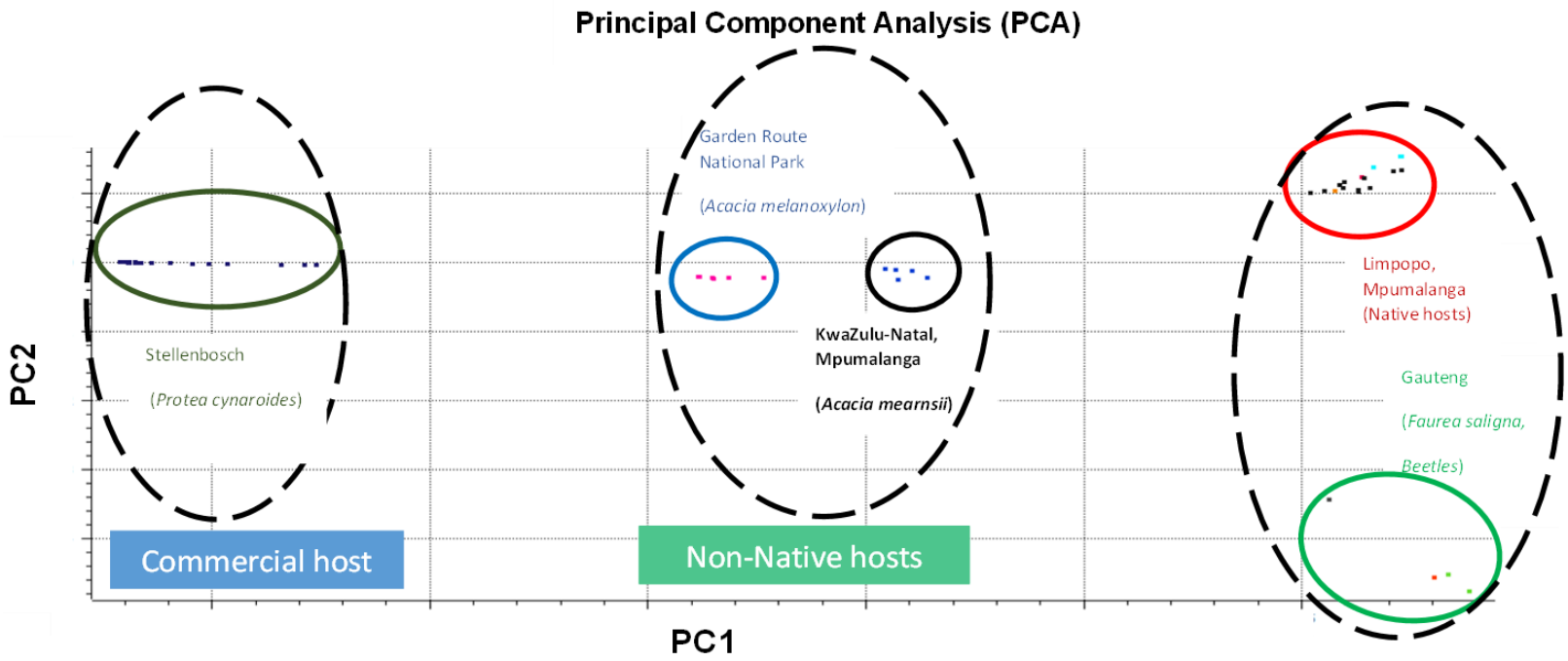


Figure 2.2. Two-dimensional principal component analysis (PCA) revealed the presence of distinct clusters within our collection of strains. Those originating from commercial, native, and non-native hosts are grouped into three main clusters. These main clusters consisted of 5 clusters. The total variance is explained by principal component (PC) 1 is 14,6981, while PC 2 is 8,05822.

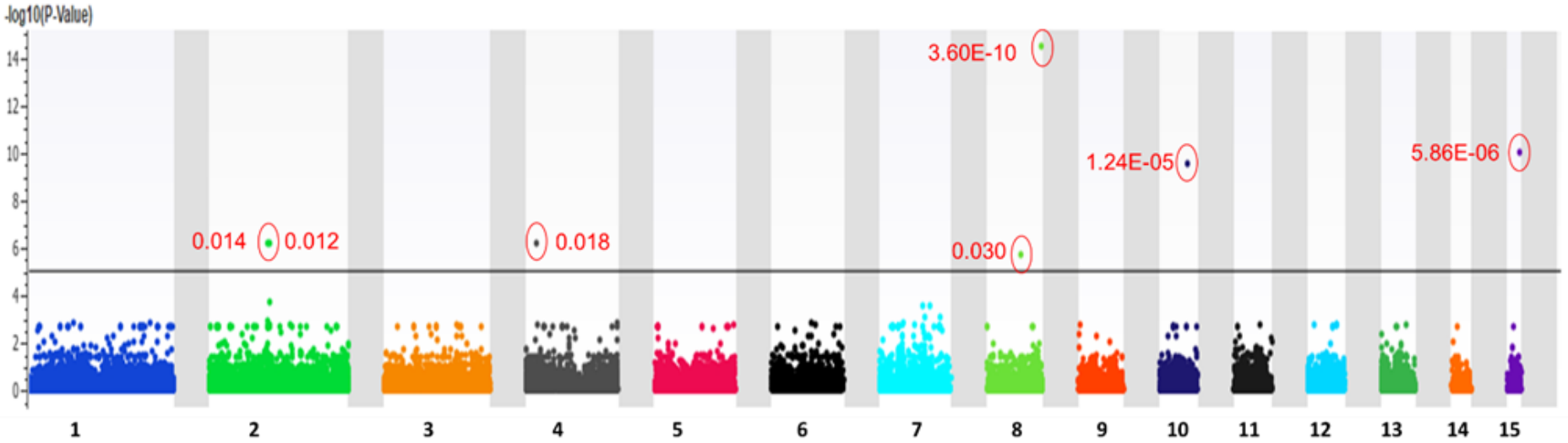
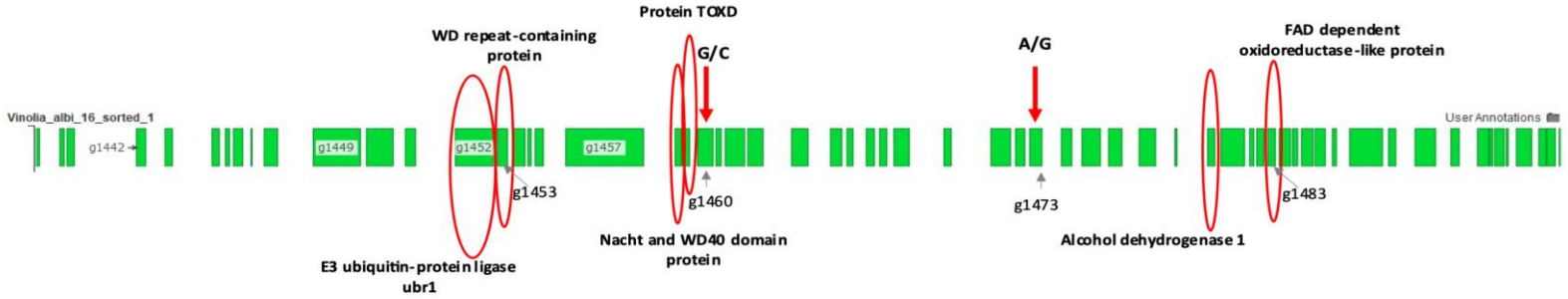
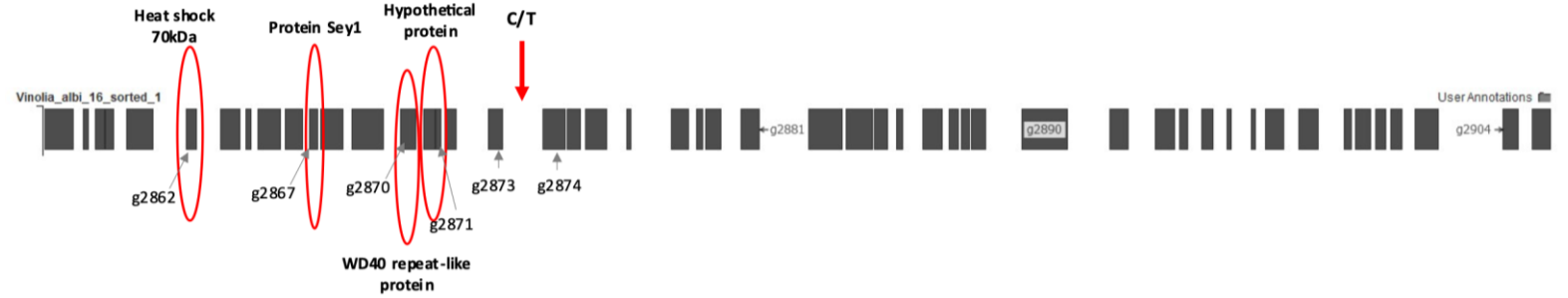


Figure 2.3. Manhattan plot of the 112 568 filtered SNPs mapped to the *C. albifundus* genome. Each data point represents one SNP (FDR value indicated) at which inheritance was tested for association with laccase expression associated with pathogenicity across the 78 isolates. The X-axis reports the SNP position on each scaffold and the Y-axis reports the –log10 (P-Value). Multi-locus Mixed Model Analyses identified 7 SNPs across 15 scaffolds, at a cut-off threshold line ($P < 0.05$).

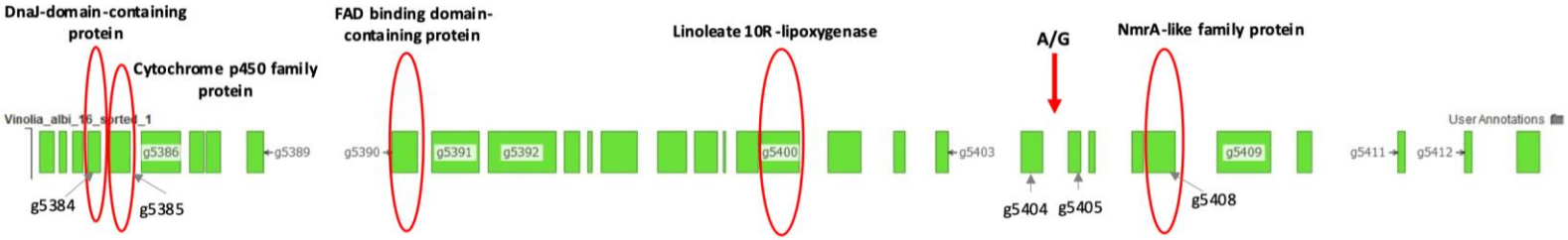
Scaffold 2



Scaffold 4



Scaffold 8



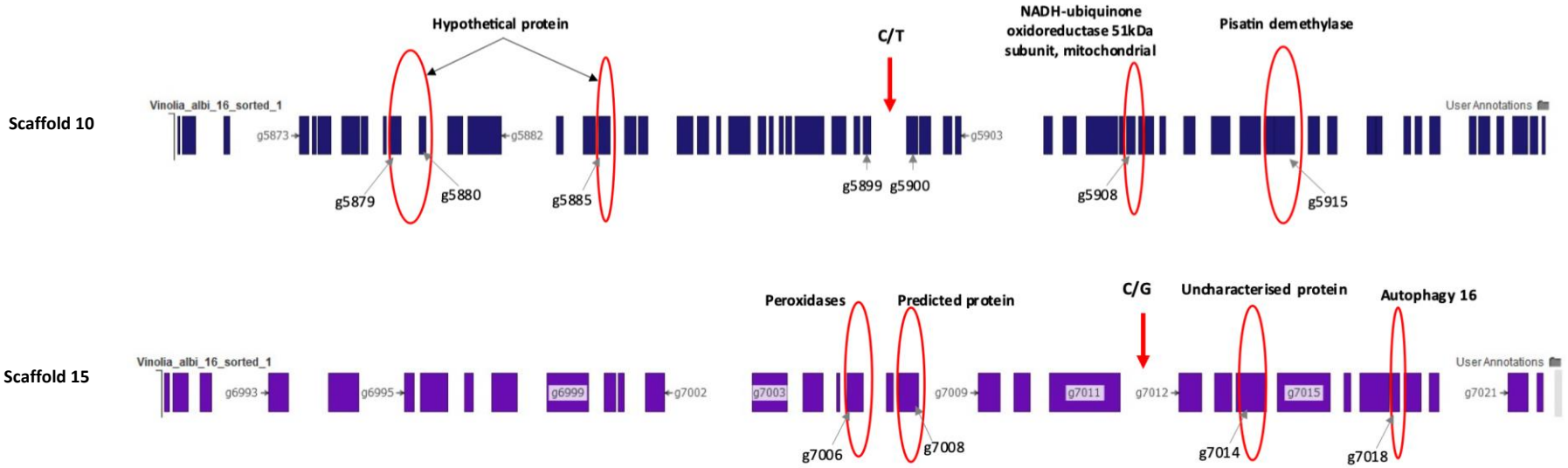
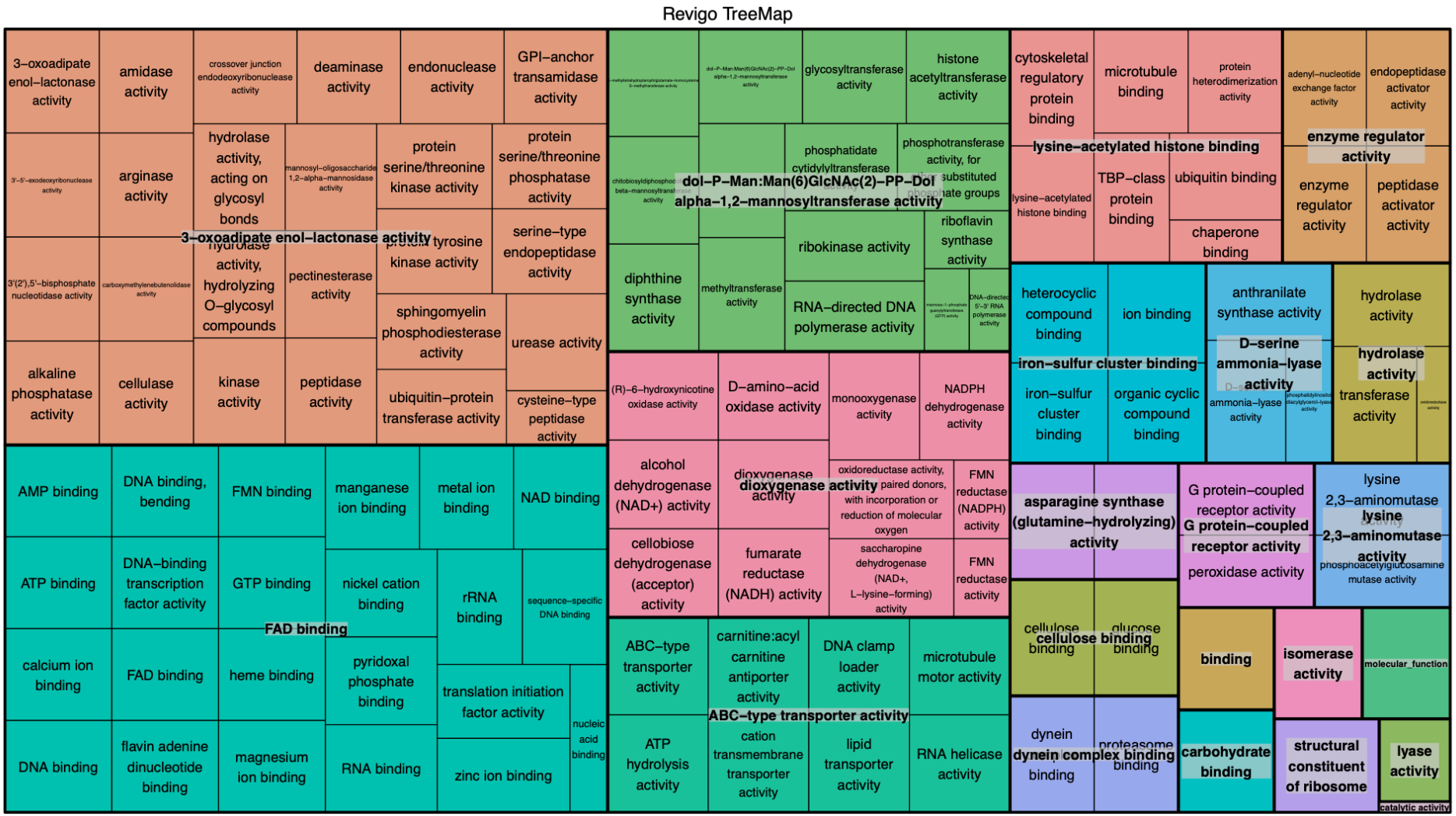
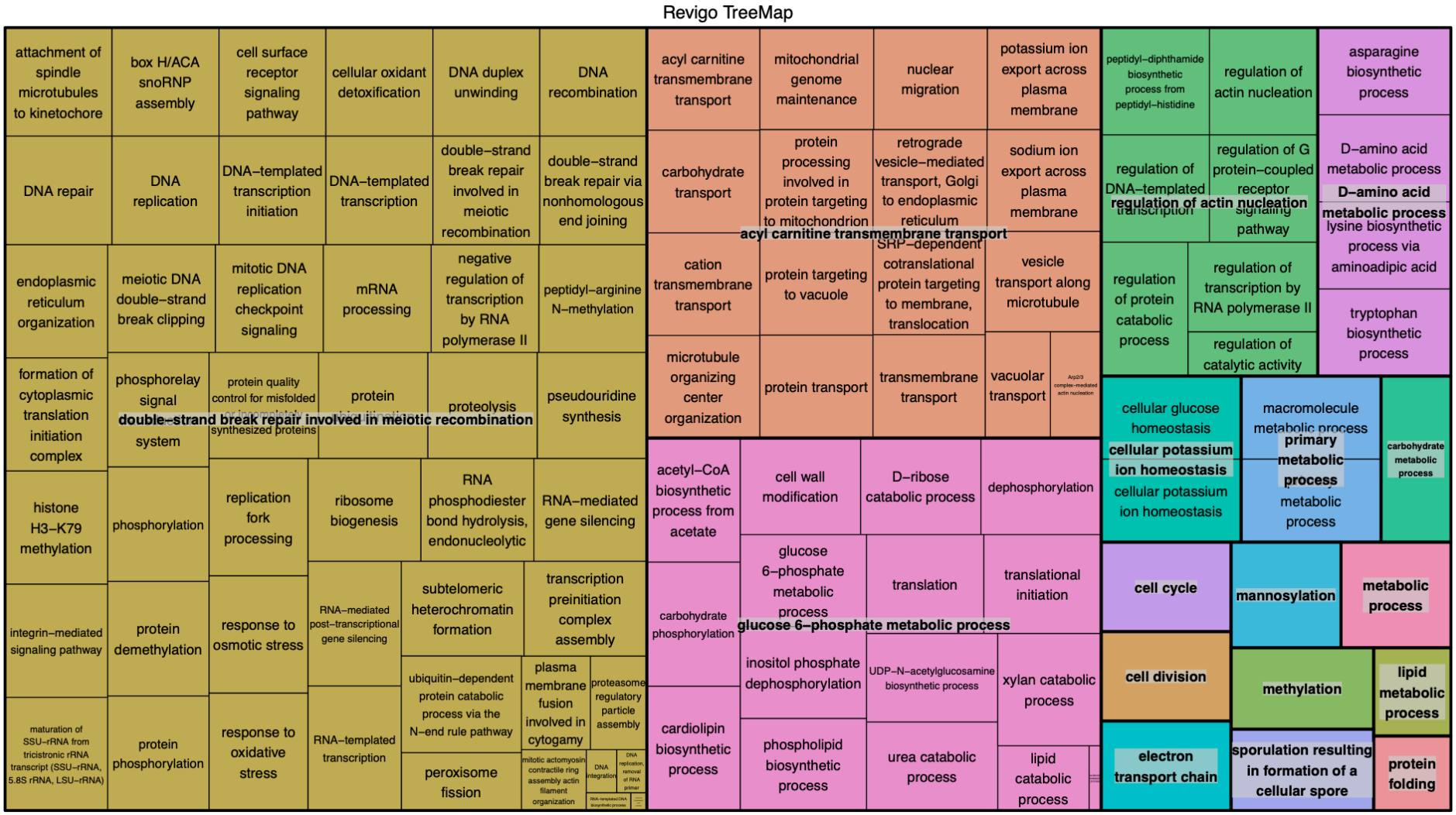


Figure 2.4. Schematic representation of the 6 SNPs (identified by the nucleotide bases e.g. A/T) associated with oxidase activity. The genes were located 10kbps up- and downstream the SNP. The red circle indicates the genes of interest.

A



B



C

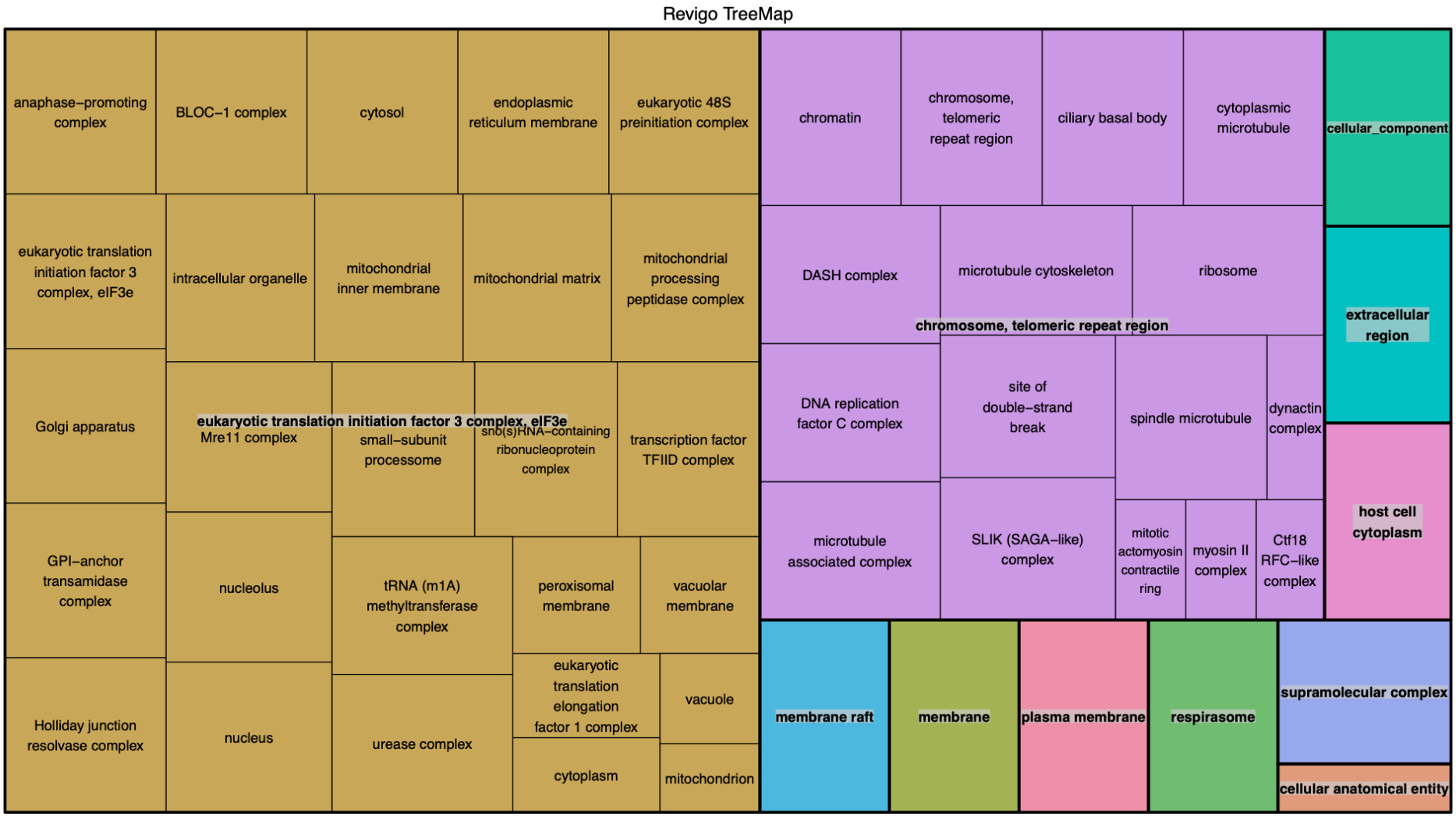


Figure 2.5. Visual summary of GO terms (Treemap in REVIGO, (http://revigo.irb.hr/) for Molecular Function **(A)**, Biological Process **(B)** and Cellular component **(C)**. Each box is a single ontology cluster, which are joined into super-clusters of loosely related terms, shown by the same color and named with a representative term. Larger boxes mean more significant enrichment.

SUPPLEMENTARY DATA

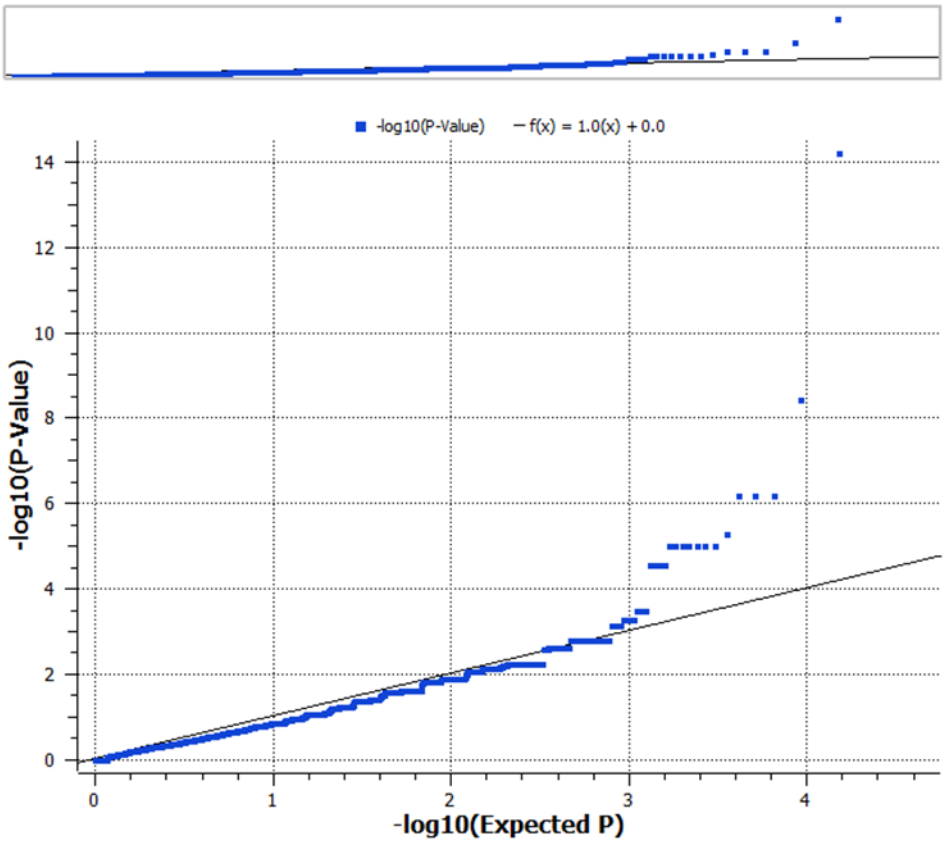


Figure S1.1. Quantile-quantile (Q-Q) plot of the observed -log10(P-value) against the -log10(expected P) values for each SNP (indicated by the blue dots) analyzed in the mixed linear model association. The black solid diagonal line represents the expected.

Supplementary Table S2.1. Genome and assembly information for the *Ceratocystis albifundus* isolates sequenced in this study.

Supplementary Table S2.2. Identity By State (IBS) values for the *Ceratocystis albifundus* isolates.

Supplementary Table S2.3. **Enriched GO terms explaining possible functionality of the genes**

***Supplementary tables S2.1, S2.2 and S2.3 are available at: bit.ly/3CTetaj**

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CHAPTER 3

Investigation of fitness characters in a collection of *Ceratocystis albifundus* isolates

Abstract

Ceratocystis albifundus is an important plant pathogen infecting a wide range of tree species in Southern Africa. It is a causal agent of cankers, wilts, and bark discolouration known as Ceratocystis wilt in commercial forests (*Acacia mearnsii*). Except for commercial *Protea cynaroides* in the Western Cape in South Africa, *C. albifundus* is not associated with disease symptoms in native hosts. Previous studies found that *C. albifundus* isolates collected from various host also differ in their ability to cause disease in the same host. Thus, this study aims (i) to compare the ability of *C. albifundus* isolates collected from a wide variety of hosts and different regions in South Africa to cause disease on *A. mearnsii* trees with the aid of inoculation trials on *A. mearnsii*; (ii) to determine if these *C. albifundus* isolates differ in growth rate in culture as an indication of fitness; and lastly, to (iii) verify if reproductive mode influences the fitness and the ability of this fungus to cause disease. This study will also shed light on how the mating genes associated with these important traits may influence the biology, ecology, and evolution of this important fungus.

3.1. INTRODUCTION

Fitness is a measurement of the match between an organism and its environment (Schoustra et al., 2010; 2009). In filamentous fungi the concept typically refers to their survival and reproductive success in a particular environment (Pringle and Taylor, 2002). However, universal fitness metrics for fungi have not been identified because optimal life-history strategies are determined by numerous traits and/or combinations of traits (Schoustra et al., 2010). Notable components of fitness in fungi include mycelial growth rate, sporulation, reproductive strategy, pathogenicity, substrate/host range, symbiotic relationships and many more (Gilchrist et al., 2006; McDonald and Linde, 2002; Newton et al., 1997). These components are typically not correlated and vary substantially across populations (Merilä and Sheldon, 1999), but some are used as proxies for fitness and the long-term survival of individuals under certain conditions (Brommer et al., 2004; Schoustra et al., 2010). For example, laboratory tests with *Aspergillus* species showed that mycelial growth rate and sporulation are good fitness predictors (Visser et al., 1997; Schoustra et al., 2010). However, despite the existence of broad commonalities regarding the traits potentially suitable as fitness metrics, empirical data are needed for understanding how these components are linked to growth and survival in particular environments (Pringle and Taylor, 2002; Visser et al., 1997).

In the current study, fitness was explored in the African fungus *Ceratocystis albifundus*. This species is classified in the *Ceratocystidaceae* together with numerous economically important plant pathogens (de Beer et al., 2014; Lee et al., 2015). In Southern and Eastern Africa, *C. albifundus* infects diverse tree species native to the region (e.g., *Protea gigantea, Burkea africana, Combretum molle*) without causing any obvious signs of disease (Roux et al., 2007). However, on wattle species (e.g., *Acacia mearnsii* and *Acacia melanoxylon*) introduced for forestry purposes, and on commercially cultivated king protea (i.e., *Protea cynaroides*), *C. albifundus* is a devastating pathogen (Lee et al., 2016; Roux et al., 2007). In the case of *Acacia*, it causes a wilting disease associated with substantial economic losses in the wattle-based forestry sector (Morris et al., 1993; Roux et al., 1999). In *P. cynaroides* orchards (planted to particular cultivars) in the Western Cape Province of South Africa, *C. albifundus* causes canker

and wilt symptoms that pose significant risks to the cut-flower industry built around *P. cynaroides* (Lee et al., 2016).

Like other members of the *Ceratocystidaceae*, *C. albifundus* is closely associated with insects (de Beer et al., 2014; Lee et al., 2015). In these associations, the fungus usually serves as a source of nutrition for the insect, while the insect in turn assists with the dispersal of the fungus (de Beer et al., 2014). Accordingly, fungi in this family are morphologically adapted for insect dispersal (e.g., having ascospores in sticky drops borne at the apices of long-necked sporocarps) and they produce various volatile compounds to attract insects (Roux and Wingfield, 2009). In the case of *C. albifundus*, specifically, sap-feeding nitidulid beetles may also vector the fungus across landscapes as the insects visit fresh wounds on trees, thereby also providing the fungus opportunity to infect its plant host (Heath et al., 2009).

A notable trait known to impact fitness of *C. albifundus* individuals is their reproductive status. Isolates of the fungus are either self-fertile or self-sterile (Wilken et al., 2014). Self-fertile isolates encode both *MAT 1-1* and *MAT 1-2* genes and are capable of producing sexual offspring without needing a compatible mating partner. By contrast, self-sterile isolates lack the *MAT 1-2*-1 gene and to complete the sexual cycle, they require interaction with a compatible mating partner. By making use of growth and pathogenicity studies, Lee and co-workers (2015) demonstrated that self-fertile isolates grow faster and are more aggressive (i.e., the quantitative component of pathogenicity) than self-sterile isolates. In other words, self-fertile isolates are superior in their ability to produce infective propagules and to rapidly spread or cause epidemics at host population scales (Pariaud et al., 2009; Schoustra et al., 2010). Combined with the ability to more easily produce fruiting structures carrying sticky spore drops for vectoring by insects, these traits afford self-fertile isolates a competitive advantage, ultimately ensuring their survival and efficient reproduction. This is also consistent with the significant bias towards self-fertile isolates that has been observed among single ascospore cultures derived from spore drops taken from ascomata produced by haploid-selfed cultures or from those collected in the field during disease outbreaks (Lee et al., 2018).

Despite the growing body of knowledge regarding the biology and evolution of *C. albifundus* (e.g., Kanzi et al., 2020; van der Nest et al., 2019; Simpson et al., 2018), little is known regarding the variation that might be expected when growth and pathogenicity of isolates from different locations and hosts are compared. Most studies investigated these aspects only in small numbers of isolates (Roux et al., 1999; Lee et al., 2015, 2016; Heath et al., 2010). Also, those studies reporting the influence of self-fertility on growth and pathogenicity of *C. albifundus* utilized no more than five strains collected from the field (Lee et al., 2015; 2016).

In this study the aim was to provide a population-wide framework for *C. albifundus* regarding the variation that could be expected regarding growth, pathogenicity and reproductive status of the fungus. This was achieved by investigating the growth and pathogenicity on *A. mearnsii* of a large collection of isolates from diverse sources and locations. Secondly, the growth and pathogenicity data were examined for possible structuring according to the geographic origin and source/host from which isolates were obtained, as well as their reproductive status and ability to produce laccase in culture (data obtained from Chapter 2 of this dissertation). The latter was included because laccase production is known to impact attributes involved in fitness (Janusz et al., 2020). The last objective was to determine whether any of the traits examined are correlated with one another to identify possible proxies for the overall fitness of individual *C. albifundus* isolates.

3.2. MATERIALS AND METHODS

3.2.1. Fungal isolates

A set of 61 *C. albifundus* isolates were included in this study. They were obtained from a wide host range, including both native (i.e., *P. cynaroides, P. gaguedi, Lannea schweinfurthii, Peltophorum africanum, Combretum zeyheri, C. apiculatum, Faurea saligna, and Terminalia serecia*) and non-native hosts (i.e., *A. mearnsii* and *A. melanoxylon*), as well as two isolates obtained from beetle species (Table 3.1). These isolates were collected from a wide geographic range in South Africa, including various locations in the summer rainfall region of South Africa (i.e., in KwaZulu-Natal, Gauteng, Limpopo, and Mpumalanga provinces) and in the winter

rainfall region from the Western Cape Province (http://pza.sanbi.org/vegetation; Table 3.1). The isolates were obtained from the CMW culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria (South Africa).

3.2.2. Growth medium and routine culturing

The fungi were grown on 2% (w/v) malt extract agar (MEA) medium, which was prepared by suspending 20 g of malt extract powder (Biolab, Midrand, South Africa) and 20 g of bacteriological agar (Biolab) in 1000 mL of distilled water. After autoclaving the mixture at 1.1 kgf/cm^2 (121 °C) for 20 min, 10 mL of the sterile mixture were aseptically poured into 90 mm Petri dishes. The medium was then left to solidify and dry at room temperature, after which the plates were incubated at 28 °C for 2 to 7 days to ensure sterility of the prepared growth medium. Isolates were then inoculated onto the prepared medium and incubated in the dark at 25°C, and routinely sub-cultured onto fresh MEA medium every 2-3 weeks.

3.2.3. Growth study

For each *C. albifundus* isolate, the radial mycelial growth on culture medium was determined as previously described (Lee et al., 2015; Pringle and Taylor, 2002). For this purpose, 2-3-weeks-old cultures were prepared, and used to inoculate MEA medium. This was done by using a sterile 5 mm cork borer to take a mycelial plug from the actively growing margin of a culture and transferring it to the centre of a sterile 90 mm Petri dish with MEA. Following sealing with Parafilm M® (Sigma-Aldrich, St. Louis, Missouri, United States), the inoculated plates were incubated at 25°C and 30°C in the dark for 14 days. Three plates were inoculated for each isolate at 25°C and three plates were inoculated for each isolate at 30°C. Colony diameter on each plate was then determined by taking the average of two perpendicular measurements (mm). The entire experiment was repeated.

3.2.4. Pathogenicity study

Data for the relative aggressiveness of the *C. albifundus* isolates included were available from a previous pathogenicity study conducted in a FABI greenhouse facility (van der Nest et al., unpublished). This was done using 2-years-old *A. mearnsii* trees as described previously (Fourie et al., 2019). Briefly, this involved preparation of inoculum by growing the isolates on

MEA at 25°C in the dark for 14 days. A 7 mm mycelial plug (taken from the actively growing margin of the culture) was then placed on a wound created on the plant's stem (with the mycelium facing the stem) and covered with Parafilm M® to prevent cross-contamination and desiccation. The wounds were created at 20 cm above ground level by using a sterile 10 mm cork borer to remove the bark and expose the underlying plant tissue to infection by the fungus. For the control treatment, inoculations were performed with sterile MEA plugs. A total of 10 trees were inoculated with each isolate and the control. The inoculated trees were then kept in a greenhouse at 25°C for 3 weeks. Virulence of the isolates was then estimated by removing the bark around the point of inoculation and measuring the length (mm) of the lesions. Due to limited availability of trees, the experiment could not be repeated.

3.2.5 Reproductive status

The reproductive status of each isolate was determined by using their available genome sequences (Chapter 2 of this dissertation). This involved determining whether the isolates are likely self-fertile (i.e., capable of sexual reproduction without needing a partner) or self-sterile (i.e., requiring a compatible mating partner). Here, self-fertility is conferred by the presence of both MAT1-1 and MAT1-2 mating type genes in the same genome, while self-sterility results from the absence of MAT1-2-1 gene (Wilken et al., 2014). Therefore, to assess the isolates' reproductive potential, their genome sequences were examined for the presence of the known MAT1-2-1 gene of *C. albifundus*. The sequence for this gene was obtained from GenBank® (Benson et al., 2013) using the accession number KF033902 (Wilken et al., 2014). It was compared against the translated sequences of each genome, using local tBLASTn searches in CLC Genomics Workbench v.10 (CLC bio, Aarhus, Denmark).

2.2.6 Statistical analyses

Mycelial growth and the virulence estimate for the *C. albifundus* isolates were subjected to various analyses. The mean colony diameter and mean lesion length measurements for each individual were used to construct histograms to investigate the distribution of these traits amongst particular isolate group using Microsoft® Excel®. These groups were defined based on the isolates' host, geographic origin, reproductive status and ability to produce laccase (data obtained from Chapter 2 of this dissertation).

To test for normality in the growth and pathogenicity datasets, the Kolmogorov-Smirnov test was used. The growth and pathogenicity data were also subjected to various statistical analyses to determine if there are patterns linked to the various user-defined isolate groups. For this purpose, relevant data were subjected to one-way ANOVA (analysis of variance) or two-tailed Student's *t*-test using a confidence level 95% ($\alpha = 0.05$). Here, the null hypothesis assumed no differences between the means of the different groups. Tukey's Honestly Significant Difference (HSD) tests were performed to identify groups that differed significantly from each other. All ANOVAs and Tukey's HSD analyses were performed using the online tools available respectively at https://acetabulum.dk/anova.html. The Kolmogorov-Smirnov and Student's tests were performed with the tools available at https://www.socscistatistics.com.

A Chi$(X)^2$ squared test was done to determine if the observed ratio of self-fertility to sterility departed significantly ($\alpha = 0.05$) from what was reported previously (Lee et al., 2018), by making use of the online tool available at https://www.socscistatistics.com. Also, the growth and pathogenicity data were subjected to Pearson's product-moment correlation analyses ($\alpha = 0.05$). Correlations between these continuous data and the categorical data for laccase production and fertility status were done using point-biserial correlation analysis. The latter represents a derivation of Pearson's product-moment correlation where one of the variables is continuous and the other dichotomous (i.e., laccase production or non-production, and self-fertile or self-sterile). To explore a possible correlation between laccase production and reproductive status, the Phi (Φ) coefficient was determined. This coefficient is analogous to Pearson's product-moment correlation coefficient ($r_{Pearson}$), but estimates the degree of association between two binary variables. All correlation analyses were conducted using online statistics resources (https://www.socscistatistics.com).

3.3. RESULTS

3.3.1. Mycelial growth of *C. albifundus* isolates

The mean colony diameter following two weeks of growth at 25°C ranged widely across the *C. albifundus* examined, ranging from as low as 14 mm to as high as 54 mm (Figure 3.1). At 30°C, the diameter ranged from 21 mm to 55 mm (Figure 1). The isolates often grew faster at the higher temperature, but this was not always the case (e.g., isolates CMW4084 and CMW4090 with means of 49 mm at 25°C, and at 30°C their respective mean colony diameters were 39 and 30 mm). This overall trend was reflected in the moderate positive correlation ($r = 0.5458$, $P < 0.00001$, $\alpha = 0.05$) observed between growth at the two temperatures (Figure 3.2).

The Kolmogorov-Smirnov statistic (D) for the two sets of colony diameters showed that both datasets were normally distributed. At 25°C, the value for D was 0.08581 ($P = 0.74529$) and at 30°C it was 0.10411 ($P = 0.51145$), showing that the data did not differ significantly from that which is normally distributed. Accordingly, ANOVA and t-tests were used to evaluate whether groups of means for growth at the two temperatures differed significantly. Specifically, this was in terms of the host species from which the isolates were obtained, the disease status of their host species and the isolates' geographic origin, as well as the isolates' potential to produce laccase. For the analyses involving geographic origin, the limited number of isolates originating from certain provinces in the summer rainfall region of South Africa precluded reliable statistical treatments. To account for this, isolates were grouped as being from the Western Cape Province or not (i.e., non-Western Cape, containing all isolates from Kwa-Zulu Natal, Mpumalanga, Limpopo and Gauteng). However, no significant differences were observed between or among colony diameter means for the user-define isolate groups, at both temperatures (Figure 3.3, Tables 2 and 3).

3.3.2. Pathogenicity of *C. albifundus* isolates

The data obtained from the pathogenicity assay were normally distributed, as indicated by the results of the Kolmogorov-Smirnov test ($D = 0.0637$, $P = 0.94892$). The mean lesions lengths obtained ranged from 16 mm for isolate CMW42450 to 134 mm for isolate CMW4090 (Figure 3.1). However, ANOVA results indicated that 31 of the isolates included in the pathogenicity

study did not differ significantly from the control treatment that produced a mean lesion length of 14 mm (see isolates indicated with green asterisks in Figure 3.1A).

As with mycelial growth, ANOVA and t-tests were used to evaluate whether user-defined groups of lesion length mean differed significantly (i.e., the isolates' host species, host disease status, geographic origin and potential to produce laccase). Limited numbers of isolates from particular locations were again accounted for by grouping isolates as being from the Western Cape Province or not (Figure 3.3, Tables 2 and 3). Statistically significant differences in mean lesion length were lacking for the comparisons involving laccase production and mycelial growth at 30°C. Although ANOVA indicated significant differences among means in the comparison involving lesion length and the isolate's host species, no significant differences were found with Tukey's HSD tests. However, when the isolates were grouped based on the disease status of their original hosts species (diseased vs. non-diseased/healthy), significant differences were observed. Isolates obtained from trees with disease symptoms produced significantly ($P < 0.05$) longer lesions than those originating from trees without obvious signs of disease. The same was also true when isolates were grouped based on geographic origin, where isolates from the Western Cape province mostly formed significantly ($P < 0.05$) longer lesions than those outside this province in the summer rainfall region (i.e., Limpopo, Mpumalanga, Gauteng and KwaZulu-Natal).

No significant correlations were observed between the pathogenicity data and mycelial growth at either 25°C ($r_{Pearson}$ = -0.0725, P = 0.5769) or 30°C ($r_{Pearson}$ = 0.071, P = 0.5741) (Figure 3.2). Comparisons of lesion length to categorical data for the isolates' potential to produce laccase and their reproductive status also yielded no significant correlations (Table 3.4). However, when the isolates were grouped based on their ability to cause lesions on *A. mearnsii* that were significantly longer than those observed for the control treatment (i.e., the were scored as either pathogenic or non-pathogenic), significant correlation was found with mycelial growth at 25°C ($\Phi = 0.2790$, P = 0.5741). As expected, this grouping also correlated perfectly with the lesion lengths obtained, although it did not correlate with the isolates' original host species (Table 3.4).

3.3.3. Reproductive status

The genomes for 46 of the isolates examined encoded the MAT1-2-1 gene and were scored as self-fertile. The gene was absent from 11 genomes, and the relevant isolates were scored as self-sterile. The reproductive status of three isolates could not be determined due to genome unavailability. This ratio of 4:1 self-fertile to self-sterile differed significantly (X^2 = 20.287; P < 0.00001) from the 1:1 ratio expected following Mendelian segregation. However, it did not differ significantly (X^2 = 2.606; P = 0.10648) from the average ratio (*ca*. 7:1) reported previously for instances where self-fertility dominated among isolates collected from native hosts or *P. cynaroides* orchards in Western Cape (Lee et al., 2018).

Self-fertile and self-sterile isolates did not differ significantly in terms of mycelial growth at 25°C and 30°C (Figure 3.3; Table 3.3). However, the self-fertile isolates produced significantly (P = 0. 0005) longer lesions in the pathogenicity study on *A. mearnsii* trees. Nevertheless, this binary trait was not significantly correlated to lesion length, mycelial growth at 25°C and 30°C, nor any of the other categorical variables estimated (Table 3.4).

To determine whether self-sterility of isolates could have impacted the statistical analyses performed, all analyses were repeated by including only the isolates scored as self-fertile (i.e., those encoding the MAT1-2-1 gene). The three smaller datasets were also normally distributed (pathogenicity with D = 0.0695; P = 0.9650; growth at 25°C with D = 0.1173; P = 0.7430; growth at 30°C with D = 0.1236; P = 0.4350). However, different from analyses with the entire set of isolates (Table 3.2, Figure 3.3), the results for ANOVA with only the self-fertile isolates indicated that isolates from different hosts (i.e., *Acacia* spp., *P. cynaroides*, and the native tree species) caused significantly different lesion lengths on *A. mearnsii* and grew significantly differently in culture 25°C (Table 3.2). In terms of pathogenicity, the isolates from *Acacia* spp. made significantly longer lesions than those from the native hosts (Figure 3.3). The isolates from *Acacia* spp. and the native hosts grew significantly faster at 25°C than those from *P. cynaroides* (Figure 3.3). The results of the *t*-tests revealed the same patterns as was seen using the entire set of isolates (i.e., both self-fertile and self-sterile), but additionally showed that the Western Cape isolates grew significantly slower at 25°C than the isolates from outside this province (Table 3.3).

The results for the various correlation analyses were mostly similar to those obtained using both the self-fertile and self-sterile isolates (Figure 3.2, Table 3.4). Again, a moderate positive correlation ($r_{Pearson}$ = 0.4546, P = 0.0017, α = 0.05) was observed between the growth of self-fertile isolates at the two temperatures. However, neither of these data sets showed any significant correlation to the lesion length data for the self-fertile isolates (i.e., at 25°C $r_{Pearson}$ = -0.2882, P = 0.0551) and at 30°C ($r_{Pearson}$ = -0.0244, P = 0.875648). As previously with the bigger dataset, growth at 25°C for the self-fertile isolates was significantly correlated (see Table 3.4) with pathogenicity to *A. mearnsii* when this trait was scored as a binary character (i.e., pathogenic, or non-pathogenic/eliciting the same effect as the control treatment).

3.4 Discussion

By making use of isolates all originating from collections in the field, the current study sought to explore how reproductive potential might influence growth and aggressiveness of *C. albifundus*. This was previously investigated by Lee et al. (2015) using a small number of isolates representing field-collected self-fertile isolates and laboratory-generated self-sterile progeny. As found in this previous study (Lee et al., 2015), inoculations of *A. mearnsii* with self-sterile isolates produced significantly (P < 0.05) shorter lesions as those using self-fertile isolates. Unexpectedly, however, significant differences between self-fertile and self-sterile isolates were not observed regarding their growth in culture at 25°C (Roux 1996; Lee et al., 2015). This could likely be attributed to the different types of samples used in the current study and by Lee et al. (2015) and the possibility that reproductive fertility has a much larger effect on the aggressiveness of *C. albifundus* than its growth in culture. Contrast to Lee and co-workers (2015) being able to directly compare the growth and aggressiveness between self-fertile parent and self-sterile progeny isolates, the current study had to contend with the traits' natural variation across populations. Although differences in pathogenicity between self-sterile and self-fertile could be visualized, the growth differences between them likely overlapped strongly with the variation found across populations of the fungus (e.g., compare mean colony diameter of self-fertile isolates in Figures 3.1B and 3.1C). Figure 3.1A and Figures 3.2. 3.3

Results of this study confirmed that reproductive potential is an important component of fitness in *C. albifundus* populations. The ratio of self-fertile to self-sterile were strongly skewed towards self-fertility (Lee et al., 2015, 2018). As suggested previously, this situation might arise because fewer ascospores bearing the self-sterile trait originate in an ascus or, alternatively, ascospores bearing the trait have lower germination (Lee et al., 2015). Why self-sterility is retained in *C. albifundus* populations is unclear, but the presence of self-sterile isolates potentially promotes outcrossing, thereby allowing the fungus to fully exploit the benefits of sexual recombination (Billiard et al., 2011; Lin and Heitman, 2007). Outcrossing would lead to the formation of new genotypes, thereby facilitating effective adaptation to rapidly changing environments (Heitman, 2010; Lee et al. 2015; McGuire et al., 2004; Wilken et al., 2014). The presence of self-sterile isolates in populations could thus mediate re-assortment of advantageous alleles, or lead to the introduction of new alleles, while self-fertile isolates ensure through selfing the multiplication and transmission of advantageous allelic combinations that are locally adapted without the process of outcrossing (Giraud et al., 2008, 2010; Gandon et al., 1996; McDonald and Linde 2002). Nevertheless, to exclude the influence that reproductive potential might have on the variables examined in the current study, all analyses were also conducted using the self-fertile isolates only.

Geographic origin of the *C. albifundus* examined and the host species from which they were collected, particularly for the self-fertile isolates, explain some of the variation observed in growth at 25°C and pathogenicity to *A. mearnsii*. Isolates from the summer rainfall region grew faster and produced shorter lesions on *A. mearnsii* than those collected in the Western Cape Province with its winter rainfall. Also, isolates obtained from native hosts produced shorter lesions on *A. mearnsii* than those isolated from *Acacia* species and from orchard-grown *P. cynaroides*, while the latter isolates grew the slowest. Such geographic differentiation in populations of microorganisms are not uncommon, as the associated climatic conditions influence their distribution and as well as that of their plant hosts (Bernardo-Cravo et al., 2020; Roberts and Wiedmann, 2003). In other words, the fitness of both *C. albifundus* and its various plant hosts are likely governed by geography and the climatic conditions associated with it.

The *C. albifundus* isolates varied widely in their response to *A. mearnsii* in the pathogenicity assay, similar to previous studies (Lee et al., 2015, 2016). Overall, the disease status of the isolates' original host appeared to be an important factor. Substantially longer lesions were produced by isolates obtained from symptomatic tissues on diseased plants (i.e., *Acacia* spp. and *P. cynaroides*) than those isolated from apparently healthy native trees. This pattern was further explored by scoring isolates based on results of the pathogenicity assay – pathogenic isolates produced significantly longer lesions than the control, while those of non-pathogenic isolates did not. This binary trait emphasized the highly significant differences between isolates originating from diseased and healthy hosts. A possible explanation may be that *C. albifundus* co-evolved with its plant hosts (Lee et al., 2016; Roy, 2001; Woolhouse et al., 2002), and accordingly do not cause disease to host species with which it shares a native range (Barnes et al., 2005; Lee et al., 2015; Roux et al., 2001, 2007; Wingfield et al., 2017). The ability to cause disease on non-native *Acacia* species and on commercially cultivated *P. cynaroides* is thought to be due to host range expansion and/or host shift (Barnes et al., 2005; Lee et al., 2016; Roux et al., 2001). The clear separation among isolates from native and these "new" hosts regarding their ability to infect *A. mearnsii* suggest a strong genetic basis. This is because genotypes with high levels of aggressiveness to the "new" hosts would be better adapted and preferentially selected for (Desprez-Loustau et al., 2007; Pariaud et al., 2009).

Growth in culture and pathogenicity to *A. mearnsii* both displayed continuous distributions. This was despite the *C. albifundus* isolates originating from various hosts and locations. Therefore, both characters represent quantitative traits, governed by multiple genes. In terms of growth, this is consistent with the results of growth studies (e.g., van der Nest et al., 2019; Schoustra, S., Punzalan, D. 2012; Olson, 2006). In terms of growth, differences may also be expected in the loci governing mycelium growth at 25°C and 30°C, because the two variables were not strongly correlated and that is broadly in agreement with reports from other fungi (e.g., Brasier, 1970; Olson, 2006). In terms of pathogenicity of *C. albifundus*, similar findings were also reported for other fungal pathogens (e.g., Idnurm and Howlett, 2001; Li et al., 2012). It would be interesting to determine whether there are any overlaps in the loci underpinning pathogenicity to *A. mearnsii* and *P. cynaroides*, and these might be involved during asymptomatic infection of native tree hosts.

The variables examined in this study were mostly not correlated with one another, irrespective of whether self-sterile isolates were included or not. The only exception was growth at 25°C or 30°C that showed a moderately positive correlation. These included analyses of growth at either 25°C or 30°C and correlation with the lesion lengths obtained from the pathogenicity assay on *A. mearnsii*. Such lack of correlations between mycelial growth in culture and pathogenicity data has also been reported for fungi such as *Fusarium*. For example, De Vos et al. (2011) did not find an association between growth and pathogenicity, despite previous suggestions that the two characters would be correlated (Doohan et al., 2003). However, in other fungi such as certain *Alternaria* species, fast mycelial growth was linked to high aggressiveness, which suggested growth might be key to aspects of pathogenicity (Harteveld et al., 2014).

None of the correlation analyses involving reproductive potential or laccase production as a binary variable yielded significant results. This is despite previous studies showing reduced growth rates in isolates lacking the *MAT 1-2-1* gene, albeit only in a handful of individuals (Lee et al., 2015). Also, a previous gene-knockout study found that the absence of *MAT 1-2* genes significantly reduced growth rate in culture (Wilson et al., 2020). However, our findings are similar to those reported for a worldwide collection of *F. circinatum* isolates (Mullett et al., 2017), where there was also not an association between mating type and mycelial growth rate in the laboratory. In terms of laccase production, there was no evidence of an association between mode of reproduction and laccase production that is known to influence fitness (Janusz et al., 2020).

Among the variables examined in this study, only mycelial growth at 25°C and the ability to induce lesions on *A. mearnsii* (i.e., pathogenic or non-pathogenic) can potentially serve as proxies for one another. However, whether one or both of these two traits are sufficiently informative to serve as fitness metrics is not clear. The results from this study showed that growth in culture and pathogenicity (as measured using inoculation studies of *A. mearnsii*) are polygenic traits, each likely determined by different sets of loci. The data presented in Chapter 2 of this dissertation demonstrated that this is likely also the case for laccase production in

culture. Additionally, other traits such as host preferences and aggressiveness are probably also complex, quantitative traits in *C. albifundus*. Therefore, studies into the pathogen's fitness should consider multiple traits, as no single property currently explains the distribution and ecological success of *C. albifundus* in nature.

3.5. CONCLUSIONS

This work investigated mycelial growth and pathogenicity to *A. mearnsii* of *C. albifundus* isolates. Inoculation trials and radial mycelium growth are valuable tools to study the aggressiveness and fitness respectively, of plant pathogens under controlled conditions. These approaches are more fundamental to the genetics of the interaction. Results of this study has elevated our understanding of mycelial growth and virulence plant-pathogen interactions, factors that influence pathogen fitness, and the outcome of host-pathogen interactions. Data obtained from this study therefore provided a strong foundation for studying pathogenicity in this fungus. However, further research is needed to explain the high variability observed for pathogenicity among the *C. albifundus* individuals. This is because the high variability might due to a lack of distinct taxonomic species delineation of the isolates or that other additional mechanisms (e.g., types or quantity of metabolites produced) may be involved in causing the differences between the isolates (Harteveld et al., 2014).

3.6. REFERENCES

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Table 3.1. Geographic origin and host of *C. albifundus* isolates screened for growth rate and pathogenicity.

Isolate Number [a]	Hosts	Province	Collector, date
CMW21146	*Acacia grandicornula*	Limpopo	Heath RN, 2005
CMW4090	*Acacia mearnsii*	Kwa Zulu Natal	Roux J, 1997
CMW23823	*Acacia mearnsii*	Mpumalanga	Heath RN, 2006
CMW4075	*Acacia mearnsii*	Kwa Zulu Natal	Roux J, 1997
CMW4084	Acacia mearnsii	Kwa Zulu Natal	Roux J, 1997
CMW42100	*Acacia melanoxylon*	Western Cape	Misse A, 2014
CMW42101	*Acacia melanoxylon*	Western Cape	Misse A, 2014
CMW42102	*Acacia melanoxylon*	Western Cape	Misse A, 2014
CMW42103	*Acacia melanoxylon*	Western Cape	Misse A, 2014
CMW42104	*Acacia melanoxylon*	Western Cape	Misse A, 2014
CMW42117	*Acacia melanoxylon*	Western Cape	Misse A, 2014
CMW21150	*Acacia nigrescens*	Limpopo	Heath RN, 2005
CMW22302	*Brachypephus* beetle	Gauteng	Heath RN, 2006
CMW37949	*Combretum apiculatum*	Mpumalanga	Mbenoun M, 2010
CMW37313	*Combretum zeyheri*	Mpumalanga	Mbenoun M, 2010
CMW17628	*Faurea saligna*	Gauteng	Heath RN, 2005
CMW41551	*Lannea schweinfurthii*	Mpumalanga	Lee DH, 2014
CMW41552	*Lannea schweinfurthii*	Mpumalanga	Lee DH, 2014
CMW41550	*Lannea schweinfurthii*	Mpumalanga	Lee DH, 2014
CMW21473	*Nitulid* beetle	Gauteng	Heath RN
CMW41421	*Peltophorum africanum*	Mpumalanga	Heath RN, 2006
CMW42416	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42427	*Protea cynaroides*	Western Cape	Roux J, 2015
CMW42408	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42409	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42417	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42419	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42420	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42423	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42425	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42429	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42435	*Protea cynaroides*	Western Cape	Roux J, 2014
CMW42436	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42437	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42438	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42444	*Protea cynaroides*	Western Cape	Farm owner, 2013
CMW42445	*Protea cynaroides*	Western Cape	Farm owner, 2013
CMW42446	*Protea cynaroides*	Western Cape	Farm owner, 2013
CMW42447	*Protea cynaroides*	Western Cape	Farm owner, 2013
CMW42476	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42477	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW43681	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW43682	*Protea cynaroides*	Western Cape	Roux J, 2014
CMW43683	*Protea cynaroides*	Western Cape	Roux J, 2015
CMW42432	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42474	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42415	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42428	*Protea cynaroides*	Western Cape	Roux J, 2013
CMW42442	*Protea cynaroides*	Western Cape	Farm owner, 2013
CMW42450	*Protea cynaroides*	Western Cape	Farm owner, 2013
CMW42478	*Protea cynaroides*	Western Cape	Roux J, 2013

CMW40625	*Protea gaguedi*	Western Cape	Roux J, 2013
CMW17620	*Terminalia serecia*	Limpopo	Roux J, 2005
CMW23839	*Terminalia serecia*	Limpopo	Heath RN, 2006
CMW37312	*Terminalia serecia*	Mpumalanga	Mbenoun M, 2010
CMW41580	*Terminalia serecia*	Mpumalanga	Hyeon DL, 2014
CMW41601	*Terminalia serecia*	Mpumalanga	Misse A, 2014
CMW42118	*Terminalia serecia*	Mpumalanga	Lee DH, 2014
CMW42119	*Terminalia serecia*	Mpumalanga	Lee DH, 2014
CMW42120	*Terminalia serecia*	Mpumalanga	Lee DH, 2014
CMW42123	*Terminalia serecia*	Mpumalanga	Lee DH, 2014

[a] All isolates are available from the CMW culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria.

Table 3.2. ANOVA[a] results for seven separate datasets involving the comparison of lesion length data obtained from the pathogenicity study on *Acacia mearnsii* using the *Ceratocystis albifundus* isolates examined, as well as the comparisons involving pathogenicity and mycelial growth at 25°C and 30°C when isolates are grouped according to their original plant host species (i.e., *Acacia* species, various native hosts and *Protea cynaroides*).

Source of variance	Degrees of freedom	Sum of Squares	Mean Squares	F -value	P-value [b]
Pathogenicity results per isolate (Lesion length)					
Between-treatments	59	400198	6783	9.9871	4.1×10^{-51}
Within-treatments	433	295083	679	-	-
Including all isolates, both self-fertile and self-sterile					
Host species vs. Growth at 25°C					
Between-treatments	2	528	3099	2.8771	0. 0645
Within-treatments	57	5228	55	-	-
Host species vs. Growth at 30°C					
Between-treatments	2	227	264	1.4953	0.2330
Within-treatments	56	4244	92	-	-
Host species vs. pathogenicity					
Between-treatments	2	5497	2749	3.4334	**0.0392**
Within-treatments	56	44824	800	-	-
Including only the self-sterile isolates					
Host species vs. Growth at 25°C					
Between-treatments	2	1016	508	7.7033	**0.0014**
Within-treatments	43	2835	66	-	-
Host species vs. Growth at 30°C					
Between-treatments	2	107	53	0.6277	0.5388
Within-treatments	44	3739	85	-	
Host species vs. pathogenicity					
Between-treatments	2	5488	2744	4.3420	**0.0190**
Within-treatments	44	27806	632	-	-

[a] Conducted using the online statistical tools available at https://www.socscistatistics.com/tests/biserial/default.aspx and https://acetabulum.dk/anova.html.

[b] P-values that were significant at the 95% confidence level are indicated in bold.

Table 3.3. Results of Student's *t*-tests[a] for comparisons involving the lesion lengths obtained from a pathogenicity study on *Acacia mearnsii* and mycelial growth at 25°C and 30°C for the *Ceratocystis albifundus* isolates examined and grouped according to their reproductive status, diseases status of their original host, geographic origin and their ability to produce laccase.

Groups compared	**Treatment**	***t*-value[b]**	**P-value[c]**
Reproductive status *(self-fertile or sterile)*	Growth at 25°C	-0.6334	0.5291
	Growth at 30°C	-0.8997	0.1861
	Lesion length	-3.6790	**0. 0005**
Pathogenicity to *A. mearnsii (long lesions or same as the control)*	Growth at 25°C	2.1534 *(2.4859)*	**0.0357** ***(.01680)***
	Growth at 30°C	1.2286 *(1.5522)*	0.2243 *(0.1278)*
	Lesion length	-8.9823 *(-6.1806)*	**<.00001** ***(<.00001)***
Host's disease status *(symptoms or no symptoms)*	Growth at 25°C	1.8543 *(-1.9221)*	0.0688 *(0.0611)*
	Growth at 30°C	-1.5617 *(-0.8913)*	0.1239 *(0.3775)*
	Lesion length	-2.6186 *(2.9510)*	**0.0113** ***(0.0050)***
Geographic origin *(Western Cape Province or outside the province)*	Growth at 25°C	1.6478 *(3.0250)*	0.1047 ***(0.0041)***
	Growth at 30°C	0.7816 *(0.8315)*	0.2188 *(0.4101)*
	Lesion length	2.9718 *(-2.0892)*	**0.0043** ***(0.0424)***

Laccase production *(producer or non-producer)*	Growth at 25°C	0.0115 *(-0.8480)*	0.9909 *(0. 4015)*
	Growth at 30°C	-0.2425 *(-1.3663)*	0.8089 *(0.1793)*
	Lesion length	-1.4431 *(1.7192)*	0.1548 *(.09313)*

[a] Conducted using the online statistical tools available at https://www.socscistatistics.com/tests/biserial/default.aspx.

[b] The *t*-values in brackets were obtained when all self-sterile isolates were excluded from the analyses.

[c] The P-values in brackets were obtained when all self-sterile isolates were excluded from the analyses. Those significant at the 95% confidence level are indicated in bold.

Table 3.4. Results of correlation analyses[a] involving categorical data for fertility or laccase production for the *C. albifundus* isolates included in this study.

Variable 1	Variable 2	Coefficient [b]	P-value [c]
	Continues variable vs. Categorical variable [b]		
Lesion length	Reproductive status (self-fertile or self-sterile)	0.0449	0.7142
	Laccase production or non-production	0.7572 *(0.2593)*	0.5469 *(0.0931)*
	Pathogenicity to *Acacia mearnsii*	-0.7655 *(-0.7073)*	**<.00001 *(<.00001)***
Mycelial growth at 25°C	Reproductive status (self-fertile or self-sterile)	0.2017	0.1255
	Laccase production or non-production	0.0180 *(0.0415)*	0.8944 *(0.7943)*
	Pathogenicity to *A. mearnsii*	0.2790 *(0.3510)*	**0.0356 *(0.0168)***
Mycelial growth at 30°C	Reproductive status (self-fertile or self-sterile)	0.0913	0.4877
	Laccase production or non-production	-0.155 *(-0.2087)*	0.2386 *(0.1793)*
	Pathogenicity to *A. mearnsii*	0.1606 *(0.2278)*	0.2243 *(0.1278)*
	Categorical variable vs. Categorical variable		
Reproductive status (self-fertile or self-sterile)	Laccase production or non-production	0.1567	0.2495
	Original host's disease status (symptoms or no symptoms)	-0.1245	0.6525
	Pathogenicity to *A. mearnsii*	-0.3749	0.9954
Pathogenicity to *A. mearnsii*	Original host's disease status (symptoms or no symptoms)	-0.4086 *(-0.5125)*	0.9983 *(0.9995)*
	Laccase production or non-production	-0.0669 *(-0.0912)*	0.3804 *(0.4456)*

[a] Conducted using the online statistical tools available at https://www.socscistatistics.com/tests/biserial/default.aspx. Point biserial correlation analysis using comparisons between continuous growth or pathogenicity data and their associated categorical data for fertility or laccase production. Phi Coefficient analysis of the binary data involving reproductive status (i.e., self-fertile or sterile), host's disease status, and pathogenicity against *A. mearnsii*.

[b] Coefficient $r_{(pbc)}$ for the Point Biserial correlation analysis and Φ for Phi coefficient analysis. *V*alues in brackets were obtained when all self-sterile isolates were excluded from the analyses.

[c] The P-values in brackets were obtained when all self-sterile isolates were excluded from the analyses. Those significant at the 95% confidence level are indicated in bold.

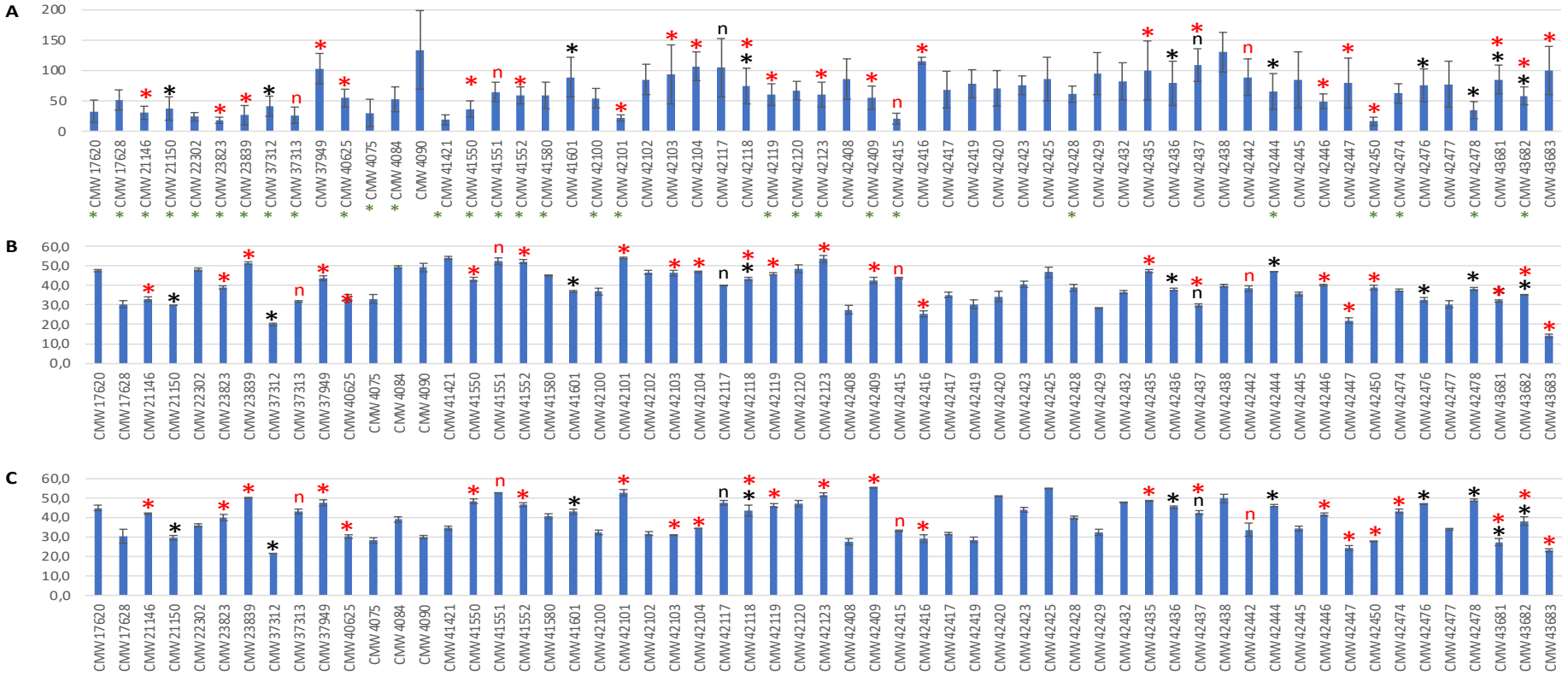


Figure 3.1. Histogram of mean lesion length (mm, y-axis) caused by *Ceratocystis albifundus* isolates included in this study (**A**) on two-years-old *Acacia mearnsii* trees under greenhouse conditions, and their mycelial growth expressed as mean colony diameter (mm, y-axis) following 14 days of incubation on MEA at 25°C **(B)** and 30°C **(C)**. In all three cases, bars indicate standard deviation. Reproductive status of isolates are indicated in red – asterisks denote female-sterility and "n" shows when its unknown. Potential for laccase production is indicated in black – asterisks denote non-producers and "n" shows when its unknown. In panel **A**, instances where mean lesion lengths did not differ significantly from that of the control treatment are indicated with green asterisks next to the isolate number.

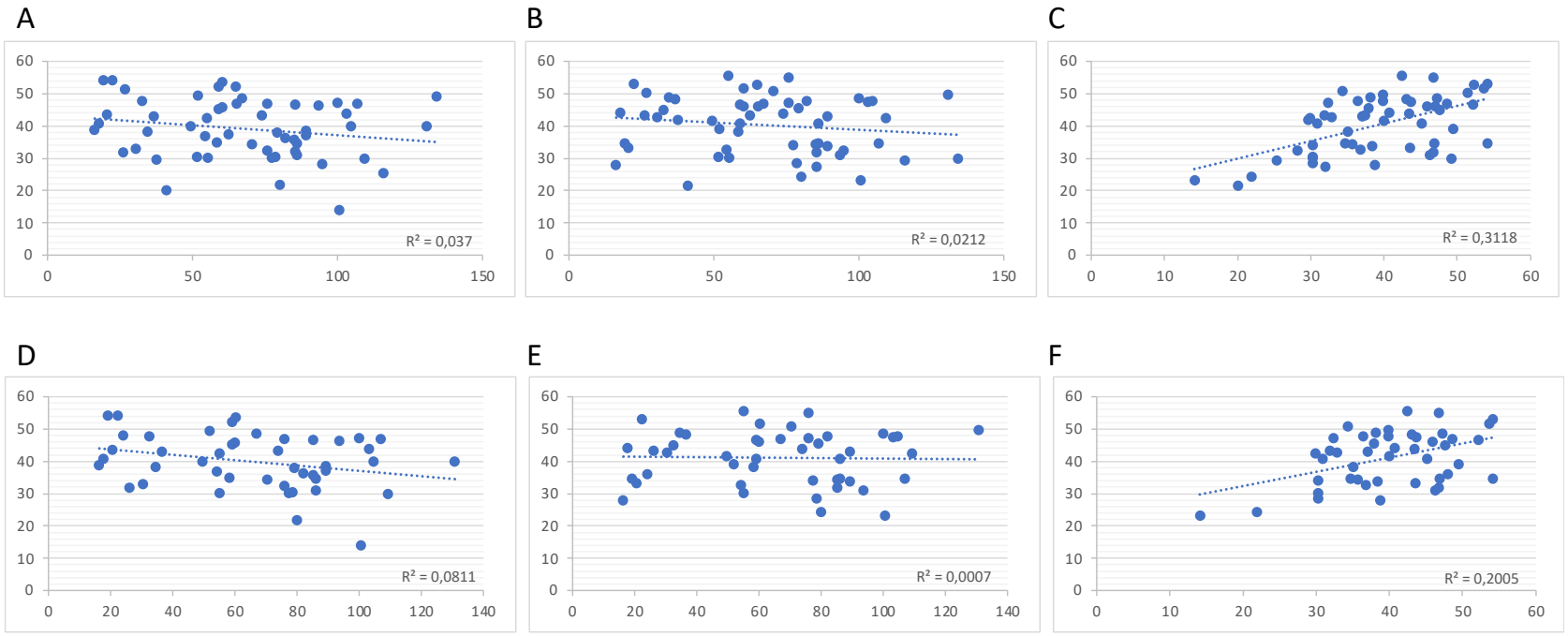
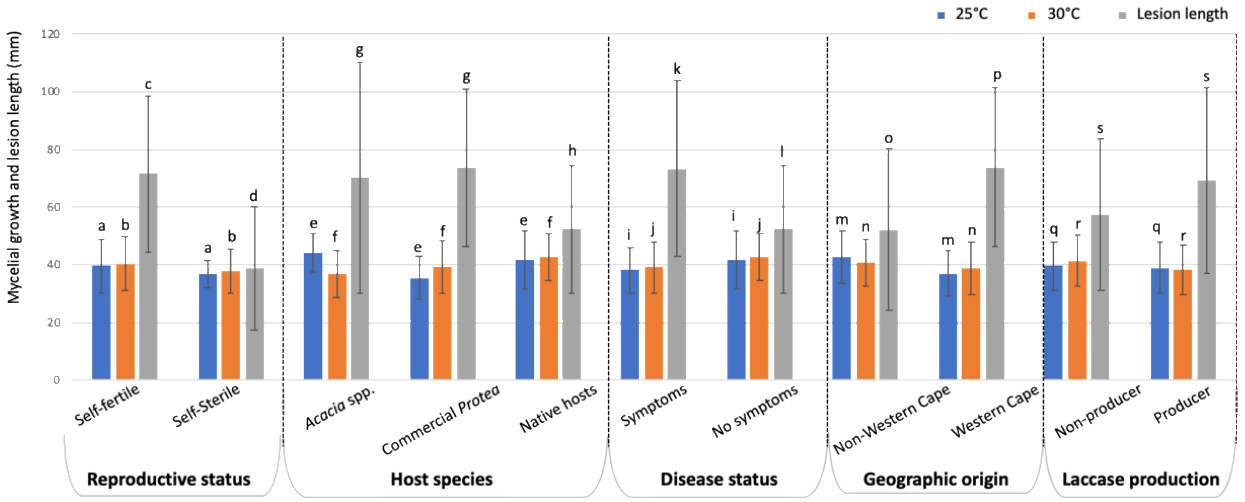


Figure 3.2. Results of Pearson's correlation analyses between the pathogenicity and mycelial growth data for the *Ceratocystis albifundus* isolates included in this study. The mean colony diameter (mm) following 14 days of incubation at 25°C (y-axis) was plotted against mean lesion length (mm, y-axis) obtained from the pathogenicity study on *Acacia mearnsii* using both self-fertile and self-sterile isolates (**A**) or only the self-fertile isolates (**D**). Likewise, the mean colony mean diameter (mm) obtained at 30°C (y-axis) was plotted against mean lesion length (mm, y-axis using both self-fertile and self-sterile isolates (**B**) or only the self-fertile isolates (**E**). Also, the mean colony mean diameter (mm, y-axis) at 25°C (x-axis) was plotted against the mean lesion length (mm, y-axis) obtained for growth at 30°C (mm, x-axis) using all the isolates (**C**) or only the self-fertile isolates (**F**).

A



B

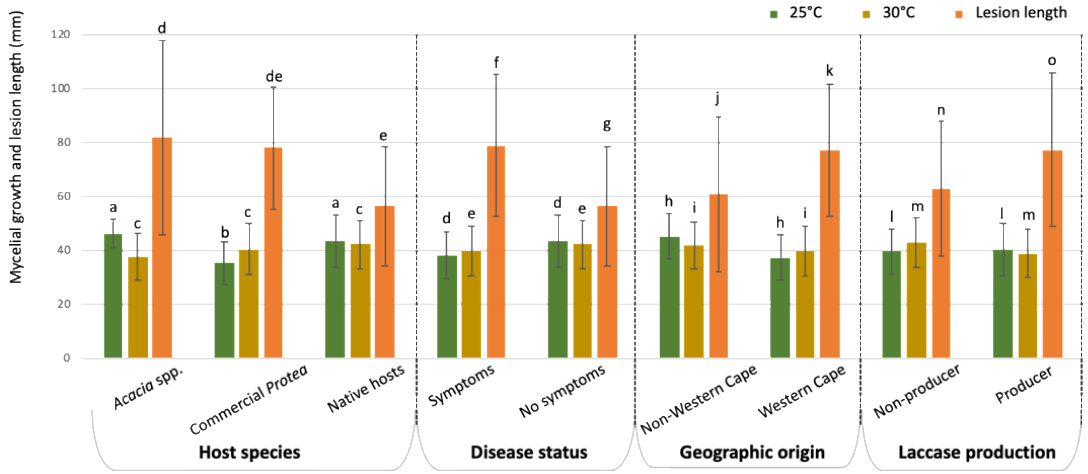


Figure 3.3. Histograms representing mean mycelial growth at 25°C (blue) and 30°C (orange), as well as and mean lesion length (grey) for the self-fertile and self-sterile *C. albifundus* isolates **(A)**. Mean mycelial growth at 25°C (green) and 30°C (brown), as well as and mean lesion length (orange) for the self-fertile *C. albifundus* isolates **(B)**. The isolates are grouped based on their host species, geographic origin, whether they were isolated from healthy or diseased hosts**,** their reproductive status and ability to produce laccase. Bars represent standard deviation, while the different letters within each set of results indicate significant ($P < 0.05$) differences between or among (see Tables 2 and 3 for details of statistical analyses).

SUMMARY

The genus *Ceratocystis* includes economically important species that are associated with a variety of tree hosts. *Ceratocystis albifundus* is an important plant pathogen that causes Ceratocystis wilt on black wattle (*Acacia mearnsii*). The main goal of this study was to increase the current knowledge fot the pathogen's pathogenicity and fitness, using a combination of genomics and laboratory-based methodologies.

The first research chapter of this dissertation exploited available genome sequences to identify genes associated with the oxidation of the phenolic compound α-naphthol. For this purpose, *C. albifundus* isolates from a wide range of hosts and geographic regions were genotyped using genome-wide association analysis (GWAS) using single nucleotide polymorphism (SNP) data. The isolates were also phenotyped by using the phenolic compound α-naphthol which undergoes a colour change when oxidized. The latter data were then combined with the genotype data in GWAS to identify genes and genomic regions underlying this phenotype. Analysis of the genes within the identified regions revealed those encoding products participating in processes and pathways associated with host-pathogen interactions. Future studies are needed functionally characterise the genes identified in this study, as well as to perform expression analyses and knock-out studies.

The second research chapter of this dissertation describes as the study in which the aim was to explore the fitness of *C. albifundus* isolates collected from different host and regions in South Africa. This was done using as the isolates' ability to cause disease on *A. mearnsii* and mycelial growth, and comparisons with other biological data, such as reproductive potential, host species and geographic origin. Results from this study shed light on how host species, reproductive potential and geographic region where collected affect the two fitness parameter measured. Future studies are needed to identify genes associated with mycelial growth and pathogenicity. This includes employing a genome-wide association study, as well as performing whole genome comparisons with the isolates used in this study, as well as isolates collected in future studies.

Overall, the findings from this study substantially increased the available knowledge of the biology, ecology and evolution of this important fungus.