Investigation into DMI and SDHI Fungicide Resistance of the Barley Net Blotches in Victoria, Australia

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List of tables

Table 1. Primer sequences used in endpoint PCR to differentiate *Pyrenophora teres*-subspecies..26
Table 2. The response of six *Pyrenophora teres* f. sp. *maculata* and six *Pyrenophora teres* f sp. *teres* isolates to discriminatory dose concentrations of 10, 20, and 40 µg mL^{-1} of tebuconazole. ..29
Table 3. Example of point mutations in the Cyp51A gene that result in DMI fungicide-resistant *Pyrenophora teres* isolates in Australia* 32
Table 4. Net form net blotch severity in the assessment of the impact of the Cyp51A F489L mutation on the effectiveness of DMI foliar fungicide control. Value for each treatment is displayed as the mean of six treatment replicates and differences between treatments assessed by two-way ANOVA set at a significance level of 95% with Fishers least significant difference test. 35
Table 5. Effect of seed-applied fluxapyroxad (Systiva) on the control of Cyp51A F489L mutants. 36

List of figures

Figure 1. Comparison between spot form net blotch (left) symptoms caused by *Pyrenophora teres* f. sp. *maculata* and net form of net blotch (right) caused by *Pyrenophora teres* f. sp. *teres*. 10
Figure 2. Diagrammatic representation of the Cyp51A genetic polymorphisms, 134 bp insertion mutation in the promoter region and point–mutations, causing an F489L amino acid change found in the Western Australian (WA) *Pyrenophora teres* f. sp. *maculata* (Ptm) and *P. teres* f. sp. *teres* (Ptt) isolates. MR (Moderately resistant to azoles); HR (highly resistant to azoles); CNV (copy number variation of Cyp51A1 and A2 gene paralogues, leading to higher Cyp51 concentration). Adapted from Mair et al. (2020). 21
Figure 3. Locations in the Wimmera and Mallee regions where crop residues 1-20 were collected. 23
Figure 4**.** Gel electrophoresis analysis of PCR product amplified from gDNA of *Pyrenophora teres* f. sp. *maculata* (Ptm) and *Pyrenophora teres* f. sp. *teres* (Ptt) isolates with primers PtmQ7_F and PtmQ7_R and PttQ4_F and PttQ4_R, respectively. PCR products appear at the expected amplicon sizes (Poudel et al. 2017). 30
Figure 5**.** Spot form net blotch symptoms caused by *Pyrenophora teres* f. sp. *maculata* isolates (a) ptm19-057 and (b) ptm19-T1009 and net form net blotch symptoms caused by *Pyrenophora teres* f. sp. *teres* isolates (c) ptt19-T16013 and (d) ptt19-T1011. 31
Figure 6. Phylogram between *Pyrenophora teres* f. sp. *maculata* (ptm), *Pyrenophora teres.* f. sp. t*eres* (ptt) and outgroup *Pyrenophora tritici*-repentis Cyp51A1 gene coding region (Accession no. JQ314404) comparing the sensitive (S) and resistant (R) isolates from Western Australia (red), South Australia (green) and Victoria (blue). The phylogram represents the Geneious Tree: consensus method built by Geneious Prime. Neighbour Joining bootstrap support percentages are indicated at branching points. Scale bar represents the average substitutions per site. 33
Figure 7. Radial growth on PDA agar of six *Pyrenophora teres* f. sp. *maculata* (a) and six *Pyrenophora teres* f. sp. *teres* (b). Averages of four replicates shown ± standard error at 48 h intervals from inoculation. 34
Figure 8. Leaves, cultivar La Trobe, representative of six replicates of treatments 1-8 where variables are *Pyrenophora teres.* f. sp. *teres* isolates and succinate dehydrogenase fungicide seed treatment fluxapyroxad (Systiva). 36

Summary

Net blotches are the most prevalent foliar diseases of barley *(Hordeum vulgare)* in Australia. There are two forms of net blotch, caused by the fungus *Pyrenophora teres* which are estimated to cause a combined cost of $62 million per annum to the Australian barley industry. The net form net blotch (NFNB) is caused by *P. teres*. f. sp. *teres* (*Ptt*) and spot form net blotch is caused by *P. teres* f. sp. *maculata* (*Ptm*). Barley growers are reliant on fungicides for control of net blotches whereas there are few barley cultivars with good *P. teres* resistance. Both *P. teres formae speciales* are genetically diverse and populations have fungicide insensitive genotypes. This has resulted in the selection for, and subsequent increase in the frequency of genotypes with mutations at either the *Cyp51* and/or *Sdh* gene loci which encode enzymes with fungicide resistance to the demethylation inhibitors (DMI) and succinate dehydrogenase inhibitors (SDHI), respectively. Such mutations have been characterized in Western and South Australia, and it was hypothesised that similarly mutated *P. teres* genotypes would be present in Victoria. This was investigated by field screening for potentially resistant *P. teres* isolates, fungicide sensitivity assays, *formae speciales* identification, and subsequent molecular analysis in comparison to known wild-type and mutant isolates. This study identified two out of 36 *P. teres* field isolates screened for fungicide sensitivity (ptt19-T1011 and ptt19-T16013) to have the *Cyp51A* F489L amino acid substitution also found in South Australian *Ptt* mutants. These two isolates displayed high resistance *in vitro* to the DMI fungicides tebuconazole and propiconazole. No DMI fungicide resistance was detected in *Ptm* isolates, and no reduced sensitivity to SDHI fungicides for either *P. teres* subspecies was identified. This is the first known discovery of mutations that have resulted in fungicide resistance within the Victorian *Ptt* population and will have implications for future fungicide use in the control of net blotches.

Keywords

Pyrenophora teres, net blotches, Demethylation inhibitors, fungicide, barley, resistance, Cyp51

Contents

List of tables....3
List of figures....3
Summary....4
Keywords....4
Statement of Authorship....6
Acknowledgments....7
Chapter 1: Net blotch: impact, control, and incidence of fungicide resistance....8
1.1. Introduction: Global presence of barley net blotches....8
1.2. *Pyrenophora teres* distribution and life-cycle....8
1.2.1. Impact across Australia....8
1.2.2. Increasing prevalence and virulence of the barley net blotches in Australia....9
1.2.3. Symptoms and life cycle....9
1.3. Current controls....11
1.3.1. Host plant resistance....11
1.3.2. Cultural control....11
1.3.3. Fungicides....11
1.4. Incidence of *Pyrenophora teres* fungicide resistance....12
1.4.1. Mechanisms of fungicide resistance....12
1.4.2. Resistance to demethylation inhibitors (DMI)....13
1.4.3. Resistance to succinate dehydrogenase inhibitors (SDHI)....14
1.4.3. Resistance to quinone outside inhibitors (QoI)....15
1.4.4. Dual resistance....16
1.4.5. Analysis of mutations for fungicide resistance....16
1.4.6. Management of fungicide resistance....18
1.5. Conclusion....18
Chapter 2. Characterization of DMI and SDHI fungicide resistance in net blotch (*Pyrenophora teres*) of barley (*Hordeum vulgare*) in Victoria, Australia....19
2.2. Introduction....19
2.2.1. Decreased sensitivity and resistance to DMI fungicides in Australia....19
2.2.2. Decreased sensitivity and resistance to SDHI fungicides in Australia....21
2.2.3. Detection of fungicide resistance....21
2.3. Materials and methods....22
2.3.1. Field screen design....22
2.3.2. Infected tissue sample collection....23
2.3.3. Single spore cultures....23
2.3.4. Propiconazole, tebuconazole and fluxapyroxad discriminatory dose screening....24
2.3.5. Genomic DNA extraction....25
2.3.6. Sub-species identification....25

2.3.6.1. Sub-species identification using species-specific primers in endpoint PCR 25
2.3.6.2. Koch's postulates 26
2.3.7. Molecular characterization of candidate fungicide resistance conferring loci 27
2.3.7.1. Endpoint PCR of Cyp51A promoter region 27
2.3.7.2. PCR of Cyp51A promoter and coding sequence analysis 27
2.3.8. In vitro assessment of colony growth 27
2.3.9. In planta assessment of fungicide resistance 28
2.3.9.1. Glasshouse assessment of foliar triazole fungicide efficacy 28
2.3.9.2. Glasshouse assessment of SDHI seed dressing fungicide fluxapyroxad 28
2.4. Results 28
2.4.1. Single-spore cultures from field experiment 28
2.4.2. Discriminatory dose assay 29
2.4.3. Subspecies identification (PCR assay) 29
2.4.4. Molecular characterization of fungicide resistance 31
2.4.4.1. Gel electrophoresis of the Cyp51A promoter region 31
2.4.4.2. Sequencing result 31
2.4.5. Phylogenetic analysis of Cyp51A gene region 33
2.4.6. Radial growth rate assessment 34
2.4.7. Fungicide control of Cyp51A mutants in planta 34
2.5. Discussion 37
2.6. Conclusion 42
Chapter 3. Conclusion 43
3.1. Key Findings 43
3.2. Implications 43
3.3. Future directions 44
References 45
Appendix 52

Statement of Authorship

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Chapter 1: Net blotch: impact, control, and incidence of fungicide resistance

1.1. Introduction: Global presence of barley net blotches

Net blotches are a significant foliar disease of barley globally. There are two common diseases caused by two genetically similar *formae speciales* of fungal pathogen *Pyrenophora teres* (anamorph *Drechslera teres*). Spot form net blotch (SFNB, causal agent *Pyrenophora teres* f. sp. *maculata* (*Ptm*)) and net form net blotch (NFNB, causal agent *Pyrenophora teres* f. sp. *teres* (*Ptt*)) produce dark necrotic spot-type and net-type symptoms respectively (McLean *et al.* 2010; McLean and Hollaway 2019). Net blotches were first described in the 1920s (Drechsler 1923) with epidemics becoming common in the 1960s and are now distributed in all barley growing regions of the world (McLean *et al.* 2009; Liu *et al.* 2011). *Ptm* and *Ptt* have been reported in Denmark (Smedgard-Petersen 1971; Liu *et al.* 2011), Morocco (Karki and Sharp 1986; Jebbouj and El Yousfi 2009), Turkey, Tunisia (Karki and Sharp 1986), France (Arabi *et al.* 1992), Japan (Sato and Takeda 1993), South-Africa (Louw *et al.* 1996; Campbell and Crous 2002), Finland (Jalli *et al.* 2008; Jalli 2010), Canada (Turkington *et al.* 2012), USA (Lartey *et al.* 2013), Czech Republic (Leišová-Svobodová *et al.* 2014) Germany (Rehfus *et al.* 2016), Estonia (Kangor *et al.* 2017), Norway (Wonneberger *et al.* 2017), and Australia (Shipton 1966; Gupta and Loughman 2001; Fowler *et al.* 2017). Net blotches are more severe where susceptible varieties are grown in close rotation. The prevalence of *Ptt* and *Ptm* is dependent on the host-resistance of varieties in a given barley-growing region (Leišová-Svobodová *et al.* 2014).

1.2. *Pyrenophora teres* distribution and life-cycle

1.2.1. Impact across Australia

Net blotches are the most economically important foliar diseases of barley which are prevalent in all barley growing regions of Australia (Murray and Brennan 2010). They are estimated to cause $19 and $43 million in Australian barley production per annum (Murray and Brennan 2010). SFNB has the highest recorded yield losses of 23-44% in Western Australia (WA) (Jayasena *et al.* 2007), while NFNB has been reported to cause yield losses of up to 30% in Victoria and Western Australia (McLean and Hollaway 2019). Yield loss is associated with reduced qualities such as; kernel number, kernel weight, grain plumpness, protein, bulk density, and is worse in barley varieties susceptible to net blotches (Jayasena *et al.* 2007; McLean and Hollaway 2015; McLean and Hollaway 2019). Australia is forecast to produce 10.6 million tonnes of barley in 2020 (ABARES 2020), therefore control of net blotches is important to maximise production by the barley industry for the Australian economy.

1.2.2. Increasing prevalence and virulence of the barley net blotches in Australia

Yield loss associated with net blotches was first assessed in the 1980s in Western Australia (Khan 1987, 1989). The spread of *Ptm* and *Ptt* into the eastern states was first confirmed in the 1990s (Wallwork 2000; McLean *et al.* 2016; McLean and Hollaway 2019). Over the past twenty years, NFNB and SFNB have become the most economically important foliar diseases of barley in all Australian states (Murray and Brennan 2010). Within Victoria alone, SFNB has been observed in 90% of all barley paddocks surveyed (McLean *et al.* 2010). Increasing prevalence has occurred due to a shift in farming practices including retained crop residues, close rotation of barley crop, and suitable climatic conditions (McLean *et al.* 2009, 2010; Liu *et al.* 2011; Gupta *et al.* 2012). The absence of sufficient host resistance in commercial barley varieties has resulted in the dominance of *Ptm* across all regions. *Ptt* occurs sporadically where susceptible varieties are grown (McLean *et al.* 2009) and is becoming more of a widespread problem due to the breakdown of host resistance in some varieties (Hollaway 2020).

Consistent with global studies of *Ptt* and *Ptm* populations, the Australian population has abundant genetic and pathogenic diversity. Fowler *et al.* (2017) demonstrated that regional preference for barley cultivars creates selection pressure for *Ptt* virulence unique to barley growing areas throughout Australia. Using Diversity Array Technology markers, Poudel *et al.* (2019) assessed the genetic structure of *Ptt* within an Australian paddock. This demonstrated that while there is high genetic diversity between geographical locations (e.g. states) there is much less diversity in a single location (e.g. a paddock). However, genetic change is rapid, with Poudel *et al.* (2019) demonstrating that only 25% of the initial genotypes present were clones after three seasons. Virulence has also been seen to move via gene flow between states and re-emerge as cultivar preference changes (Fowler *et al.* 2017; Linde and Smith 2019). As a consequence, *Ptt* and *Ptm* virulence needs to be monitored for changes in pathogenicity (Gupta *et al.* 2012; Poudel *et al.* 2019). Pathogen diversity of *P. teres* is driven by a large population size resultant from the immense land-area sown to barley. In addition, new net blotch genotypes are introduced due to international agricultural trade and the *P. teres* sexual reproduction cycle contributes to the pathogen's genetic variation annually.

1.2.3. Symptoms and life cycle

The two *formae speciales* are morphologically identical, but symptomatically different and belong to the phylum Ascomycota. Early signs of infection are similar but distinct symptoms develop within five days of infection. *Ptm* produces circular or elliptical necrotic lesion surrounded by chlorosis, with hyphae affecting cells directly in contact with or adjacent to the fungus, developing the distinct 'spot-form' (Figure 1) (Lightfoot and Able 2010; Liu *et al.* 2011). *Ptt* has fine, elongated, crisscrossed lesions with faster-growing hyphae resulting in a 'net-type' symptom sometimes

surrounded by chlorosis (Figure 1) (Lightfoot and Able 2010). Chlorotic regions are caused by fungal toxins and plant defence compounds and are known to overtake the entire leaf in cases of severe infection (Leisova *et al.* 2006). The initial stages of infection with *Ptm* appear to be slower and hemibiotrophic with an initial latent or biotrophic infection stage, whereas *Ptt* appears necrotrophic for the duration of the apparently, more aggressive infection (Lightfoot and Able 2010). Despite this, the *formae speciales* life-cycle is almost identical.

Figure 1. Comparison between spot form net blotch (left) symptoms caused by Pyrenophora teres f. sp. maculata *and net form of net blotch (right) caused by* Pyrenophora teres f. sp. teres.

Pyrenophora teres is a crop residue-born fungus that colonizes barley residue between seasons to infect subsequent barley crops (McLean *et al.* 2009). This is a heterothallic fungus (Ellwood *et al.* 2019) with two reproductive cycles (Bogacki *et al.* 2010; Akhavan *et al.* 2017). The teleomorph or sexual stage requires the re-combination of isolates with different mating-types known as *MAT-1-1* and *MAT-2-1*. The hybridization of *Ptt* and *Ptm* mating types have been recorded under laboratory conditions (Rau et al. 2007). *Pyrenophora teres* has also been observe to have hybridized between the two forms in the field but is thought to either be uncommon or progeny generally have poor aggressivenes (Rau *et al.* 2003; Poudel *et al.* 2017). Mycelia colonize crop resides between seasons which then produce pseudothecia (sexual fruiting bodies) when conditions are conducive (Jayasena *et al.* 2004; Bogacki *et al.* 2010). *Ptt* can also survive as invasive mycelia on barley seed husk (Liu *et al.* 2011; McLean and Hollaway 2019). Pseudothecia produce ascospores within approximately six hours of wet conditions between 10-25^{o}C. Ascospores are projected up to 35 cm into the crop canopy as the primary source of infection (McLean *et al.* 2009; Bogacki *et al.* 2010). Leaves are infected by an infection peg that punctures the cell wall forming an intracellular vesicle (McLean *et al.* 2009; Lightfoot and Able 2010) and symptoms develop over 14-20 days (McLean *et al.* 2009). The asexual cycle produces polycyclic asexual conidia that are spread by wind and rain dispersal, causing secondary infections throughout the growing season (Bogacki *et al.* 2010). Symptoms are prominent on the lamina and sheath of leaves but can infect the stem and in the case of *Ptt*, the grains towards the end of a growing season. The main host of *P. teres* is barley (*Hordeum vulgare*) but it has also been found to colonise many grasses including wheat (*Triticum aestivum*) and barley grass (*Hordeum leporinum*) (Brown *et al.* 1993). However, the *P. teres* population of barley grass has been shown not to contribute to barley crop infection (Linde and Smith 2019).

Pyrenophora teres can be distinguished by molecular techniques including the use of species-specific fragment length polymorphisms (Williams *et al.* 2001) and real-time qPCR which also quantifies the amount of fungal biomass in a sample (Leisova *et al.* 2006). Species-specific markers

have also been identified for multiplex PCR reactions to detect individual sub-species as well as possible *Pyrenophora teres* hybrids (Poudel *et al.* 2017).

1.3. Current controls

1.3.1. Host plant resistance

Host-resistance towards *Ptt* and *Ptm* has been assessed by utilized using major and minor genes (McLean *et al.* 2012; Wallwork *et al.* 2016). Major gene resistance normally confers complete resistance often displaying a gene-for-gene interaction with the pathogen (Keller *et al.* 2000; Friesen *et al.* 2007). Minor gene resistance displays no direct interaction with the pathogen and are likely to reduce the impact of pathogen infection rather than prevent infection from occurring (Keller *et al.* 2000). Major gene resistance has been used primarily against *Ptt* genotypes Multiple studies have identified sources of seedling and adult plant genetic host resistance against *P. teres* (McLean *et al.* 2009). Mapping of quantitative trait loci (QTL) in barley cultivars have been done using single sequence repeats (SSRs), amplified length resistance polymorphisms (ALFP), and DArT (Friesen *et al.* 2006; Grewal *et al.* 2008; Gupta *et al.* 2012). Adult-plant resistance to *Ptt* has been found on chromosome 3H, 5H, and 7H, with resistance at the seedling stage also on 5H in addition to 6H and 2H. Resistance QTL against *Ptm* has been identified at the seedling stage on chromosome 4H and 7H with adult resistance also on 4H (Grewal *et al.* 2008). There is one locus on chromosome 6H that confers resistance to both *Ptt* and *Ptm* (Grewal et al. 2008; Burlakoti et al. 2017). *Pyrenophora teres* has high genetic variability which could result in any major-gene resistance being overcome by a single-step mutation. Therefore, it is a preferable breeding strategy to stack resistance QTLs rather than depend on major, dominant resistance genes that could be overcome with a single-step mutation (McDonald and Linde 2002).

1.3.2. Cultural control

Integrated disease management includes the cohesive use of resistant varieties, cultural and chemical practices. To decrease the pressure on host-resistance and minimize epidemics of *net blotches,* barley residue management is essential. Maintaining a three-year gap between barley crops in a paddock and sowing later in predicted wet years will reduce the impact of *P. teres*-associated losses (McLean *et al.* 2009). Any disease that occurs despite cultural controls can be managed with fungicides (McLean *et al.* 2009).

1.3.3. Fungicides

Fungicides are effective at reducing grain yield and quality losses caused by net blotches (Jayasena et al. 2002; Jayasena et al. 2007; McLean et al. 2016; McLean and Hollaway 2019). In Australia, succinate dehydrogenase inhibitors (SDHIs), demethylation inhibitors (DMIs or triazoles), and strobilurin (or Quinone outside inhibitors, QoI) fungicides are registered for the control of *P. teres.*

An initial study of ten fungicides in Western Australia found that pyraclostrobin, epoxiconazole, propiconazole, and a propiconazole/iprodione mix resulted in the best disease control and yield preservation in response to *Ptm* infection (Jayasena *et al.* 2002). A more recent study in Victoria has demonstrated that fluxapyroxad seed dressing provides effective control of *Ptt* and can increase grain yield relative to untreated controls by 8-20% (McLean and Hollaway 2019). Two applications of foliar fungicides are required for effective control of both *Ptt* and *Ptm* in years of high disease pressure (McLean *et al.* 2016; McLean and Hollaway 2019). The most effective time of application is initially at early stem elongation (Z31), and then again at flag leaf emergence (Z39) (Zadoks *et al.* 1974). Fluxapyroxad can be integrated into current control strategies of *Ptt*, but may require a follow-up foliar fungicide at flag leaf emergence (Z39) for control where disease pressure is severe (McLean and Hollaway 2019). Using fungicides when disease severity is greater than 10% on the top three canopy leaves is most likely to provide the most economic control (Jayasena *et al.* 2007).

1.4. Incidence of *Pyrenophora teres* fungicide resistance

1.4.1. Mechanisms of fungicide resistance

Fungicides are classified into modes of action (MOA) by how they inhibit fungal metabolic processes to reduce mycelial growth, or induce fungistasis, or fungal death. Fungicides registered for control of *P. teres* are systemic and classified as targeting a single site within the pathogen, and are therefore more prone to fungicide resistance (Kitchen *et al.* 2016). Selection pressure applied by fungicide can induce two types of resistance. Qualitative resistance is driven by a single-step genetic change where mutant isolates cannot be controlled using fungicide rates that are safe for the crops and the wild-type is heavily selected against within a population under fungicide selection pressure (Deising *et al.* 2008). Quantitative resistance is more common and is related to a more gradual decrease in a pathogen's sensitivity to fungicide rather than outright resistance (Deising *et al.* 2008). When this occurs in the laboratory, fungal isolates that can be inhibited *in vitro* by higher fungicide concentrations are defined as having reduced sensitivity. In a field setting, reduced sensitivity refers to a decline in fungicide performance whereas the term resistance refers to a field failure of fungicide against a pathogen population (Brent and Hollomon 1995; Ishii *et al.* 2015).

Three mechanisms of fungicide resistance are responsible for the changed interaction of fungicides with target enzymes (Hamamoto et al. 2000; Ma and Michailides 2005; Cools et al. 2012; Mair et al. 2016). Firstly, the overexpression of membrane-bound drug transporters results in the increased efflux of azole compounds from a pathogen cell. A mechanism commonly exhibited in multiple drug resistant *Botrytis cinerea* (Kretschmer *et al.* 2009) and citrus pathogen *Penicillium digitatum* (Nakaune *et al.* 1998). Secondly, overexpression of the gene increases target enzyme intracellular concentrations (Cools *et al.* 2012). This has been observed in apple (*Malus*) pathogen *Venturia inaequalis* (Schnabel and Jones 2001) and *Penicillium digitatum* (Hamamoto et al. 2000) in resistance to multiple DMI fungicides (Cools *et al.* 2012). Thirdly, point mutations to the target

gene are the primary source of resistance causing single amino acid changes that alter the fungicide binding efficiency (Parker *et al.* 2014; Mair *et al.* 2016). This is well documented in many pathogens including soil-born animal pathogen *Aspergillus fumigatus* (Liu *et al.* 2015; Berger *et al.* 2017) and wheat pathogen *Zymoseptoria tritici* (Parker *et al.* 2014). A combination of mechanisms is often observed in highly resistant isolates; for example the presence of both a point mutation and overexpression of the target enzyme as observed in grapevine (*Vitis*) pathogen *Erysiphe necator* (Rallos and Baudoin 2016) and brasicca pathogen *Pyrenopeziza brassicae* (Carter et al. 2014).

1.4.2. Resistance to demethylation inhibitors (DMI)

Demethylation inhibitor (DMI) fungicides have been on the market since 1970 and are popular due to their broad-spectrum action against multiple fungal pathogens and affordability in comparison to other registered chemistries. Demethylation inhibitors bind non-competitively to the 14α-sterol demethylase or Cyp51 enzyme in the ergosterol biosynthesis pathway essential to fungal membrane integrity (Hof 2001; Campbell and Crous 2002; Snelders *et al.* 2010; Becher and Wirsel 2012; Parker *et al.* 2014). Located in the outer membrane of the endoplasmic reticulum (Parker et al. 2014), the resultant DMI-CYP51 complex prevents the demethylation of sterol precursors lanosterol and eburicol (Price et al. 2015). The absence of ergosterols and the accumulation of toxic intermediate compounds leads to compromised fungal membrane integrity and eventual fungistasis (Snelders et al. 2010; Price et al. 2015). The widespread use of low-cost DMI fungicides has increased selection pressure on *P. teres,* reducing sensitivity (Schnabel and Jones 2001; Price *et al.* 2015; Rallos and Baudoin 2016). Multiple applications of a fungicide with the same mode of action provides strong selection pressure for the generation of resistant genotypes throughout the growing season. These genotypes then proliferated and dipareses within the population due to sexual reproduction (Campbell and Crous 2002; Akhavan *et al.* 2017; Ellwood *et al.* 2019)

While levels of reduced DMI sensitivity to *P. teres* have been recorded globally (Campbell and Crous 2002; Akhavan *et al.* 2017), molecular mechanisms were first characterized in the Australian cereal belt (Mair *et al.* 2016). A combination of mechanisms causes reduced sensitivities or outright resistance to DMI chemicals. Two mutations have been associated with changes in DMI sensitivity in *Ptm* populations. An amino acid change from phenylalanine (F) to a leucine (L) resulting from one of three different point mutation/s at codon position 489 (F489L). In addition to point mutations, a 134 bp insertion can occur in five different locations in the promoter region of the *Cyp51A*. The presence of these two types of mutations results in either or both reduced binding efficiency and overexpression of the Cyp51A target enzyme (Mair *et al.* 2020). *Ptt* mutants also display the F489L mutation and overexpression of the Cyp51 enzyme. However, in this case the overexpression is thought to be associated with copy number variation and *trans*-regulatory factors (Francisco Lopez-Ruiz, personal communication). Whilst strategic application of the majority of the DMI mode of

action remains an effective control of *P. teres* with reduced sensitivity, continued selection pressure will skew populations towards highly resistant isolates. Some instances of DMI fungicide field failure have been reported in association with highly resistant mutant in Western Australia (Lopez-Ruiz *et al.* 2018). Caution should be exercised in the use of specific DMIs since the binding to their target site is differentially affected by *Cyp51* mutation F489L. Cross-resistance within the DMI mode of action have been recorded in multiple pathogens (Cools *et al.* 2013; Jørgensen *et al.* 2017) and as all DMI's bind to the same active site (Snelders *et al.* 2012), constant monitoring of field isolates is required to prevent the loss of these important fungicides against *P. teres.*

1.4.3. Resistance to succinate dehydrogenase inhibitors (SDHI)

Succinate dehydrogenase inhibitors (SDHIs) are the most rapidly developing fungicide modes of action on the market, especially for their use as a seed treatment. Released in 1966, the first highly effective, broad-spectrum chemical within this group was boscalid in 2003. All SDHI fungicides marketed for agricultural practices bind competitively to the ubiquinone binding site of complex II of the electron transport chain. This prevents the oxidation of succinate in the Krebs cycle leading to the inhibition of respiration. Complex II, also known as the succinate dehydrogenase enzyme (Sdh), is comprised of four subunits (Horsefield *et al.* 2006). The SDHI fungicides bind tightly with highly conserved amino acids inside the catalytic pocket formed by the interface of Sdh subunits B, C and D. Therefore some point-mutations to the genes *SdhB, SdhC,* and *SdhD* will cause amino acid substitutions that can change the chemical properties of the catalytic pocket and reduce the binding efficiencies of fungicide molecules with the Sdh enzyme by up to 90% (Horsefield *et al.* 2006; Sierotzki and Scalliet 2013; Rehfus *et al.* 2016).

Despite their ability to effectively control barley net botches, reduced sensitivity was first detected in Germany in 2012, with 30% of all surveyed net blotch isolates possessing a mutation correlated with reduced SDHI activity (Rehfus *et al.* 2016). In Europe, gene mutations leading to amino acid changes in the subunits *SdhB* (B-H277Y), *SdhD* (D-D124N/E, D-H134R, D-D145G, D-E178K), and *SdhC* (C-N75S, C-G79R, C-H134R, C-S135), are distributed widely. Mutation SdhC-H134R is known to cause the greatest level of resistance with an effective concentration to inhibit by 50% of growth (EC_{50}) of 0.441 mg L^{-1} of fluxapyroxad. This is in comparison to the wild-type at approximately 0.008 mg L^{-1} fluxapyroxad (Rehfus *et al.* 2016). In 2019, this substitution was identified in South Australian *Ptt* populations in addition to SdhD-D145G (Garrard and Wallwork 2020). Mutation SdhC-H134R has also been identified in West Australian *Ptm* isolates in 2020 (Wesley Mair, personal communication). This is hypothesised to be due to the repeated use of fluxapyroxad as a seed dressing in Australian barley varieties susceptible to net-botches. Therefore integrated disease management is essential, with the requirement of any foliar application later in the season recommended to have a different chemical mode of action

The increasing distribution and frequency of *Sdh* mutations in countries with strict SDHI regulations such as France indicate that resistance accumulation is also driven by high disease loads. In climatic conditions that favour barley net blotch disease, conidia with reduced sensitivity to SDHI fungicides proliferate in a population. A recent study in Algeria assessed the net blotch population for *Sdh* mutations before the introduction of commercially available SDHI fungicides and all isolates surveyed were fungicide sensitive (Lammari *et al.* 2020). Suggesting that the continual application of SDHI fungicide is the main selection pressure for any Sdh enzyme mutants over time. It has been hypothesized that field rates of fungicide will still be effective on net blotch isolates with *Sdh* gene mutations (Rehfus *et al.* 2016). This, however, was based on glasshouse experiments and therefore cannot fully replicate field fungicide efficacy or the starting frequency of the resistant *Sdh* gene alleles in the *P. teres* population. The frequency of the South Australian SdhC-H134R and SdhD-D145G mutants is not confirmed, however, field failure of fluxapyroxad has been observed in regions where these mutations were identified (Garrard and Wallwork 2020). To maintain the longevity of SDHI fungicides in the field, the use of alternative seed-applied fungicides and rotating barley varieties with net blotch resistance between seasons is essential in combination with continual resistance monitoring across Australia and globally.

1.4.3. Resistance to quinone outside inhibitors (QoI)

Strobilurin fungicides are quinone outside inhibitors (QoI) and are a more recently developed mode of action. Released in 1996 with the first chemical active azoxystrobin (Fernández-Ortuño *et al.* 2010) resistance was quick to develop with the first instance identified in *Blumeria graminis* f. sp. *tritici* in 2002 (Grasso *et al.* 2006; Sierotzki *et al.* 2007). The QoI's also target the electron transport chain and cause ATP deficiency within the cell by binding to the outer quinone oxidation enzymatic pocket of complex III (Sierotzki *et al.* 2007). The amino acid changes G134A or F129L are found in many pathogens and result in varying levels of strobilurin resistance (Sierotzki *et al.* 2000; Grasso *et al.* 2006; Sierotzki *et al.* 2007). Reduced sensitivity of *P. teres* towards QoI was detected in 2004 (Sierotzki *et al.* 2007; FRAC 2012). Only the mutation encoding F129L substitution has been found in *P. teres* isolates and while it is known to cause variable isolate sensitivities to pyraclostrobin and picoxystrobin *in vitro* bioassays (Marzani 2011), field rates are likely to control *P. teres* isolates with this mutation. Only one known *Ptm* isolate to date has been recorded with reduced sensitivity to QoI relative to other isolates in Canada with growth inhibition of <30% at concentration 0.15 mg L^{-1} pyraclostrobin but could still be controlled at 0.45 mg L^{-1} pyraclostrobin (Akhavan *et al.* 2017). The average EC_{50} values for *Ptt* and *Ptm* are 0.015 mg L^{-1} and 0.024 mg L^{-1} pyraclostrobin in Canada respectively. It is not known if this instance of reduced sensitivity was due to point mutations or natural variation in the pathogen population. A combination of the point mutation that encodes the G134A substitution with an intron adjacent to the point mutation is lethal to *P. teres* because it results in the production of cytochrome b enzyme that is non-functional. Meaning it is unlikely to persist in the pathogen population (Semar *et al.* 2007; Sierotzki *et al.* 2007). QoI fungicide sensitivity

of *Ptt* and *Ptm* in Australia was assessed by Mair *et al.* (2016) and no cases of QoI resistance were identified at that time.

1.4.4. Dual resistance

Three instances of dual decreased sensitivity to fungicides with different modes of action are recorded for *P. teres* globally. Isolates possessing the SdhC-G79R or SdhC-H134R in combination with F129L associated with reduced sensitivity to both SDHI and QoI fungicides have been detected in Europe (Rehfus *et al.* 2016). The *formae speciales* of this isolate was not determined. A low frequency of isolates with these combined mutations has led to the hypothesis that dual reduced sensitivity is associated with a fitness cost on the pathogen, although further study is required (Rehfus *et al.* 2016). Akhavan *et al.* (2017) assessment of Canadian *P. teres* sensitivity to propiconazole and pyraclostrobin identified one *Ptm* isolates with reduced sensitivity to both the QoI and DMI fungicides. Dual resistance between the SDHI fluxapyroxad and DMI tebuconazole has also been identified in South Australian *Ptt* populations (Mair and Lopez-Ruiz 2019). Further reasearch is ongoing.

1.4.5. Analysis of mutations for fungicide resistance

Techniques for analyzing fungicide resistance mechanisms have evolved in parallel with molecular technology. Traditional methods involve pathogen isolation in pure culture followed by plating on media amended with fungicides and sequencing of individual loci to genotype mutations associated with resistant phenotypes obtained in-vitro (Ma and Michailides 2005). These methods of determining resistance is still valid and commonly used, but the development of new molecular techniques complement and accelerate the process of determining fungicide resistance. Techniques need to be cost-effective, sensitive, and rapid (Zulak *et al.* 2018). Those described in the following paragraph are in relation to *P. teres* and their potential to quickly detect mutations associated with fungicide resistance, specifically mutations associated with DMI resistance.

Most techniques revolve around the use of polymerase chain reaction (PCR) (Ishii *et al.* 2015). Endpoint PCR is a viable option for the identification of the *Ptm* 134bp insertion in the promoter region due to the size difference between resistant isolates possessing the insertion and wild-types isolates. As done to monitor *P. digitatum* DMI-resistance (Hamamoto *et al.* 2001), PCR primers based on the DNA sequence polymorphisms could be developed, where the resistant amplicon would be recognized by gel electrophoresis (Ishii *et al.* 2015). An endpoint allele-specific PCR could be used to identify the F489L point mutation by matching the resistant allele at the 3' end. This would have a specific positive or negative endpoint result if the mutation is present (Ma and Michailides 2005; Ishii *et al.* 2015). It does not, however, take into consideration other mutations

that may be contributing to resistance, the need for quantitative data, the possibility of false negatives, or allelic sequence variation (Zulak *et al.* 2018).

Quantitative PCR (qPCR) monitors the accumulation of mutation products in real time (Leisova *et al.* 2006) until reaction substrates become limiting. Allele-specific real-time qPCR has been developed to amplify both sensitive and insensitive alleles (Ma and Michailides 2005). This allows for the rapid analysis of mutant allele ratios in a sample. Plotted against a standard curve, allele-specific real-time PCR has been a successful tool in tracking QoI resistance in *Alternaria* populations (Ma and Michailides 2005). Reliance on a standard curve can be time-consuming (Zulak *et al.* 2018), a factor which is removed in digital PCR (dPCR). This highly precise method uses Poisson statistics instead of a calibration curve to measure DNA quantities (Huggett and Whale 2013). The use of dPCR in agriculture has evolved to detecting DMI resistant mutations in the *Cyp51* gene of *Blumeria graminis* f. sp. *hordei*, quantifying mutations to 0.2% accuracy (Zulak *et al.* 2018). Digital PCR assays have been developed for detecting some of the mutations associated with CYP51A F489L in *Ptm* and *Ptt* with further development underway (Lopez-Ruiz *et al.* 2018).

Previously described molecular techniques all require specialized laboratory equipment, leaving a noticeable gap for in-field testing for fungicide resistance (Ishii *et al.* 2015). LAMP (Loop-mediated isothermal amplification) is an in-field diagnostic technique that amplifies small amounts of target DNA at a constant temperature. Specifically designed, 4-6 primers are used in isothermal conditions within a single, hour-long reaction (Norihiro *et al.* 2008; Duan *et al.* 2016). LAMP has the potential to assist the identification of both the F489L-associated mutations and the promoter insertion in *Ptm.* LAMP has been successfully developed to screen fungicide-resistant point mutations in *Fusarium sp.* (Duan et al. 2016) and *Sclerotinia sclerotiorum* (Duan et al. 2015). Detection of a 120 bp promoter insertion in *Z. tritici* by LAMP has also been developed to identifying a similar overexpression of the gene encoding the CYP51 enzyme in-field (Fraaije 2014).

Microsatellite or short-sequence repeat (SSR) analysis has been used to characterize the variation of pathotypes across *Ptm* and *Ptt* populations (Bogacki *et al.* 2010; Leišová-Svobodová *et al.* 2014). Their abundance in *P. teres* DNA has led to the development of microsatellite markers (Keiper *et al.* 2007) that can be compared between *P. teres* mutant and wild-type isolates and were recently used to track DMI fungicide resistance across Western Australia (Ellwood *et al.* 2019). Diversity Array Technology (DArT) markers have also utilized microsatellites to study the hybridization and population structure of *P. teres* in Western Australia. DArT markers were employed to determine that DMI fungicide resistant isolates were *Ptm/Ptt* hybrid clones that had spread across an area of 350 km in Western Australia (Lopez-Ruiz *et al.* 2020). A similar analysis could be used to track the origin of fungicide resistance-associated mutations across the prominent barley-growing regions of Australia and the globe.

1.4.6. Management of fungicide resistance

Fungicides have become essential in maintaining the efficiency and quality of large-scale food production (Deising *et al.* 2008), with grain production dominating the demand for chemical plant protection (Jeschke *et al.* 2019). Reliance and overuse of fungicides will lead to resistance against chemicals with the same mode of action and must be considered when developing new chemistries. Factors such as a virulence, the number of host species, and mode of action will either increase or decrease the rate at which fungicide resistance will occur (Grimmer *et al.* 2015).

Fungicide resistance of crop pathogens has been traced as a global effort since the 1980s and currently, *P. teres* as a species is classified as a medium risk pathogen to develop resistance towards fungicides (FRAC 2020), while the fungicides register for its control are listed as moderately (DMIs) and highly susceptible (SDHIs and QoI) to having resistance develop towards them.

Management of resistance is vital to maintaining the longevity of fungicides. Initial detection of reduced sensitivity isolates can be difficult as they occur at low frequencies in the field until selection pressure increases and the geographical spread of mutant isolates can no-longer be contained (Cools *et al.* 2013). Strategies to delay fungicide resistance revolve around reducing applications through integrated disease management including rotating different modes of action, correct use of fungicide label rates, a three-year break crop, and the use of resistant barley cultivars (McLean *et al.* 2010; Ishii *et al.* 2015; Hollaway 2020). Kristoffersen *et al.* (2020) demonstrated the possibility of cultivar mixes within a single field reducing the frequency of mutated *Cyp51A* genes of Z*ymoseptoria tritici* within a field by up to 73% under high disease pressure. While this method requires further study and has the potential to manage fungicide resistant isolates of *P. teres* in the future, it is currently impractical for use by producers.

1.5. Conclusion

Ptm and *Ptt* are agriculturally important pathogens in Australia. The current frequency of fungicide-resistant mutants is moderate within both *formae speciales* populations in Australia and in the majority of cases, can currently still be controlled by recommended field rates of fungicide. Regardless, the heterothallic nature of the pathogen and rapid accumulation of resistant genotypes in Europe due to selection pressure provides a warning that fungicides are prone to the evolution of resistance worldwide. The development of molecular techniques to assess levels of fungicide resistance, proper rotation of break crops, alternating modes of action, and investment into breeding resistant high-yielding barley varieties will increase the longevity of fungicides against *P. teres*.

Chapter 2. Characterization of DMI and SDHI fungicide resistance in net blotch (*Pyrenophora teres*) of barley (*Hordeum vulgare*) in Victoria, Australia.

2.2. Introduction

Net blotches are Australia's most damaging foliar diseases of barley (*Hordeum vulgare*). The two forms are SFNB and NFNB caused by *Pyrenophora teres* f. sp. *maculata* (*Ptm*) and *Pyrenophora teres* f. sp. *teres* (*Ptt*). Fungicide application is known to reduce yield losses associated with net blotch epidemics (Jayasena *et al.* 2002; Jayasena *et al.* 2007; McLean *et al.* 2016; McLean and Hollaway 2019). Intensive close-crop rotation and an absence of barley cultivars with good resistance to *P. teres* has led to increased net blotch disease incidence and reliance on fungicide for control (Liu *et al.* 2011; Price *et al.* 2015). Decreased sensitivity and resistance to DMI and SDHI fungicides have been detected in populations of *Ptm* and *Ptt* in Western and South Australia (Mair *et al.* 2016; Mair and Lopez-Ruiz 2019; Mair 2020; Mair *et al.* 2020). The use of similar fungicide control practices raises concerns about selection pressures driving the evolution of fungicide-resistant genotypes in Victoria.

2.2.1. Decreased sensitivity and resistance to DMI fungicides in Australia

Demethylation inhibitor fungicides bind to the Cyp51 enzyme which inhibits fungal growth. Mutations in the *Ptt Cyp51* gene were first identified in 2013 in Western Australia (Mair *et al.* 2016). This study found that *Ptt* has three paralogues of the *Cyp51* gene: *Cyp51A1*, *Cyp51A2*, and *Cyp51B*, all encoding a Cyp51 enzyme. The point mutation results in phenylalanine (F) to a leucine (L) substitution at amino acid position 489 (designated as F489L). This causes the site where DMI molecules bind to change conformation, thereby reducing the DMI fungicide binding efficiency with the Cyp51 enzyme (Mair *et al.* 2016). Similar genetic variation in the *Cyp51* gene has been found in multiple fungal species (Mellado *et al.* 2001; Becher *et al.* 2011). Movement of resistant *Ptt* genotypes across WA was determined by comparing genetic variation in microsatellite markers closely associated with the *Cyp51A* F489L mutation loci. This found that the codon change occurred in a single mutation event and distributed as a result of gene flow and regional spore dispersal (Ellwood *et al.* 2019). A different point mutation leading to the same amino acid substitution (F489L) was identified in South Australia in 2019 (Mair and Lopez-Ruiz 2019). This implies that in this case separate mutation events have occurred rather than gene flow across state borders.

Moderately resistant (MR) isolates in the above studies are defined as having reduced sensitivity to fungicide but can still be inhibited by higher chemical concentrations *in vitro*. Moderately resistant *Ptt* isolates possess only the point mutation at the *Cyp51A* F489L locus. In addition, MR isolates upregulate their *Cyp51A1* and *Cyp51A2* gene paralogues by 3 to 5.3 fold in the presence of DMI fungicide, when compared to wild-type isolates (Mair *et al.* 2016). However, the mechanism of upregulation remains unknown. In comparison, highly resistant (HR) isolates can only be inhibited by very high fungicide concentrations *in vitro*. Highly resistant *Ptt* isolates also possess the *Cyp51A* F489L mutation and higher degrees of overexpression thought to be associated with increased gene copy number of *Cyp51A1* and *A2* paralogues. This results in increased intracellular concentrations of Cyp51 enzyme, out-competing DMI fungicide molecules, however, the extent of this mechanism remains unpublished (Wesley Mair, Personal communication) (Figure 2).

Fungicide resistance towards DMIs in the Western Australia *Ptm* population was identified in 2016 (Mair *et al.* 2020). *Ptm* also possesses point mutation F489L at the *Cyp51A*. However, it is caused by three different point mutations in the coding sequence, hypothesised to have emerged independently by parallel evolution (Mair *et al.* 2020). Unlike *Ptt*, the upregulation of the *Ptm Cyp51A* gene is constitutive regardless of the presence of DMI fungicide and is caused by a 134 bp insertion upstream of the start codon in the promoter region of the gene (Mair *et al.* 2020). Similar insertions have been linked to overexpression of *Cyp51* in multiple fungal pathogens (Hamamoto *et al.* 2001; Schnabel and Jones 2001; Ishii *et al.* 2015). Moderately resistant *Ptm* isolates possess either of the individual fungicide resistance mechanisms, whereas HR *Ptm* isolates contain both F489L mutation and the insertion in the *Cyp51* promoter (Mair *et al.* 2020). For both *Ptt* and *Ptm,* the resistance mechanisms identified are known to correlate with cross-resistance to multiple DMI fungicides including; tebuconazole, epoxiconazole, propiconazole, and prothioconazole to varying sensitivities (Mair *et al.* 2020).

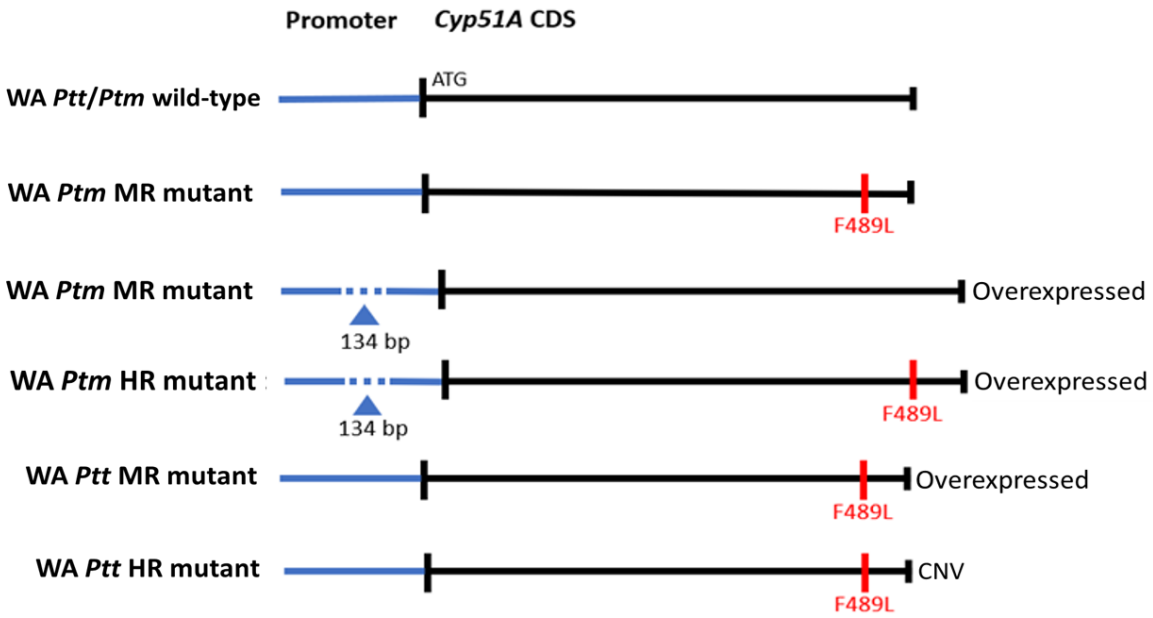


Figure 2. Diagrammatic representation of the Cyp51A genetic polymorphisms, 134 bp insertion mutation in the promoter region and point–mutations, causing an F489L amino acid change found in the Western Australian (WA) Pyrenophora teres f. sp. maculata *(*Ptm*) and* P. teres f. sp. teres *(*Ptt*) isolates. MR (Moderately resistant to azoles); HR (highly resistant to azoles); CNV (copy number variation of Cyp51A1 and A2 gene paralogues, leading to higher Cyp51 concentration). Adapted from Mair et al. (2020).*

2.2.2. Decreased sensitivity and resistance to SDHI fungicides in Australia

Succinate dehydrogenase inhibitors bind with the complex II or the succinate dehydrogenase (Sdh) enzyme (Horsefield *et al.* 2006; Sierotzki and Scalliet 2013). Amino acid changes to SdhD-D145G and SdhC-H134R were identified in South Australian *Ptt* populations in 2019 (Mair and Lopez-Ruiz 2019). Both mutations have also been identified in European *Ptt* populations (Rehfus *et al.* 2016) and are associated with a reduction in the efficacy of SDHI fungicide fluxapyroxad by altering the binding efficiencies. Dual resistance towards DMI and SDHI fungicides has also been identified within the South Australian, York Peninsula *Ptt* population, with 4% of isolates tested as resistant *in vitro* to both modes of action (Mair and Lopez-Ruiz 2019; Garrard and Wallwork 2020). No such dual resistance has yet been identified in Western Australia or Victoria (Francesco Lopez-Ruiz, personal communication).

2.2.3. Detection of fungicide resistance

Sensitivity assays are used to establish pathogen fungicide sensitivity baselines and routine analysis (Ma and Michailides 2005; Bolton and Thomma 2012). *In vitro* radial growth assays and, more recently, microtiter bioassays, which measure growth using optical density (OD), have been used to calculate the sensitivity of *P. teres* to fungicides (Campbell and Crous 2002; Serenius and Manninen 2006; Mair *et al.* 2016; Akhavan *et al.* 2017). The half-maximal effective concentration to inhibit growth (EC_{50}) is the most commonly used indicator of plant-pathogen sensitivity to a fungicide (Liang *et al.* 2015). Discriminatory dose (DD) concentrations are concentrations of a particular fungicide that are used as a standard scale to discriminate between fungicide sensitive, resistant,

moderately resistant and highly resistant phenotypes in screening large isolate collections. The DD will vary depending on the particular fungal species and may even vary between populations of the same species. The means of determining DD concentration of the *P. teres* population varies between researchers (Campbell and Crous 2002; Mair *et al.* 2016; Rehfus *et al.* 2016; Akhavan *et al.* 2017) . However, all DD concentrations must be low enough to allow mutants with marginal levels of resistance to grow, while still been high enough to inhibit the growth of fungicide-sensitive isolates (Ishii *et al.* 2015). These methods complement molecular analyses to identify specific mutations encoding different degrees of fungicide resistance (Bolton and Thomma 2012).

Molecular techniques to detect fungicide resistance can be high-throughput, rapid, accurate, affordable, and quantitative (Zulak *et al.* 2018). Multiple molecular assays have been developed to interrogate the gene regions associated with fungicide resistance for both *Ptt* and *Ptm* (Mair *et al.* 2016; Poudel *et al.* 2017; Ellwood *et al.* 2019; Mair *et al.* 2020). However, frequent surveillance has not detected any fungicide resistant strains in either *P. teres* population in Victoria. Approximately 890,000 ha of barley was sown in Victoria alone in 2020. Given the absence of resistant varieties and reliance on fungicide for production, there is a need for continued sampling to determine the presence or absence of both Cyp51 or Sdh enzyme mutants.

This study aimed to determine the presence or absence of DMI and SDHI fungicide resistance in *P. teres* populations in Victoria. It was hypothesized that fungicide resistant genotypes were present within the Victorian *P. teres* population. This was achieved using a field screen experiment to collect potentially resistant isolates, subsequent *in vitro* analysis of sensitivity, and molecular characterization of Cyp51 or Sdh loci to determine a possible causal link for fungicide resistance of *Ptt* or *Ptm*.

2.3. Materials and methods

2.3.1. Field screen design

P. teres isolates were collected in 2019 from a field site with the purpose of producing and obtaining axenic cultures of potentially fungicide resistant isolates. Barley residues were collected from 20 paddocks remaining after the 2018 harvest in the Wimmera and Mallee regions of Western Victoria during March and April 2019. Residue collected was used as fungal inoculum in the field nursery. Paddocks were selected with a history of propiconazole use in the previous year. In each paddock, 2 kg of dry, crop residues were collected and stored at 20 °C.

The field site was sown on 13 May 2019 on a paddock at Agriculture Victoria's Plant Breeding Centre (PBC) near Horsham (Appendix 1, Appendix 2). Forty-eight 1.9 × 4 m plots of barley cultivar Spartacus CL (*P. teres*-susceptible cultivar) were sown in rows at 150 seeds m^{-2}. Plots of wheat cultivar Wallup were sown around the edge of the field trial to act as buffer rows to reduce the occurrence of spores from outside the collection area. Barley and wheat seed were treated with

triadimenol (150 g L^{-1} active ingredient applied at 100 mL 100 kg $^{-1}$ seed) and penflufen (240 g L^{-1} active ingredient, 40 mL 100 kg^{-1} respectively prior to sowing to control seed-borne smut diseases. Approximately 1 kg of barley residue from each single paddock collection site (Figure 3) was split between fungicide treatments and spread on corresponding plots (1-20, Appendix 3). Plots were allowed to become infected naturally for 4 weeks. Double the manufacturer's recommended rates of tebuconazole (430 g L^{-1} active ingredient at 580 mL ha $^{-1}$) or propiconazole (625 g L $^{-1}$ active ingredient at 400 mL ha^{-1}) were applied to twenty plots each (Appendix 3). Fungicide was applied with a Hardi 3PL 400 L spray unit at 8 km h^{-1} with Hardi yellow 02 mini drift nozzles, pressure 2.1 bar, and a water rate of 100 L ha^{-1}. Fungicide treatments were applied when the barley plants had reached Z15 (Zadoks *et al.* 1974) on 21 June 2019. Control plots did not have barley residues or fungicide applied.

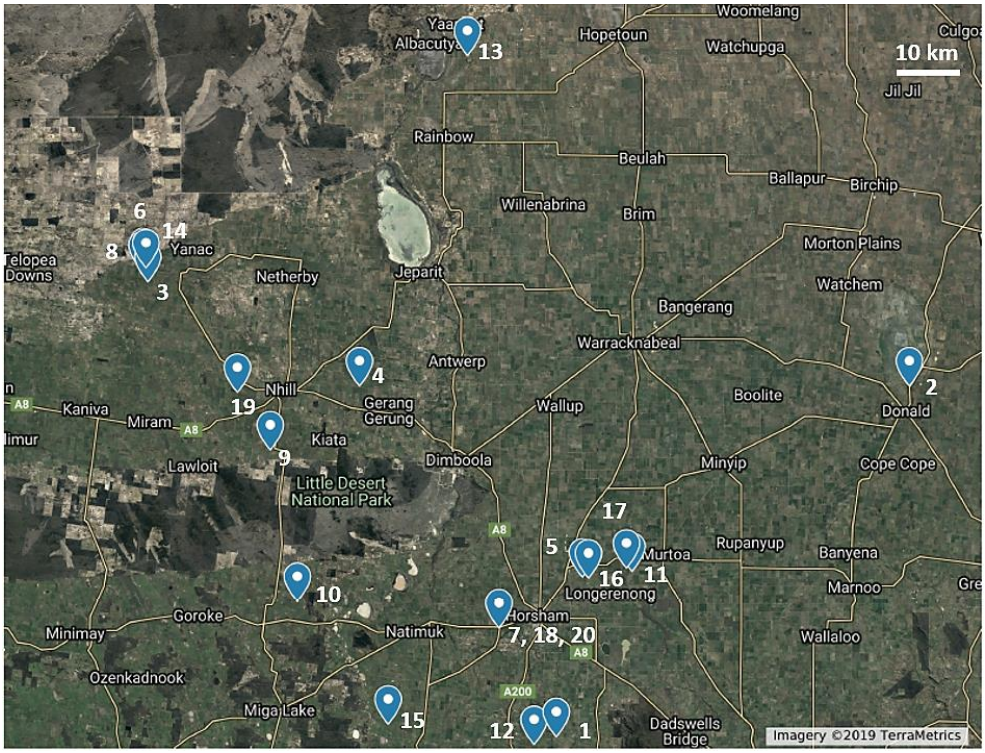


Figure 3. Locations in the Wimmera and Mallee regions where crop residues 1-20 were collected.

2.3.2. Infected tissue sample collection

Ten leaves displaying barley net blotch symptoms were collected per plot. Infected leaf samples were oven-dried at 36 °C for 24-48 h and stored in a dry sealed container at ambient temperature for single spore isolation and molecular analysis.

2.3.3. Single spore cultures

Barley leaves with net blotch symptoms were cut into 1 cm^2 segments. Approximately ten lesions per plot were immersed in 70% ethanol for 30 s, transferred to 25% bleach solution (25% NaOCl,

75%, sterile water) for 30 s then washed with sterile distilled water for 30 s. Leaf segments were then placed onto sterile Petri dishes containing potato dextrose agar media (PDA, 15.6 g PDA, 400 mL RO water) (McLean *et al.* 2010). Leaf segments were incubated under white fluorescent and Gro-Lux lights at 19±1 °C for 3 days until conidiophores were present. To induce sporulation, cultures were incubated at 16 °C for 12-24 h in darkness. A sterile needle was used to transfer single conidia onto Petri dishes with PDA media. Once germinated, single spores were sub-cultured onto V8 juice agar (1.6 g $CaCO_3$, 80 mL V8 juice, 6.4 g technical agar (Difco Laboratories), 320 mL distilled water) and grown for 7-10 d after which plugs of mycelium were freeze-dried in ampoules (Appendix 4).

2.3.4. Propiconazole, tebuconazole and fluxapyroxad discriminatory dose screening

A total of 36 isolates (source: 17 isolates from this study, 2019 field screen, 15 isolates from Horsham collection, Agriculture Victoria, 4 isolates from SARDI 2018 collection) were screened against discriminatory dose (DD) concentrations based on concentrations defined by Mair *et al.* (2016) for tebuconazole and Akhavan *et al.* (2016) for propiconazole (see below and Appendix 4). HR defining concentrations were determine as 10 and 30 µg mL^{-1} higher than the MR tebuconazole concentration and as 10 µg mL^{-1} higher than the MR concentration for propiconazole. Isolates were plated on V8-PDA media (75 mL V8 juice, 5 g PDA, 1.5 g $CaCO_3$, 7.5 g technical agar (Difco Laboratories), 42 mL sterile H_2O). Fungicide discriminatory dose (DD) assays were performed as per Mair et al. (2020). A 4 mm-diameter mycelium plug was taken from the edge of 7 d old cultures and placed on Yeast Bacto Acetate agar (YBA, 5 g yeast extract, 5 g Bacto tryptone (Difco Laboratories), 5 g CH_3NaO_2, 7.5 g technical agar (Difco Laboratories), 500 mL sterile H_2O). YBA Agar plates were amended with fungicide concentrations used to define isolates as either moderately resistant (reduced sensitivity, MR) or highly resistant (HR) to either propiconazole or tebuconazole. Concentrations used for tebuconazole were 10 (MR) and 20 µg mL^{-1} (HR), and 5 (MR) or 15 µg mL^{-1} (HR) for propiconazole. The unamended medium was used as a no fungicide control. Three replicates of each isolate were tested. Isolates that grew were considered resistant to that concentration (+) and colony radius measured. Isolates that did not grow were considered sensitive to the fungicide (-). Isolates that grew only on the infection plug were also considered sensitive (*). Isolates that grew at either 10 or 20 µg mL^{-1} tebuconazole or 5 or 15 µg mL^{-1} propiconazole amended media were included in molecular analysis.

The DD assay was repeated on twelve isolates using concentrations 10 (MR), 20 and 40 µg mL^{-1} (HR) tebuconazole and 5 (MR), and 10 µg mL^{-1} (HR) fluxapyroxad after *Formae speciales* confirmation with PCR. The DD assay was done to directly compare *formae speciales* of confirmed wild-type isolates SG1 (*Ptm*), 9254 (*Ptt*) (source; WA), and fungicide resistant isolates 16FRG073 (*Ptt*), 17FRG001 (*Ptt*), Ko103 (*Ptt*) (source; WA). Also included were isolates of unknown

sensitivity ptm19-057, ptm19-T18005, ptm18-134, ptt19-062, pt19-T16013, pt19-T1011. Growth was compared to an unamended control that did not contain a fungicide and scored as above in the initial DD assays.

2.3.5. Genomic DNA extraction

Fourteen isolates (Appendix 4) were grown on PDA for 7-10 days under 12 h light conditions at 20 °C. Approximately 20 mg wet weight of mycelium were freeze-dried, pulverized with a mortar and pestle, and homogenised in 550 µL CTAB buffer (2% CTAB, 100 mM Tris, pH 7.5;1.4 M NaCl; 20 mM EDTA). Tubes were incubated at 65 °C for 1 h, after which 400 µL of phenol-chloroform-isoamyl alcohol mixture ($C_{12}H_{19}Cl_3O_2$ (24:1)) was added, and then centrifuged at 13000 rpm for 15 min. The supernatant was then transferred to a 2 mL Eppendorf tube, and 50 µL ammonium acetate (7.5 M $NH_4CH_3CO_2$) and 600 µL cold propanol (C_3H_8O) added. Tubes were then incubated for 1 h at -20°C. Tubes were then centrifuged at 13 000 rpm for 10 m, and the supernatant discarded before adding 1mL cold 70% Ethanol (C_2H_5OH) and incubated for 30 m at -20°C. Tubes were centrifuged at 13 200 rpm for 5 m, then supernatant discarded before drying pellet. DNA was dissolved in 100 µL sterile double-distilled H_2O, then stored at 4 °C.

2.3.6. Sub-species identification
2.3.6.1. Sub-species identification using species-specific primers in endpoint PCR

Subspecies-specific primer pairs were used to distinguish between Ptm *and* Ptt *isolates Poudel* et al. *(2017) (*

Table 1). Isolates pt19-T16013, pt19-T13011, pt19-T16009 were compared to *Ptt* and *Ptm* positive controls ptt19-226 and ptm19-057. Reaction mixtures were prepared to 20 µL using 0.1 µL of MyTaq DNA Polymerase (Bioline), 4 µL of 5 × reaction buffer, 1 µL each forward and reverse primer (5 µM), 2 µL of gDNA template (30 ng μL^{-1}), and 11.9 µL sterile H_2O. Samples were incubated at 95 °C for 7 min, followed by 35 cycles; at 95 °C for 30 s, annealing according to the specific primer pairs for 30 s, and extension at 72 °C for 20 s, with a final extension at 72 °C for 7 min. Products were mixed with 6× gel loading dye, purple (New England Biolabs) then separated by gel electrophoresis on 2.5% SYBER safe agarose gel at 70 V against a 25/100 bp ladder (Bioneer).

Table 1. Primer sequences used in endpoint PCR to differentiate Pyrenophora teres-*subspecies*

Primer[b]	Primer sequence 5'– 3'	Annealing temperature (°C)	Product size[a] (bp)
PttQ4_F	CGTCCCGCCGAAATTTTGTA	60	150
PttQ4_R	CAAGGACTTACGCGCTCAAA		
PtmQ7_F	GTAGAGGCTGTAGGAGATGTGATT	60	140
PtmQ7_R	CATGGCAAATTGTTCGTAATCCTG		

[a] Expected DNA band sizes in base pairs with primer mixes and annealing temperatures
[b] (F) indicates the forward primer sequence and (R) the reverse primer sequence. *Ptt* indicates primer binding specificity to *Pyrenophora teres f. sp. teres* and *Ptm Pyrenophora teres f. sp. maculata* (Poudel et al. 2017).

2.3.6.2. Koch's postulates

Koch's postulates (Koch 1876) were conducted on isolates pt19-T16013, pt19-T13011, pt19-T16009 to confirm formae speciales *based on characteristic symptom differences. Isolates were grown on PDA for 7 days in 12 h light conditions at 20 °C. Two 5 mm plugs were sub-cultured onto fiveV8 PDA media plates and grown for 7 days to acquire sufficient inoculum. Approximately 5 mL of sterile H_2O was added to each plate, and mycelium agitated before the mycelial suspension was filtered through a 2 mm wire sieve, keeping isolates separate. Spore suspension concentrations were determined using a hemocytometer and concentrations adjusted to 5×10^3 spores mL^{-1}. Barley cultivar Spartacus CL was sown in 15, 7 cm pots per isolate. Isolates were inoculated using a spray applicator until run-off at growth stage Z14 (Zadoks* et al. *1974) and maintained at 95-100% humidity in darkness for 32 h. Symptoms were left to develop in a glasshouse with natural light at 20 ± 2℃ for 8 days. Single-spore isolates were obtained from symptomatic leaves as per the method described above. Resulting cultures had gDNA extracted and the species re-confirmed by species-specific primers as above (*

Table 1). All isolates identified were re-named with the prefix ‘ptt’ or ‘ptm’ (replacing the original, ‘pt’ prefix) before the isolate unique code identifier.

2.3.7. Molecular characterization of candidate fungicide resistance conferring loci

2.3.7.1. Endpoint PCR of Cyp51A promoter region

The upstream promoter region of the *Cyp51A* gene was amplified with primer sets PtmCyp51A_Pro and PttCyp51A_Pro (Appendix 13) depending upon the *forma specialis* as determined by prior analysis (Mair *et al.* 2016; Mair *et al.* 2020), as per Mair *et al.* (2020). The *Ptm* and *Ptt*-promoter PCR products were mixed with 6× gel loading dye purple and 5 µL loaded into a 1.5% agarose gel that was run for 100 min at 90 V.

2.3.7.2. PCR of Cyp51A promoter and coding sequence analysis

For C*yp51A* coding sequencing (CDS) amplicon, samples sf18/18a, pt19-T16013, pt19-T1011, pt19-T1009, ptm18-134, sf39/18a, ptt19-062, ptm19-057, and pt19-T18005 were amplified with primers as in Mair et al. (2020) (Appendix 6), using PCR conditions as per Mair *et al.* (2016) and (Mair *et al.* 2020). Amplification of the *Sdh* gene locus was done by Wesley Mair (Fungicide Resistance Group, Centre for Crop and Disease Management, Curtin University, Perth). Sequencing of the *Cyp51A* coding region and the *SdhB, C and D* genes was obtained by Sanger sequencing (Macrogen, Seoul, South Korea). All sequences were aligned and a phylogram was produced by Geneious Tree consensus method with default settings in Geneious Prime (version 2020.1.1) with the *Cyp51A* gene region of *Pyrenophora tritici-repentis* (Accession no. JQ314404) included as an outgroup.

2.3.8. In vitro assessment of colony growth

Radial growth was measured for isolates pt19-T16013 and pt19-T13011 to determine whether there was any apparent fitness cost associated with high resistance *in vitro* to DMI fungicide compared to wild-types. This was compared to known fungicide-resistant isolates 16FRG073 (*Ptm*), 19FRG001 (*Ptm*), Ko103 (*Ptt*) and wild-type isolates SG1 (*Ptm*), 9254 (*Ptt*), ptm19-057, ptm19-T18005, ptm18-134, and ptt19-062. Each experiment was replicated four times. A 4 mm mycelial plug was placed in the centre of a plate of PDA media. The diameter of the colony was measured every 48 h for 6 d.

2.3.9. In planta assessment of fungicide resistance

2.3.9.1. Glasshouse assessment of foliar triazole fungicide efficacy

Two seeds of barley cultivar RGT Planet (*P. teres*-susceptible cultivar) were sown in ninety-7 cm pots into the fertilised potting mix and grown under natural light at 20 ± 5 °C for four weeks or until the plants reached the 4-5 leaf stage (Z14-15). Isolates pt19-T13011, pt19-T16013, ptt18-023, and ptt19-062 were individually inoculated onto plants using the same method as above (see *Koch's postulates*). Inoculated plants were then moved from the humidity chamber to a glasshouse for symptom development for 3 days. Mock inoculated (water only) control plants were exposed to the same conditions. Plants were divided into 3 treatments and had either field recommended rates of propiconazole (625 g L^{-1} active ingredient at 200 mL ha^{-1}) or a mixture of tebuconazole (210 g L^{-1}) and prothioconazole (210 g L^{-1})(at 300 mL ha^{-1}), applied with a 1 m hand boom. The third treatment had no fungicide applied. All experimental treatments were replicated six times and organized in a complete randomized block design after fungicide application. Buffer pots were used to prevent edge effects. Disease severity on the exposed leaves prior to triazole foliar fungicide applications was assessed as a percentage of leaf area affected (%LAA). Percentage leaf area assessment was completed 4 and 7 days after inoculation. Treatments were compared by two-way analysis of variance (ANOVA) in the Genstat 18 user interface with isolate and fungicide treatment as factors assuming a 95% level of significance. Fisher's least significant difference was used to test significant differences between means.

2.3.9.2. Glasshouse assessment of SDHI seed dressing fungicide fluxapyroxad

Fluxapyroxad (333 g L^{-1}, at 150 mL 100 kg^{-1} seed) was applied to 500 g of barley seed (cv. La Trobe) at label rate 150 mL 100 kg^{-1} of seed. Two lots of twenty-four 10 cm pots were sown with either three treated or untreated seeds. Three isolates; ptt19-T16013, ptt19-T13011, ptt19-062 were inoculated separately and compared to the mock inoculated (H_2O) control. Pots with treated and untreated seeds were inoculated as above (see *Koch's* postulates). Each treatment was replicated 6 times and randomised in a complete block design. Nine days after inoculation, total disease severity was assessed as a percentage leaf area of the penultimate leaf. Treatments were compared using two-way ANOVA as above.

2.4. Results

2.4.1. Single-spore cultures from field experiment

All plots with crop-residues added developed net blotch type lesions. However, only lesions from plots with tebuconazole applied yielded conidia during the single-sporing process. A total of 33 single-spore isolates were collected from 11 of the 20 plots with corresponding sample locations (Figure 1) and 19 of these isolates were characterised using discriminatory dose analysis. Single-spores were verified as *P. teres* by spore morphology (McLean *et al.* 2009) and specific isolates of interests were further characterized as *Ptt* or *Ptm* by species-specific PCR.

2.4.2. Discriminatory dose assay

Of the 36 isolates screened (Appendix 4), eight isolates were classified as MR to propiconazole at 5 µg mL^{-1} (Appendix 7). Isolate ptt19-T1011 was classified as HR and grew on concentrations of both 5 and 15 µg mL^{-1} propiconazole, and isolate ptt19-T16013 was MR to propiconazole and only grew on the inoculation plug at 15 µg mL^{-1} (Appendix 7, Appendix 8). This experiment was repeated to compare isolates ptt19-T1011 and ptt19-T16013 with known *Ptt* propiconazole-sensitive isolate 9254 and known *Ptt* propiconazole MR mutant isolate Ko103. Isolates ptt19-T1011 and ptt19-T16013 were also confirmed to be HR to tebuconazole, growing at tebuconazole concentrations 10, 20, and 40 µg mL^{-1} (Table 2, Appendix 9). No 2019 isolates of *Ptm* were considered MR or HR to propiconazole or tebuconazole in comparison to known *Ptm* mutant isolates 16FRG073 and 17FRG001, and sensitive isolate ptm19-057, with isolates ptm19-T18005, ptm18-134, and SG1 growing only on the infection plug at 10 µg mL^{-1}. No isolates screened against fluxapyroxad grew on amended media (Appendix 10). All isolates considered MR or HR were selected for further *in vitro* screening and molecular analysis.

Table 2. *The response of six* Pyrenophora teres f. sp. maculata *and six* Pyrenophora teres f sp. teres *isolates to discriminatory dose concentrations of 10, 20, and 40 µg mL^{-1} of tebuconazole.*

	Concentration (µg mL^{-1})			
Isolates	0 µg mL^{-1}	10 µg mL^{-1}	20 µg mL^{-1}	40 µg mL^{-1}
Ptm				
ptm19-057	+	-	-	-
ptm19-T18005	+	*	-	-
ptm18-134	+	*	-	-
SG1	+	*	-	-
16FRG073	+	+	+	*
17FRG001	+	+	+	+
Ptt				
ptt19-199	+	-	-	-
ptt19-062	+	-	-	-
ptt19-T16013	+	+	+	+
ptt19-T1011	+	+	+	+
9254	+	-	-	-
Ko103	+	+	-	-

(+) reduced fungicide sensitivity radial growth on YBA agar media), (-) no growth and sensitivity,
(*) growth on inoculation plug only and sensitivity.

2.4.3. Subspecies identification (PCR assay)

Ptm positive control (ptm19-057) and previously unidentified isolates ptm19-T1009 yielded fragments at the expected amplicon size of 140 bp in the subspecies PCR assay. This is compared to the negative control (ptt19-226) which did not amplify with the *Ptm*-specific primer. The *Ptt* positive control ptt-226 also amplified at the expected amplicon length at 150 bp along with isolates ptt19-T1011 and ptt19-T16013. The negative *Ptt* control isolate (ptm19-057) did not amplify with

the *Ptt*-specific primer set (Figure 4) confirming highly resistant isolates (*in vitro*) ptt19-T1011 and ptt19-T16013 as members of the *Ptt forma specialis*.

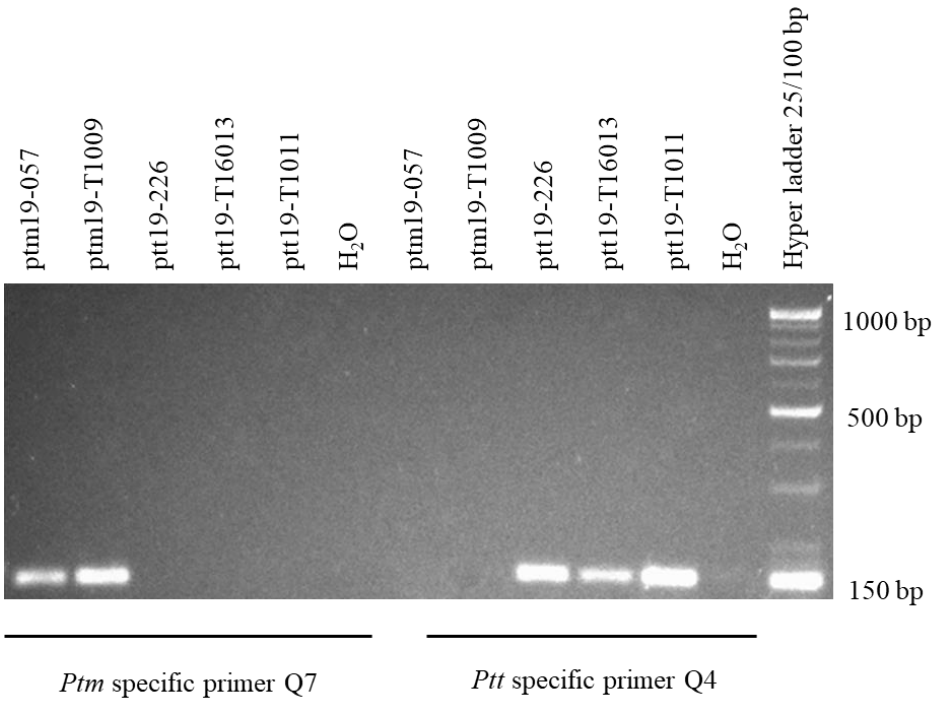


Figure 4. Gel electrophoresis analysis of PCR product amplified from gDNA of Pyrenophora teres f. sp. maculata (Ptm) *and* Pyrenophora teres f. sp. teres (Ptt) *isolates with primers PtmQ7_F and PtmQ7_R and PttQ4_F and PttQ4_R, respectively. PCR products appear at the expected amplicon sizes (Poudel* et al. *2017).*

PCR results were consistent with the spot-type symptom exhibited by isolates ptm19-057, and ptm19-T1009 and the net-type symptom displayed by isolates ptt19-T16013 and ptt19-T1011 when inoculated onto susceptible cultivar Spartacus (Figure 5). Pathogens from infected leaf tissue were re-isolated and single-spored. These isolates were renamed by adding an extra decimal number, i.e. ptm19-057.1 and ptm19-T1009.1. These isolates were then re-confirmed to be *Ptm* and *Ptt* respectively using the same primers as above (Appendix 12).

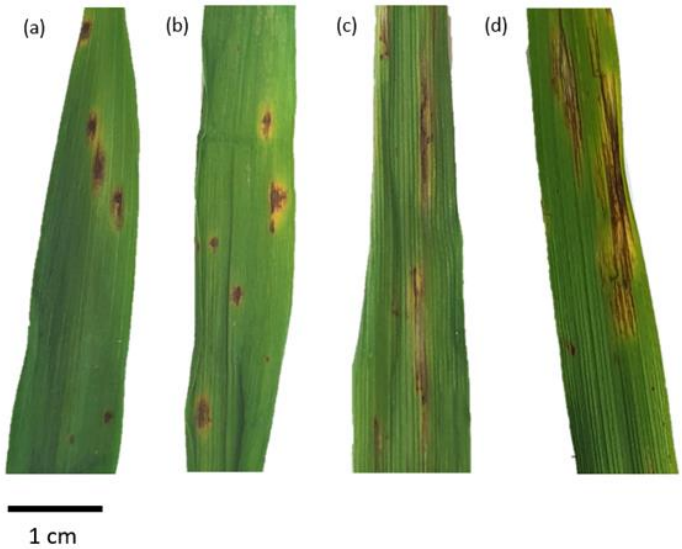


Figure 5. Spot form net blotch symptoms caused by Pyrenophora teres f. sp. maculata *isolates (a) ptm19-057 and (b) ptm19-T1009 and net form net blotch symptoms caused by* Pyrenophora teres f. sp. teres *isolates (c) ptt19-T16013 and (d) ptt19-T1011*.

2.4.4. Molecular characterization of fungicide resistance

2.4.4.1. Gel electrophoresis of the Cyp51A promoter region

There was no evidence of an insertion mutation in the promoter region of *Ptt* isolates ptt19-T1011 and ptt19-T16013 which yielded products consistent with the expected promoter fragment size of the *Ptt* reference isolate Ko103 previously reported to have no insertions in the *Cyp51A* promoter. Wild-type isolate 9254 and all other *Ptt* samples amplified a product of approximately the expected fragment size. Promoter insertion positive template controls for *Ptm* primer set (PtmCyp51A_Pro) amplified *Ptm*-mutant isolates at expected product length of 1143 bp (19FRG001, 16FRG073) and all remaining amplicons to *Ptm*-wild-type expected length of 1009 bp (Appendix 13).

2.4.4.2. Sequencing result

Victorian isolates ptt19-T1011 and ptt19-T16013 were found to have high resistance *in vitro* to propiconazole and tebuconazole (*See discriminatory dose assays* above). The crop residue inoculum samples for these two isolates were from locations 1 and 16, approximately 30 km apart near Longerenong and Laharum in the Wimmera region of Victoria. A non-synonymous thymine (t) to cytosine (c) point mutation was detected at residue 1465, t1465c, in these two isolates as compared to Victorian wild-type isolates ptt19-062, ptm18-13, ptm19-T1009, and ptm19-T18005, which retained wild-type nucleotide (t1465) at that locus (Appendix 11).

The *SdhB*, *SdhC*, and *SdhD* genes were sequenced for isolate ptt19-T1011, ptt19-T16013, and ptt19-062, and compared to the same gene sequences of wild-type isolates. All sequences were consistent with the wild type (i.e. no mutations previously characterized to confer SDHI resistance were identified).

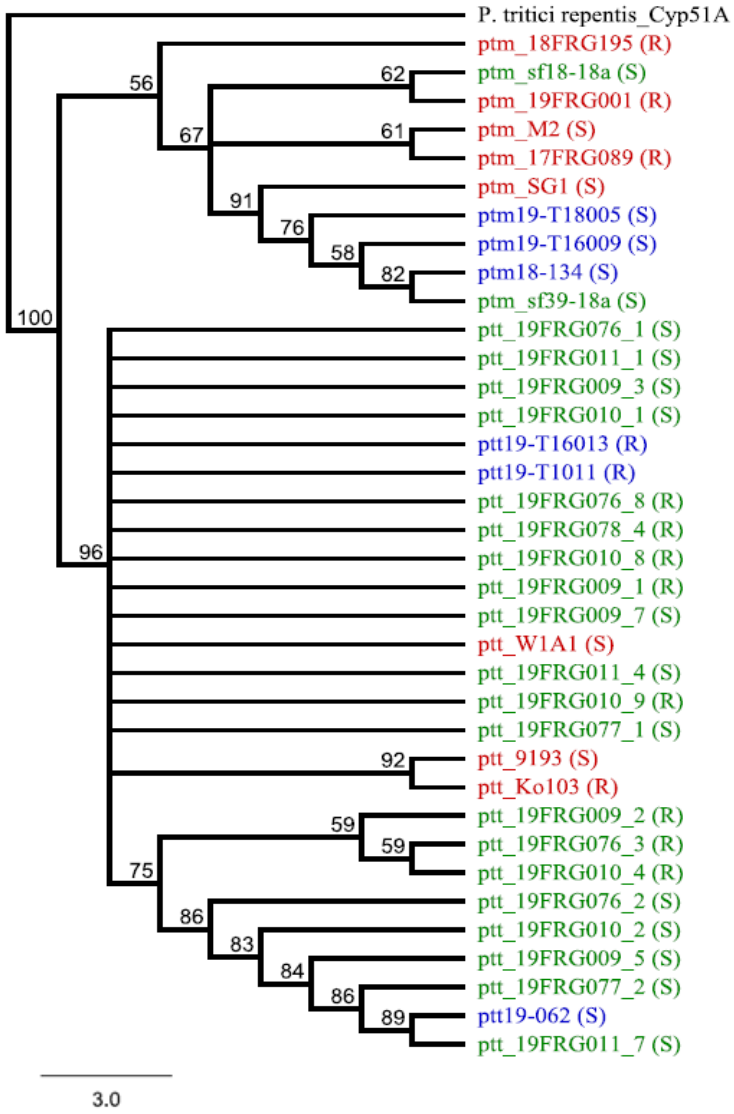
Table 3. Example of point mutations in the Cyp51A gene that result in DMI fungicide-resistant Pyrenophora teres *isolates in Australia*[*]

P. teres formae species	Isolate	First year of detection	Australian state	Codon WT > codon Cyp51A F489L mutant
Ptm	17FRG089[a]	2017	Western Australia	TTC > TTA
Ptm	18FRG195[a]	2018	Western Australia	TTC > CTC
Ptm	19FRG001[a]	2019	Western Australia	TTC > TTG
Ptt	Ko103[b]	2013	Western Australia	TTC > TTA
Ptt	19FRG010[c]	2019	South Australia	TTC > CTC[d]
Ptt	ptt19-T1011	2019	Victoria	TTC > CTC
Ptt	ptt19-T16013	2019	Victoria	TTC > CTC

[a] Published in Mair *et al.* (2019)
[b] Published in Mair *et al.* (2016)
[c] Unpublished sequence data (personal communication)
[d] Codon change representative of all F489L associated mutations in South Australian isolates
*All point mutations lead to a phenylalanine (F) to Leucine (L) substitution at residue 489 in Cyp51A (*Cyp51A* F489L)

2.4.5. Phylogenetic analysis of Cyp51A gene region

Polymorphisms between sequences clearly clustered within the *Ptt* and *Ptm* genotypes. There appears to be greater variation among the *Ptm Cyp51A* genes and no clear distinction between the *Ptt Cyp51A* genes from Western Australia, South Australia, or Victoria. No sequence pattern was evident in the distribution of DMI-resistant or -sensitive isolates (Figure 6).



*Prefix ptt or ptm were added to some isolate identification (eg. Ko103) to clarify *forma specialis* clustering.

Figure 6. Phylogram between Pyrenophora teres f. sp. maculata (ptm), Pyrenophora teres. f. sp. teres (ptt) and outgroup Pyrenophora tritici-repentis Cyp51A1 gene coding region (Accession no. JQ314404) comparing the sensitive (S) and resistant (R) isolates from Western Australia (red), South Australia (green) and Victoria (blue). The phylogram represents the Geneious Tree: consensus method built by Geneious Prime. Neighbour Joining bootstrap support percentages are indicated at branching points. Scale bar represents the average substitutions per site.

2.4.6. Radial growth rate assessment

There was no significant difference between the radial growth rate (all at approximately 12 mm d^{-1}) of azole-resistant *Cyp51A* mutant *Ptt* isolates (ptt19-T16013, ptt19-T1011, Ko103) and azole-sensitive wild-type isolates (ptt19-199, ptt19-062, 9254, Figure 7. b.) This trend is also consistent with the *Ptm* mutants (16FRG073, 19FRG001) and wild-type isolates ptm19-057, ptm19-T18005, and ptm18-134. The exception was *Ptm* Western Australian isolate SG1, which had a radial growth diameter of approximately 15 mm less than all other isolates at 4 and 6 d after inoculation and a growth rate of 9 mm d^{-1} from day 2 (Figure 7.a.).

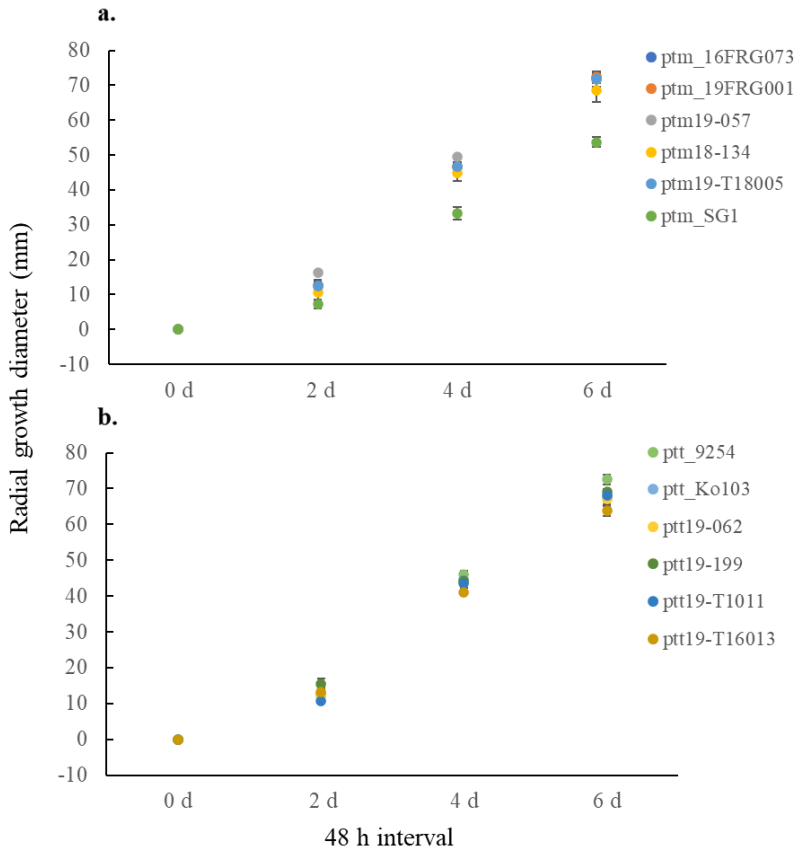


Figure 7. Radial growth on PDA *agar of six* Pyrenophora teres f. sp. maculata *(a) and six* Pyrenophora teres f. sp. teres *(b). Averages of four replicates shown ± standard error at 48 h intervals from inoculation.*

2.4.7. Fungicide control of Cyp51A mutants in planta

The direct impact of *Cyp51A* F489L mutations (2019 field screen isolates ptt19-T1011 and ptt19-T16013) on the control of NFNB by foliar-applied azole fungicides propiconazole and Prosaro (tebuconazole 210 g L^{-1}, prothioconazole 210 g L^{-1}) could not be confirmed due to a non-significant interaction (p=0.993) driven by low levels of infection in untreated plants inoculated with wild type

isolates ptt18-023 and ptt19-062 (Table 4). However, fungicide treatments failed to reduce leaf area infected in isolates ptt19-T1011 and ptt19-T16013 relative to the untreated controls.

Table 4. Net form net blotch severity in the assessment of the impact of the Cyp51A F489L mutation on the effectiveness of DMI foliar fungicide control. Value for each treatment is displayed as the mean of six treatment replicates and differences between leaf infected and wil-type treatments assessed by two-way ANOVA set at a significance level of 95% with Fishers least significant difference test.

	NFNB severity (Percentage lead area affected)[#]			
Fungicide*	Propiconazole (625 g L^{-1} active)	Prothioconazole + Tebuconazole (210 g L^{-1} and 210 g L^{-1} active)	Untreated (H_2O)	**Mean**
Isolate				
ptt18-023[a]	2	4	4	**3**
ptt19-062[a]	4	6	6	**5**
ptt19-T1011[b]	24	30	24	**26**
ptt19-T16013[b]	21	26	22	**23**
Uninoculated	0	1	1	**1**
Mean	**10**	**13**	**11**	
P-value				
Isolate	<.001			
Fungicide	0.328			
Isolate × fungicide	0.993			

[a] Wild-types isolates
[b] *Cyp51A* F489L mutant isolates highly resistant to propiconazole and tebuconazole *in vitro*
[*] All fungicide treatments applied three days post barley (variety; La Trobe) inoculation
[#] Average of percentage leaf area affected for six treatment replicates ten days after isolate inoculation

The seed-applied fungicide fluxapyroxad (Systiva) significantly reduced NFNB severity for all isolates tested (Figure 8). Both mutated isolates ptt19-T1011 and ptt19-T16013 exhibited no net-form symptoms in comparison to untreated seed which displayed net-form symptoms at a percentage infected leaf area of 31% and 37%, respectively. Isolate ptt19-062 (found to be sensitive to azoles *in vitro*) was also controlled by fluxapyroxad seed treatment, however, it was not completely inhibited (percentage leaf area; 2%, Table 5).

Table 5. Effect of seed-applied fluxapyroxad (Systiva) on the control of Cyp51A F489L mutants.

Isolate	NFNB severity (percentage leaf area affected)*	
	Fluxapyroxad (333 g L^{-1} active at 150 mL 100 kg^{-1})	Untreated
ptt19-T1011[a]	0	31
ptt19-T16013[a]	0	37
ptt19-062[b]	2	57
uninoculated	0	1
P-value (fungicide × isolate)	<.001	
LSD	13	

[a] *Cyp51A* F489L mutant isolates highly resistant to propiconazole and tebuconazole *in vitro*
[b] Wild-types isolates
* Values for each treatment displayed as the mean of six treatment replicates and differences between treatments assessed by two-way ANOVA set at a significance level of 95% with Fishers least significant difference test (variety; La Trobe)

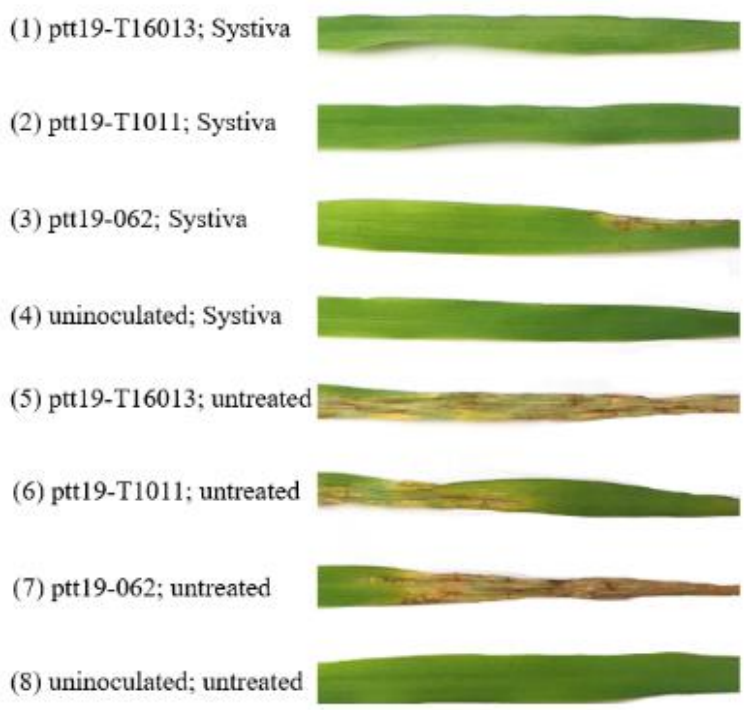


Figure 8. Leaves, cultivar La Trobe, representative of six replicates of treatments 1-8 where variables are Pyrenophora teres. f. sp. teres *isolates and succinate dehydrogenase fungicide seed treatment fluxapyroxad (Systiva).*

2.5. Discussion

This study has confirmed that strains with high *in vitro* resistance to DMI fungicides tebuconazole and propiconazole are present in Victorian *P. teres* populations. No *P. teres* strains were identified with resistance to SDHI fungicides. Of the 36 single-spore field isolates screened for fungicide resistance, two *Ptt* isolates (ptt19-T1011 and ptt19-T16013) were characterized to have a point mutation in the *Cyp51A* gene that encodes a phenylalanine to a leucine amino acid substitution (F489L). It is likely that this point mutation causing F489L amino acid change is contributing to a decrease in DMI fungicide sensitivity, as it has been recorded previously in *Ptt* isolates collected in Western Australia (Mair *et al.* 2016). The *in vitro* azole-resistant phenotype caused by this mutation was determined by fungicide discriminatory dose analysis and subsequently confirmed by genomic sequencing of the *Cyp51A* locus. This is the first known discovery of decreased sensitivity to DMI fungicides within the Victorian *P. teres* population and will impact the future monitoring and management of DMI fungicides to maintain the longevity of these chemistries on-farm.

The two *P. teres* pathogens are genetically diverse pathogens due to their sexual reproduction cycle (McLean *et al.* 2009). Variable sensitivities of *P. teres* isolates to fungicides have been recorded previously (Campbell and Crous 2002; Akhavan *et al.* 2017). The analysis of *P. teres* sensitivity to fungicide using EC_{50} values in a representative group of isolates contributes towards the establishment of discriminatory dose concentrations. This in turn differentiates between sensitive, moderately resistant, or highly resistant isolates. In this study isolates were screened on agar with concentrations of tebuconazole defined in Mair *et al.* (2016) and Mair *et al.* (2020). *Ptt* isolates ptt19-T1011 and ptt19-T16013 grew on higher concentrations *in vitro* than West Australian *Ptt* isolate Ko103 on all tebuconazole concentrations. Isolate Ko103 has been classified as moderately resistant to DMI fungicides (Mair *et al.* 2016). The EC_{50} value of ptt19-T1011 and ptt19-T16013 has yet to be assessed, but it can be inferred to be higher than 3.9 µg mL^{-1} tebuconazole which is the EC_{50} of isolate Ko103 (Mair *et al.* 2016). This is in comparison to the *Ptt* wild-type 9254, used in this study, with an of EC_{50} of 0.28 ± 0.05 µg mL^{-1} tebuconazole (Mair *et al.* 2016). Isolate Ko103 reported a lower level of resistance to propiconazole with an EC_{50} value of 0.65 ± 0.13 µg mL^{-1}. Isolate ptt19-T16013 may have a similar EC_{50} to Ko103 because it is also MR to propiconaozle. Isolateptt19-T1011 can be predicted to have a higher EC_{50} than Ko103, as it is highly resistant to propiconazole in this study. Other EC_{50} DMI assessments of propiconazole have been completed for *P. teres* populations globally, providing baseline comparisons. Canadian *Ptt* populations have an average EC_{50} of 1.5 µg mL^{-1} for propiconazole (Akhavan *et al.* 2017), with the highest individual isolate EC_{50} assessed at 2.989 µg mL^{-1}. Algerian *P. teres* populations have a EC_{50} range of 0.113-0.216 µg mL^{-1} propiconazole and 0.086-0.149 µg mL^{-1} tebuconazole (Lammari *et al.* 2020). The *Ptt* population in South Africa has also been assessed with EC_{50} values ranging between 0.004-7.693 µg mL^{-1} for propiconazole (Campbell and Crous 2002). All studies used the microtiter bioassay method with the exception of the South African analysis which used the radial growth method, meaning not

all EC_{50} values are entirely comparable due to differences in assay sensitivities (Tremblay *et al.* 2003).

Recent Australian and Canadian studies suggest greater DMI fungicide insensitivities are accumulating in the *Ptt* population in these countries due to historical use of fungicides (Akhavan *et al.* 2017, Mair *et al.* 2016). No resistance in the Victorian *Ptm* population was detected in this study (Table 2). However, caution should be exercised as resistant *Ptm* isolates have beingidentified in Western Australia with higher levels of tebuconazole resistance been detected in *Ptm* than in *Ptt* populations (Mair *et al.* 2020). There is potential for *Ptm* resistance to emerge in Victoria and continued monitoring of *P. teres* populations should be conducted to determine potential reductions in *P. teres* sensitivity to DMI fungicides.

Demethylation inhibitors are one of three mode of actions registered in Australia for foliar use against *P. teres* and all have a single target site. The 14α-sterol demethylase (Cyp51) enzyme catalyses an essential step within the fungal sterol biosynthesis pathway, which is responsible for the production of membrane sterols. Demethylation inhibitor fungicides bind non-competitively to amino acid residues that border the active site, resulting in a catalytically inactive enzyme and fungistasis (Mullins *et al.* 2011). *Ptt Cyp51* F489L mutation results in structural changes in the enzyme active site, which alters the binding of some DMI fungicides (Mair *et al.*, 2016). This reduces the efficacy of DMI fungicides against the Cyp51 enzyme, allowing the fungus to continue to produce ergosterol. The F489L mutation found in ptt19-T1011 and ptt19-T16013 is consistent with constriction of the enzyme binding site in West Australian isolates of *Ptm* and *Ptt* caused by the F489L substitution. The point mutation that causes the F489L change is also equivalent to the F506I point mutation in the *Cyp51A* locus that has been modelled in *Aspergillus fumigatus*, with isoleucine instead of a leucine substitute for phenylalanine (Mair *et al.* 2016). In comparison to *A. fumigatus*, *P. teres* does not require additional point mutations to cause fungicide resistance (Liu *et al.* 2015). This restriction of the binding site has also been observed in *Mycosphaerella graminicola* to cause different levels of reduced sensitivity to various DMI chemistries (Cools *et al.* 2011; Cools and Fraaije 2013). In this study, isolates ptt19-T1011 and ptt19-T16013 were only tested against two DMI fungicides, tebuconazole and propiconazole. However, Western Australian isolate Ko103, which acted as a positive control in this study also possesses the F489L mutation and has been confirmed as having reduced sensitivity to DMI's epoxiconazole and prothioconazole (Mair *et al.* 2016). All DMI fungicides have a slightly different binding mechanism with the Cyp51 enzyme. However, difenoconazole, epoxiconazole, tebuconazole, and propiconazole have a similar docking mechanism with the Cyp51 enzyme (Snelders *et al.* 2012). This suggests that strains that already have the *Cyp51* F489L gene mutation may also already have cross resistance to a range of azoles. Additional research would be required to validate this.

Sequence analysis of the *Cyp51A* locus confirmed that both isolates ptt19-T1011 and ptt19-T16013 had a point mutation at codon 489, causing a phenylalanine to a leucine substitution in the Cyp51

enzyme. Fungicide-resistant *Ptm* isolates from Western Australia have three different codon changes TTA, CTC, or TTG in comparison to the wild-type (TTC) present in all *P. teres* DMI sensitive isolates. In contrast, the Western Australian *Ptt* mutant isolates only possess a TTA codon at the F489L locus. Victorian mutants ptt19-T1011 and ptt19-T16013 possessed the same codon change, from TTC > CTC, as South Australian *Ptt* mutants (Table 3). It is also possible that because ptt19-T1011 and ptt19-T16013 are highly resistant *in vitro* the Cyp51 enzyme may be overexpressed. It has been found that Western Australian *Ptt* DMI-moderately resistant mutants (Ko103) overexpress the Cyp51A enzyme in the presence of a DMI fungicide in addition to having the *Cyp51A* F489L mutation (Mair *et al.* 2016). The higher concentrations of Cyp51A require increased DMI levels to maintain fungal inhibition. Such overexpression is often caused by insertion mutations in the promoter region of the *Cyp51* genes (Ishii et al. 2015). In *Ptm*, 134 bp insertions at different positions in the *Cyp51* promoter region were shown to cause *Cyp51* overexpression in West Australian mutant *Ptm* isolates (Mair et al. 2020) (Appendix 13, Figure 2). The expression levels of *Cyp51* in the *Ptt* DMI-resistant isolates, ptt19-T1011 and ptt19-T16013, is yet to be established, however no promoter length polymorphisms were detected in this study (Appendix 12). This suggests that, unlike *Ptm*, promoter insertions do not seem to be associated with the resistance found in *Ptt* isolates ptt19-T1011 and ptt19-T16013. The cause of overexpression in West Australian *Ptt* highly resistant mutants is currently under investigation. However, copy number variation (CNV) of *Cyp51* has been found in pathogen *Erysiphe necator* in combination with point mutation (Y136F) to the CDS and is correlated with fungal growth and upregulation in the presence of DMI fungicide (Jones *et al.* 2014). An increase in copy number of the *Cyp51* gene could produce an overexpression-type effect, which would translate into more DMI molecules being required to inhibit the additional Cyp51 targets. This may explain the continued growth of isolates ptt19-T1011 and ptt19-T16013 on high azole concentrations. However, the potential of CNV is concerning because, unlike resistance caused by point mutations, overexpression of the Cyp51 enzyme could potentially affect all compounds within the DMI fungicide group regardless of how they bind to the enzyme (Cools *et al.* 2013). Gene expression analysis of the Victorian isolates is needed to fully understand their potential impact upon the on-farm use of fungicides.

Gene flow may have occurred between SA and Victoria, as *P. teres* has the ability to spread conidia over 100 km from the original mutation event (Ellwood *et al.* 2019). The phylogenetic analysis of the *Cyp51A* gene displays high bootstrap values (>50%) between the Western Australian, South Australian, and Victorian *Cyp51* CDS region of *Ptt* isolates (Table 3). This means that no separation between the isolates from different states could be made and conclusions about the origin of the F489L mutation are difficult to make. Mair *et al.* (2020) suggest that the three F489L point mutations present in *Ptm* have occurred as independent mutations, then spread throughout Western Australia. *P. teres* populations in Australia are distinct between states with high levels of genetic variability (Serenius *et al.* 2007; Bogacki *et al.* 2010; Fowler *et al.* 2017). Therefore, it is possible that Victorian mutations have arisen independently under similar DMI selection pressures as in the Western and

South Australian populations. Despite the likelihood of parallel evolution occurring, gene flow cannot be as easily discounted in *Ptt* as in *Ptm.* This is firstly due to the ability of *Ptt* to become seed born and national grain movement could potentially spread resistant *Ptt* across state borders. Secondly, there is evidence of Western Australian virulence profiles in South Australian *P. teres* isolates, which suggests that genotypes have the potential to move over large distances (Fowler *et al.* (2017).

To study the geographical origin and potential movement of the *Cyp51A* F489L mutation, Ellwood *et al.* (2019) used single sequence repeat (SSR) markers to trace the Western Australian *Cyp51A* F489L change to a single mutation event in Western Australian. To clarify gene flow between *P. teres* populations within Australia, further analyses could include the use of high-throughput techniques such as diversity array technology (DArT) markers or sequencing (DArTseq) (Jaccoud 2001). Poudel *et al.* (2019) used DArT markers to compare and trace the genetic variability of *Ptt* isolates within a field location over time, indicating the rapid change in genetic diversity of *P. teres* within a single geographical location. Markers produced by DArTseq have also been linked with the *Cyp51* gene locus in the fungus *Pseudocercospora fijiensis* in a study of DMI resistance (Chong *et al.* 2019). Phylogenetic analysis (Edet *et al.* 2018) using a DArT approach could be used to compare the *P. teres* fungicide-resistant and -sensitive isolates in relation to the *Cyp51* F489L locus. This would allow the determination of whether *Cyp51A* F489L mutation originated from a single event or if it has emerged independently under similar selection pressures by parallel evolution with higher precision.

Of interest is the observation that no isolates could be obtained from any of the lesions collected from the field screen plots where propiconazole was applied. A repetition of the single-spore process from similarly treated *P. teres* lesions is required. Despite this, mutations to the *Cyp51* locus or CNV as observed in *E. necator,* are observed to have greater fitness in the presence of fungicide compared to wild-type isolates (Cools *et al.* 2011; Jones *et al.* 2014; Wieczorek *et al.* 2015; Mair *et al.* 2016). There are also no reports of pathogenic fitness penalties associated with *P. teres Cyp51A* gene mutation. This was demonstrated in this study as the *Cyp51A* mutation did not impede radial growth rate *in vitro* of isolate ptt19-T1011 or ptt19_T16013 in comparison to wild-type isolates Figure 7). As observed among many pathogens, DMI fungicide application can cause dramatic shifts towards a higher frequency of *Cyp51A* mutations in a population (Brunner *et al.* 2008; Wieczorek *et al.* 2015; Jørgensen *et al.* 2017; McDonald *et al.* 2018; Mair *et al.* 2020). However, the frequency of fungicide-resistant alleles is often low when resistance is first detected (Cools *et al.* 2006; Cools *et al.* 2013). Of the 32 Victorian *P. teres* isolates screened in the DD assays, approximately 6% of these were confirmed to have mutation F489L. Isolates ptm19-T16013 and ptm19-T1011 were sourced from barley leaf spot lesions in plots treated with crop residues sourced approximately 30 km apart (Figure 3). There is also the possibility that isolates sourced from these plots were due to random infection by wind and rain-dispersed at the field experiment location and further analysis of

samples from locations 1 and 16 is required to confirm the origin of *P. teres Cyp51* F489L mutant alleles in Victoria. The single isolate that originated from the control plots with no barley-residue applied was not resistant to DMI fungicides. The level of azole resistance detected in *Ptt* was low in this screen, however further screening would be required to predict the occurrence of azole-resistance more confidently in Victorian populations of *Ptt*. No DMI-fungicide resistance was identified in Victorian *Ptm* isolates in this study, however variation within the Victorian *Ptm* population towards DMI fungicides has been previously assessed (Nathaniel Clarke, November 2009, unpublished).

In vitro sensitivity results are not an accurate representation of the impact of mutations on DMI control programs in the field. The direct release of mutant isolates into the field is not practical or acceptable. Therefore, an *in planta* glasshouse assessment was done to determine the potential impact of *Cyp51* F489L mutation on controlling isolates ptt19-T1011 and ptt19-T16013 with DMI fungicides propiconazole and tebuconazole/prothioconazole. Unfortunately, poor infection by control isolates (ptt19-062, ptt18-023) made the results of the experiment inconclusive, but the fungicides used did not control infection of either mutant isolate (ptt19-T1011 and ptt19-T16013, Table 4). Repetition of this experiment is required to make inferences about the potential impact of *Cyp51A* F489L mutations on DMI fungicide use in the field.

Fluxapyroxad resistance has recently been detected in Western and South Australian states (Mair and Lopez-Ruiz 2019; Mair 2020). The azole-resistant isolates were screened against a seed-applied SDHI fungicide to determine whether they displayed dual fungicide resistance. An *in planta* glasshouse experiment had a significant fluxapyroxad fungicide by isolate interaction effect (p-value: 0.05, Table 5), confirming that isolates ptt19-T1011 and ptt19-T16013 were sensitive to fluxapyroxad (SDHI) seed dressing. From the glasshouse and DD experiments conducted, it can be concluded that fluxapyroxad seed dressing is likely to remain effective against these Victorian *Ptt* isolates. This is consistent with the sequencing results of the *Sdh* gene for isolates ptt19-T1011, ptt19-T16013, and ptt19-062, that contain no known mutations previously seen to result in SDHI resistance (Rehfus *et al.* 2016). More extensive screening of Victorian *P. teres* field populations and the establishment of sensitivity baselines for SDHI chemistries is recommended for Victoria *P. teres* populations. This would determine current levels of possible SDHI resistance/sensitivity as the frequency of SDHI fungicide resistance in a population can rapidly increase if selection pressure is maintained (Rehfus *et al.* 2016).

Despite inconclusive data regarding the use of DMIs against barley infected with Victorian mutants it can be stated that field failure of fungicides where a high frequency of outright resistance isolates occurs (Brent and Hollomon 1995) is unlikely in the short term. However, given the levels of insensitivity identified in the laboratory, caution should still be exercised in the use of products containing tebuconazole and propiconazole. Ongoing field screening of *P. teres* isolates will also be essential in the collection and monitoring of potentially fungicide-resistant isolates. Upon repetition

of the field screen used in this study, the use of a highly susceptible barley variety such as La Trobe would be more efficient in collecting a greater range of *P. teres* isolates in comparison to Spartacus CL, which is only moderately susceptible to *Ptt* (Brown and Harris 2019). There is the continuing need for molecular tools to diagnose the presence or absence of point mutations that encode fungicide resistance. Digital PCR can be used to calculate the proportion of mutant alleles relative to the wild-type alleles, thereby quantifying the presence and amount of mutant alleles in a sample (Huggett *et al.* 2015). This technique has been successful used in quantifying DMI-resistant mutations of the *Cyp51* gene in *Blumeria graminis* f. sp. *hordei* (Zulak et al. 2018). This highly precise method has also been designed to detect the codon change in West Australian *Ptt* populations (CCT > TTA, Table 3, Wesley Mair, Personal Communication). Further work is currently underway to develop primers and probes specific to other codon changes present in Australian *Ptt* and *Ptm* populations (Wesley Mair, unpublished), contributing towards informing growers to the potential risk associated with deploying fungicides.

2.6. Conclusion

To conclude, the *Cyp51A* F489L mutation that confers decreased sensitivity to DMI fungicides has been reported in the Victorian *Ptt* population for the first time. No decrease in DMI sensitivity to the *Ptm* population or SDHI fungicide sensitivity for either subspecies of *P. teres* was identified in this study. Short barley crop rotations, an absence of cultivars resistant to *P. teres*, and selection pressure caused by DMI fungicides have the potential to increase mutant allele frequency in the Victorian *P. teres* population (Rau *et al.* 2003; Akhavan *et al.* 2017). Barley growers and advisors in Victoria need to carefully manage the use of these fungicides within an integrated disease management program and should consider ongoing monitoring of DMI and SDHI fungicide resistance on-farm to allow reactive management.

Chapter 3. Conclusion

3.1. Key Findings

This study screened isolates from the Victorian *P. teres* population for their response to two different fungicide mode of action groups (DMI and SDHI). Chapter 1 is presented as a review of the impact of *P. teres* as an agriculturally important pathogen globally in prominent barley growing regions and the increasing threat of DMI, SDHI, and QoI fungicide resistance. Chapter 2 outlines the scientific process that resulted in the characterisation of mutation *Cyp51A* F489L in isolates ptt19-T1011 and ptt19-T16013 that confers DMI resistance *in vitro* to tebuconazole and propiconazole. This is the first known instance of mutations associated with fungicide resistance in Victoria. Further investigation is required to determine the impact of DMI fungicide usage on the field control of isolates existing resistance *in vitro*. Integrated disease management strategies and continual monitoring for fungicide resistance is needed to minimize the spread of mutated fungicide resistance *P. teres* isolates in the Victorian population.

3.2. Implications

An integrated disease management approach is the most sustainable option to manage the spread of fungicide resistance. The control of *P. teres* using cultural practices and the use of host plant resistance reduces the risk of epidemics and the need to rely on fungicides. Cultivars such as Fathom and Scope CL which are classified as moderately resistant, and ensuring a gap of at least three years between barley crops will potentially minimize the build-up of fungicide resistant genotypes (Turkington *et al.* 2012; McLean *et al.* 2016; Hills *et al.* 2018; Brown and Harris 2019; McLean and Hollaway 2019) . *Pyrenophora teres* virulence alleles can re-emerge in a population after many seasons of low frequency (Fowler *et al.* 2017) meaning that there is potential for fungicide resistance alleles to follow the same pattern. These alleles can accumulate rapidly due to the multiple asexual cycles of *P. teres* within a growing season (Akhavan et al. 2017) and the annual sexual cycle. Therefore, industry investment in breeding *P. teres*-resistance into high yielding barley lines is a priority (Burlakoti *et al.* 2017). While host plant resistance is inadequate in some current commercially available varieties, the judicious deployment of a range of fungicides with different modes of action can also be used to reduce the evolution of fungicide resistance. This strategy should only be employed to protect the potential crop yield in seasons where *P. teres* is a threat to productivity (Berg and Rossnagel 1990; Jayasena *et al.* 2002; McLean *et al.* 2016; McLean 2019). This is the case in years where average or above-average rainfall occurs in a region (McLean *et al.* 2016). In the Wimmera and Mallee regions where this experiment was conducted, when such rainfall conditions occur, the current recommended practice is to apply foliar fungicide at growth stages Z31

and Z39 (Zadoks *et al.* 1974; McLean and Hollaway 2015). A seed dressing can also replace one foliar application in the control of NFNB, with effective control up to inflorescence emergence (Z55) (Zadoks *et al.* 1974; McLean and Hollaway 2019). The use of tebuconazole alone is not recommended in a fungicide rotation as is has been found in this, and other studies (Mair *et al.* 2016; Mair *et al.* 2020) to be less effective than other DMIs in the control of *P. teres.* However, fluxapyroxad seed-applied fungicides will likely maintain effective control of net blotch in Victoria as dual DMI-SDHI resistance has not been identified yet in this state. Therefore, the use of other chemistries (i.e. propiconazole, epoxiconazole) in rotation, or in mixtures, with other registered fungicides of different modes of action, such as the quinone outside inhibitor (QoI) and SDHI's will help to slow the accumulation and spread of fungicide resistance (Ishii *et al.* 2015; Rehfus *et al.* 2016; Akhavan *et al.* 2017; Lopez-Ruiz *et al.* 2018).

3.3. Future directions

This study has highlighted multiple future directions that could contribute to on-farm management preventing the increase and spread of fungicide-resistant *P. teres* genotypes. Firstly, further fungicide resistance screening of *P. teres* isolates collected from barley residues in this study and isolates from Agriculture Victoria's (GIP, Horsham) collection, would further quantify reduced fungicide sensitivity or resistance among the Victorian populaiton. The distinction between *Ptt* and *Ptm* in such screening would also be beneficial providing a ratio of *formae speciales* more likely to develop resistance to change on-farm management for that pathogen. A broader survey of other Victorian regions would also be beneficial to identify the potential size of the problem. Sensitivity baselines defined by an EC_{50} for popular DMI and SDHI fungicides need to be established to determine the continuing effectiveness of chemistries in Victoria. Complimentary to this would be the repetition of glasshouse assessment of DMI-resistant isolates ptt19-T1011 and ptt19-T16013 to determine the likely impact of the *Cyp51A* F489L mutation should it accumulate in the field. Tracing the potential cross-border movement of mutation in the *Cyp51* CDS using DArTseq would assist in comparing isolates between states. High throughput molecular techniques to detect mutant allele frequency. Using molecular technology, the role of gene flow and independent emergence in the origin and frequency of *Cyp51A* F489L can be determined by the sequencing of fungicide target genes and detection of mutant alleles from field tissue and isolate samples. The information generated will inform the management of net blotches on-farm to minimize the impact of fungicide resistance in *P. teres.*

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Appendix

Appendix 1. Location of the Plant Breeding Centre, Horsham at latitude -36.7418 and longitude 141.11476 indicated at marker (B).

Appendix 2. Barley residue sample number with date collected GPS location and the nearest town.

Sample number	Collection date	Latitude	Longitude	Nearest town
1	3 March 2019	-36.9266	142.2373	Laharum
2	20 March 2019	-36.32845	142.99	Donald
3	18 April 2019	-36.15146	141.36676	Yanac
4	20 March 2019	-36.32828	141.8174	Nhill North
5	16 April 2019	-36.5521	142.29309	Longerenong
6	18 April 2019	-36.12607	141.35655	Yanac
7	18 April 2019	-36.7418	141.11476	Horsham
8	18 April 2019	-36.13154	141.3529	Yanac
9	20 March 2019	-36.43798	141.6274	Nhill
10	3 March 2019	-36.69637	141.6852	Gororke
11	16 April 2019	-36.64554	142.39862	Jung
12	19 March 2019	-36.94022	142.1904	Wonwonda
13	20 March 2019	-35.76253	142.0476	Rainbow
14	18 April 2019	-36.12814	141.36213	Yanac
15	3 March 2019	-36.43798	141.8789	Clearlake
16	16 April 2019	-36.65659	143.30598	Longerenong
17	16 April 2019	-36.63975	142.38708	Jung
18	18 April 2019	-36.7418	141.11476	Horsham
19	18 April 2019	-36.33923	141.5578	Nhill
20	16 April 2019	-36.7418	141.11476	Horsham
Control	No stubble	-36.7418	141.11476	Horsham

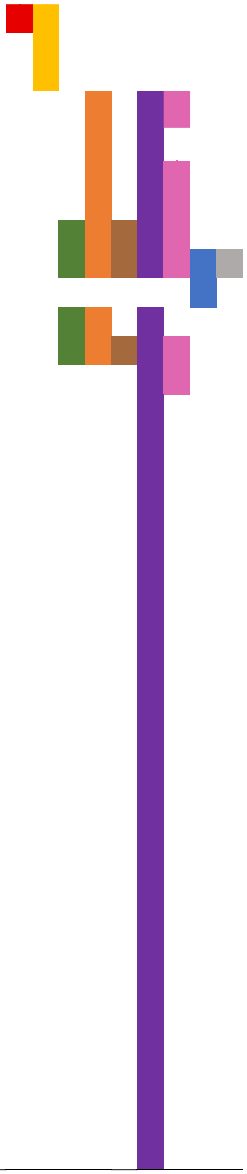
Appendix 3. Field experiment design. Plot number represent barely residue sample number, which was divided between fungicide treatments propiconazole (grey) and tebuconazole (black).

Row	Plot number = collected stubble samples										
1	Buffer										
2	1	2	3	4	5	6	7	8	9	10	No stubble/no fungicide
3	11	12	13	14	15	16	17	18	19	20	
4	1	2	3	4	5	6	7	8	9	10	No stubble/no fungicide
5	11	12	13	14	15	16	17	18	19	20	
6	Buffer										

Appendix 4. Table representing all Pyrenophora teres *isolates used for experiments and sequence analysis in this study.*

Isolate	Species	Source/State	Resistant or sensitive DMI *in vitro*	*Cyp51A* F489L mutants	Experiments using specific isolates*
pt19-T1007	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T1023	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T7012	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T7017	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T7018	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T7022	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T1029	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T11014	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T11025	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T1127	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T11020	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T16015	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T16019	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T16030	Unidentified	Victoria[a]	Unknown	Unknown	
pt19-T16031	Unidentified	Victoria[a]	Unknown	Unknown	
sf17/18a	*Ptm*	South Australia[b]	Sensitive	Wild-type	
pt19-C021	Unidentified	Victoria[a]	Sensitive	Wild-type	
pt19-T11026	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptm18-019	*Ptm*	Victoria[c]	Sensitive	Wild-type	
sf39/18a	*Ptm*	South Australia[b]	Sensitive	Wild-type	
pt19-T5003	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptm19-T1009	*Ptm*	Victoria[a]	Sensitive	Wild-type	
ptm18-134	*Ptm*	Victoria[c]	Sensitive	Wild-type	
pt19-T5001	Unidentified	Victoria[a]	Sensitive	Wild-type	
pt19-T2006	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptm18-160	*Ptm*	Victoria[c]	Sensitive	Wild-type	
ptm18-018	*Ptm*	Victoria[c]	Sensitive	Wild-type	
sf18/18a	*Ptm*	South Australia[b]	Sensitive	Wild-type	
ptm18-025-D	*Ptm*	Victoria[c]	Sensitive	Wild-type	
pt19-T14024	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptm19-025-B	*Ptm*	Victoria[c]	Sensitive	Wild-type	
pt19-T4032	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptm18-143	*Ptm*	Victoria	Sensitive	Wild-type	
pt19-T4028	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptm19-T18005	*Ptm*	Victoria[a]	Sensitive	Wild-type	
sf38/18a	*Ptm*	South Australia[b]	Sensitive	Wild-type	
ptm18-026	*Ptm*	Victoria[c]	Sensitive	Wild-type	
pt19-T8034	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptm18-018-C	*Ptm*	Victoria[c]	Sensitive	Wild-type	
pt19-T5002	Unidentified	Victoria[a]	Sensitive	Wild-type	
pt19-T1008	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptt19-T1011	*Ptt*	Victoria[a]	Resistant	Mutant	
ptm18-018-B	*Ptm*	Victoria[c]	Sensitive	Wild-type	
ptm18-016	*Ptm*	Victoria[c]	Sensitive	Wild-type	
ptm19-033	*Ptm*	Victoria[c]	Sensitive	Wild-type	
pt19-T5004	Unidentified	Victoria[a]	Sensitive	Wild-type	
ptt19-T16013	*Ptt*	Victoria[a]	Resistant	Mutant	
pt19-T16016	Unidentified	Victoria[a]	Sensitive	Wild-type	

pt19-T14010	Unidentified	Victoria[a]	Sensitive	Wild-type
ptm18-136	*Ptm*	Victoria[c]	Sensitive	Wild-type
ptm18-145	*Ptm*	Victoria[c]	Sensitive	Wild-type
9254	*Ptt*	Western Australia[d]	Sensitive	Wild-type
Ko103	*Ptt*	Western Australia[d]	Resistant	Mutant
19FRG001	*Ptm*	Western Australia[d]	Resistant	Mutant
16FRG073	*Ptm*	Western Australia[d]	Resistant	Mutant
ptm19-057	*Ptm*	Victoria[c]	Sensitive	Wild-type
ptm19-062	*Ptm*	Victoria[c]	Sensitive	Wild-type
ptm18-023	*Ptm*	Victoria[c]	Sensitive	Wild-type
ptt19-226	*Ptt*	Victoria[c]	Sensitive	Wild-type
ptt19-199	*Ptt*	Victoria[c]	Sensitive	Wild-type
SG1	*Ptm*	Western Australia[d]	Sensitive	Wild-type
18FRG195	*Ptm*	Western Australia[d]	Resistant	Mutant
M2	*Ptm*	Western Australia[d]	Sensitive	Wild-type
17FRG089	*Ptm*	Western Australia[d]	Resistant	Mutant
19FRG076_1	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG011_1	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG009_3	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG010_1	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG076_8	*Ptt*	South Australia[d]	Resistant	Mutant
19FRG078_4	*Ptt*	South Australia[d]	Resistant	Mutant
19FRG010_8	*Ptt*	South Australia[d]	Resistant	Mutant
19FRG009_1	*Ptt*	South Australia[d]	Resistant	Mutant
19FRG009_7	*Ptt*	South Australia[d]	Sensitive	Wild-type
W1A1	*Ptt*	Western Australia[d]	Sensitive	Wild-type
19FRG011_4	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG010_9	*Ptt*	South Australia[d]	Resistant	Mutant
19FRG077_1	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG009_2	*Ptt*	South Australia[d]	Resistant	Mutant
19FRG076_3	*Ptt*	South Australia[d]	Resistant	Mutant
19FRG010_4	*Ptt*	South Australia[d]	Resistant	Mutant
19FRG076_2	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG010_2	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG009_5	*Ptt*	South Australia[d]	Sensitive	Wild-type
19FRG077_2	*Ptt*	South Australia[d]	Sensitive	Wild-type
9193	*Ptt*	Western Australia[d]	Sensitive	Wild-type
19FRG011_7	*Ptt*	South Australia[d]	Sensitive	Wild-type



[*]Red: single spored isolates, yellow: all discriminatory dose analysis, green: species identification experiments, orange: isolates with DNA extracted and used in *Cyp51A* endpoint promoter PCR experiments, brown; isolates sequenced in this study, purple: sequences used for phylogenetic analysis, pink: radial growth experiment, blue: glasshouse assessment of DMI fungicides against *Cyp51A* mutants, grey: glasshouse assessment of fluxapyroxad seed dressing against *Cyp51A* mutants.

[a] Sourced from this study.

[b] Sourced from SARDI collection.

[c] Sourced from plant pathology group, Agriculture Victoria, Horsham collection.

[d] Sourced from fungicide resistance group, Centre for Crop and Disease Management, Curtin University.

Appendix 5. Thirty-three Pyrenophora teres *isolates collected from tebuconazole-applied and control (no-fungicide, no barley-residue) from barley (Spartacus) plots in the field screen for DMI fungicide resistance with corresponding residue sample number to locations detailed in Appendix 2.* All isolates screened for fungicide resistance in further analysis are bolded.

Residue sample number	Name* of isolates collected
1	pt19-T1007, **pt19-T1008**, **ptm19-T1009**, **ptt19-T1011**, pt19-T1023
2	**pt19-T2006**
3	-
4	**pt19-T4028**, **pt19-T4032**
5	**pt19-T5001**, pt19-T5002, **pt19-T5003**, **pt19-T5004**
6	-
7	pt19-T7012, pt19-T7017, pt19-T7018, pt19-T7022
8	**pt19-T8034**
9	-
10	pt19-T10029
11	pt19-T11014, pt19-T11025, **pt19-T11026**, pt19-T11027, pt19-T11020
12	-
13	-
14	**pt19-T14010**, **pt19-T14024**
15	-
16	**ptt19-T16013**, pt19-T16015, **pt19-T16016**, pt19-T16019, pt19-T16030, pt19-T16(
17	-
18	**pt19-T18005**
19	-
20	-
Control[#]	pt19-C021

*Where no isolates were collected from a particular plot, it is represented as (-). Isolate names indicate: the pathogen *Pyrenophora teres* (*pt*), for isolates that were not characterised to f. sp. level, *Pyrenophora teres* f. sp. *maculata* (ptm), or *Pyrenophora teres* f sp. *teres* (ptt); year of collection e.g. 2019 (19); fungicide applied (T = tebuconazole); residue sample/plot number 4 (4); and isolate number, in the order that it was collected in, for example of the 28th isolate for this plot (028); resulting in the name: ptm19-T4028. [#]No stubble residue and no fungicides applied

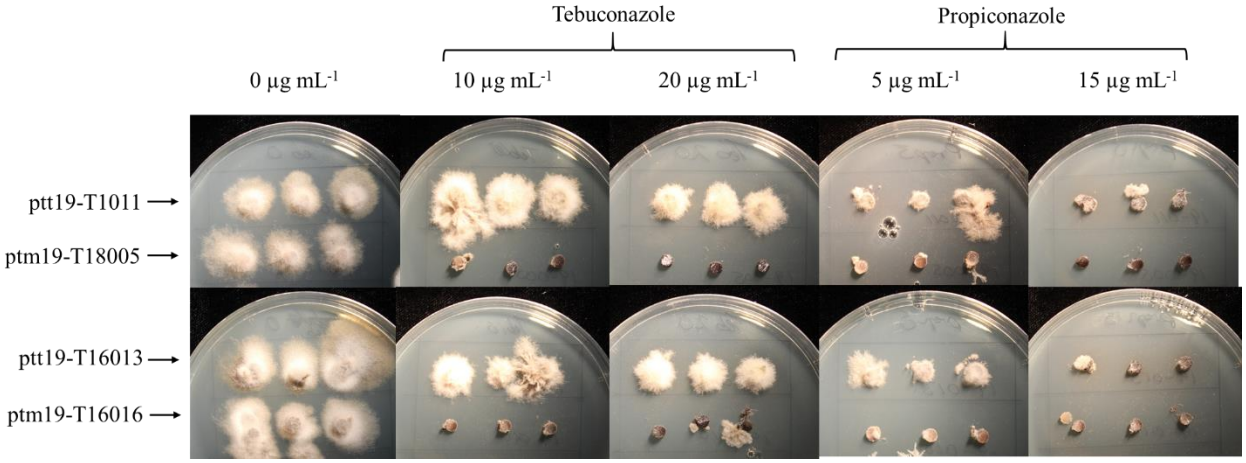
Appendix 6. Primers sequences used in promoter PCR, and PCR amplification for sequencing.

Name	Description	Sequence (5'-3')	Source
PtmCyp51A_Pro_F	Forward primer for amplifying Cyp51A promotor region	GAACAAGCACTGGAGGAGAG	(Mair *et al.* 2019)
PtmCyp51A_Pro_R	Reverse primer for amplifying Cyp51A promotor region	GATGGGTGGTTCTTTGGTGT	(Mair *et al.* 2019)
PttCyp51A_Pro_F	Forward primer for amplifying Cyp51A promotor region	GGCTCATAAATGGCGGAAC	(Mair *et al.* 2016)
PttCyp51A_Pro_R	Reverse primer for amplifying Cyp51A promotor region	AGGAAGAGGAGGGAGAGCAT	(Mair *et al.* 2016)
PttCyp51A1-F:	Forward primer for amplifying Cyp51A1 promotor region	TAGATGTCACGGATTGATTGAT	unpublished
PttCyp51A1-R:	Reverse primer for amplifying Cyp51A1 promotor region	GGCCAAATAGGAGGAAGAGG	unpublished
PttCyp51A2-F:	Forward primer for amplifying Cyp51A2 promotor region	ATTATCAGCTCGCCTTCCTACT	unpublished
PttCyp51A2-R:	Reverse primer for amplifying Cyp51A2 promotor region	GTTCTTTGGTGTTGGGGAGA	unpublished
Pt Cyp51A_1F	Forward primer for amplifying Cyp51A	ATGCTCTCCCTCCTCTTCCTC	(Mair *et al.* 2016)
PtCyp51A_2F	Primer for sequencing *Cyp51A*	TACGACTGATTGAGCAAGAGGT	(Mair *et al.* 2016)
PtCyp51A_1R	Primer for sequencing *Cyp51A*	GAGATCGTGGTACAGGCTTG	(Mair *et al.* 2016)
PtCyp51A_3F	Primer for sequencing *Cyp51A*	GCATTCCAACGTCGTCAAAG	(Mair *et al.* 2016)
PtCyp51A_2R	Primer for sequencing *Cyp51A*	TTCGCTGTTGGCTGAGATAC	(Mair *et al.* 2016)
PtCyp51A_3R	Primer for sequencing *Cyp51A*	TTACCGCCTCTCCCAGC	(Mair *et al.* 2016)
PttCyp51A_3R	Reverse primer for amplifying Cyp51A	TTACCGCCTCTCCCAGC	(Mair *et al.* 2016)

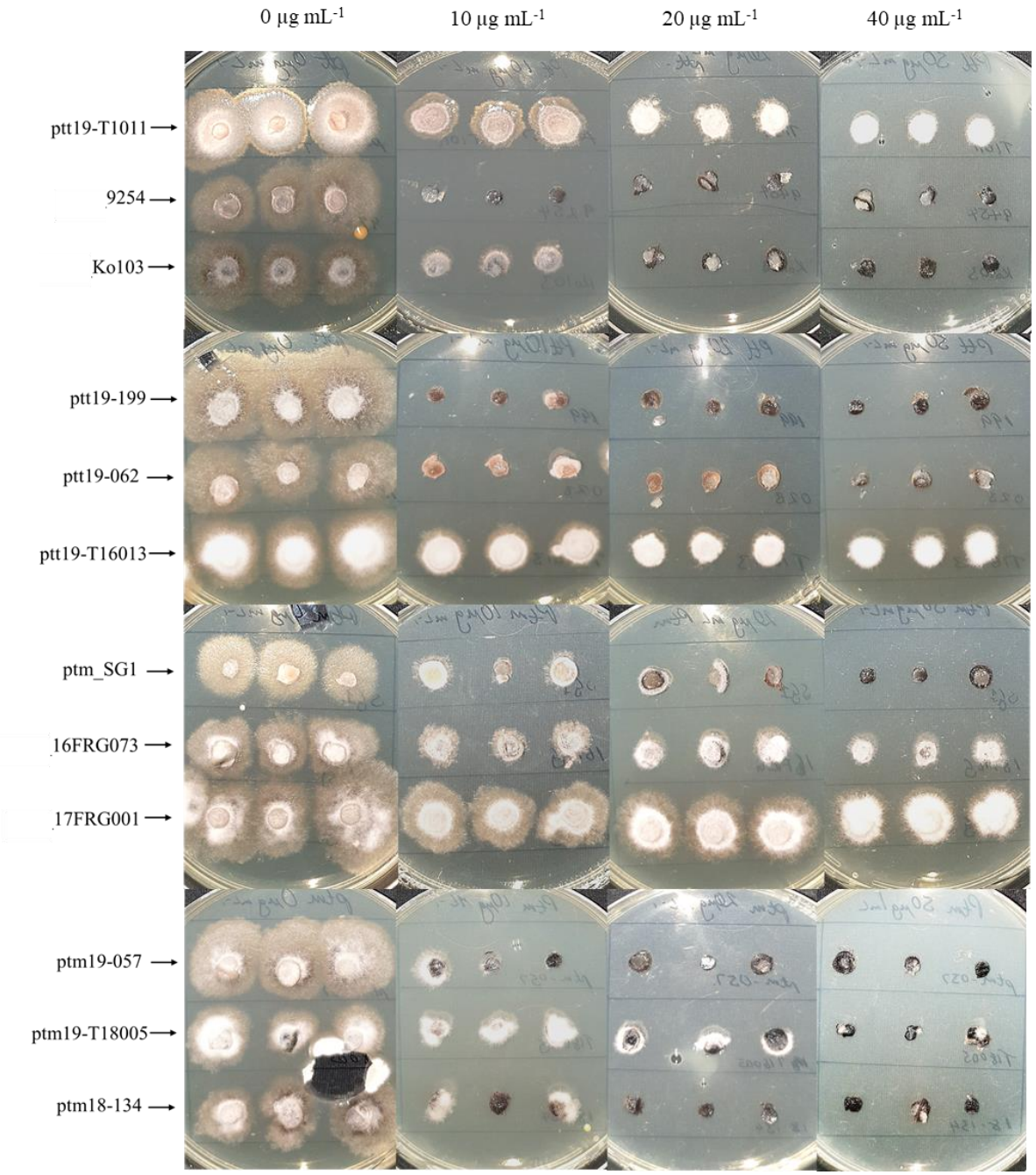
Appendix 7. Response of 34 Pyrenophora teres f. sp. maculata *isolates to discriminatory dose concentrations. (5 mL^{-1} and 10 mL^{-1} for propiconazole and tebuconazole respectively). Concentrations that define High resistance are at 15 mL^{-1} and 20 mL^{-1} highly resistant for propiconazole and tebuconazole respectively, all in comparison to unamended PDA agar media. (+, reduced fungicide sensitivity radial growth on YBA agar media), (-) no growth and sensitivity, (*) growth on inoculation plug only and sensitivity.*

	Concentration (µg mL^{-1})				
Isolate	Control	Propiconazole		Tebuconazole	
	0	5	15	10	20
sf17/18a	+	+	-	-	-
ptm19-C021	+	-	-	-	-
pt19-T11026	+	*	-	-	-
ptm18-019	+	-	-	-	-
sf39/18a	+	+	-	-	-
pt19-T5003	+	-	-	*	-
ptm19-T1009	+	-	-	-	-
ptm18-134	+	-	-	-	-
pt19-T5001	+	*	-	-	-
pt19-T2006	+	*	-	-	-
ptm18-160	+	+	-	*	-
ptm18-018	+	-	-	-	-
sf18/18a	+	-	-	-	-
ptm18-025-D	+	+	-	-	-
pt19-T14024	+	+	-	-	-
ptm19-025-B	+	-	-	-	-
pt19-T4032	+	-	-	-	-
ptm18-143	+	-	-	-	
pt19-T4028	+	+	-	-	-
ptm19-T18005	+	+	-	-	-
sf38/18a	+	*	-	-	-
ptm18-026	+	-	-	-	-
pt19-T8034	+	-	-	-	-
ptm18-018-C	+	-	-	-	-
pt19-T1008	+	*	-	-	-
ptt19-T1011	+	+	+	+	+
ptm19-T18005	+	+	-	-	-
ptm18-018-B	+	*	-		
ptm18-016	+	-	-	-	-
ptm19-033	+	-	-	-	-
pt19-T5004	+	*	-	-	-
ptt19-T16013	+	+	*	+	+
pt19-T16016	+	*	-	*	-
pt19-T14010	+	+	-	-	-
ptm18-136	+	-	-	-	-
ptm18-145	+	*	+	-	-

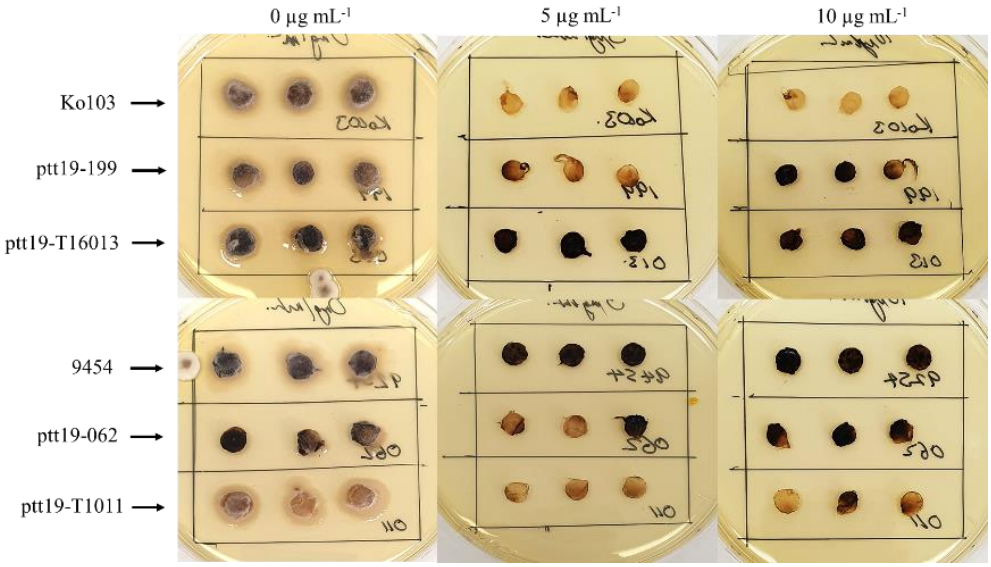
Appendix 8. Discriminatory dose assays showing Pyrenophora teres f. sp. teres *isolates ptt19-T1011 and ptt19-T16013 growing at moderately resistant and highly resistant concentrations of tebuconazole and propiconazole, in comparison to isolates ptm19-T18005 and ptm-T16016 sensitive to the same fungicides*



Appendix 9. Discriminatory dose assays showing Pyrenophora teres f. sp. teres *isolates ptt19-T1011 and ptt19-T16013 growing at moderately resistant (10 µg mL-1) and highly resistant (20 , 40 µg mL µg mL-1) concentrations of tebuconazole, in comparison to moderately resistant isolate Ko103 (Ptt)(source, Western Australia) and sensitive isolates 9254 (Ptt)(source, Western Australian, Centre for Crop and Disease Management) and ptt19-062, ptt19-199 (source, Victoria). Also shown, isolates of* Pyrenophora teres f. sp. maculata *on the same concentrations of tebuconazole. Showing highly resistant isolates isolate 1FRG073, 17FRG001 (*Ptm*) (source, Western Australia), compared to sensitive isolates SG1 (*Ptm*) (source, Western Australia) and ptm19-T18005, ptm18-134, ptm19-057 (source, Victoria). All isolates growth compared to unamended control (0 µg mL^{-1}).*

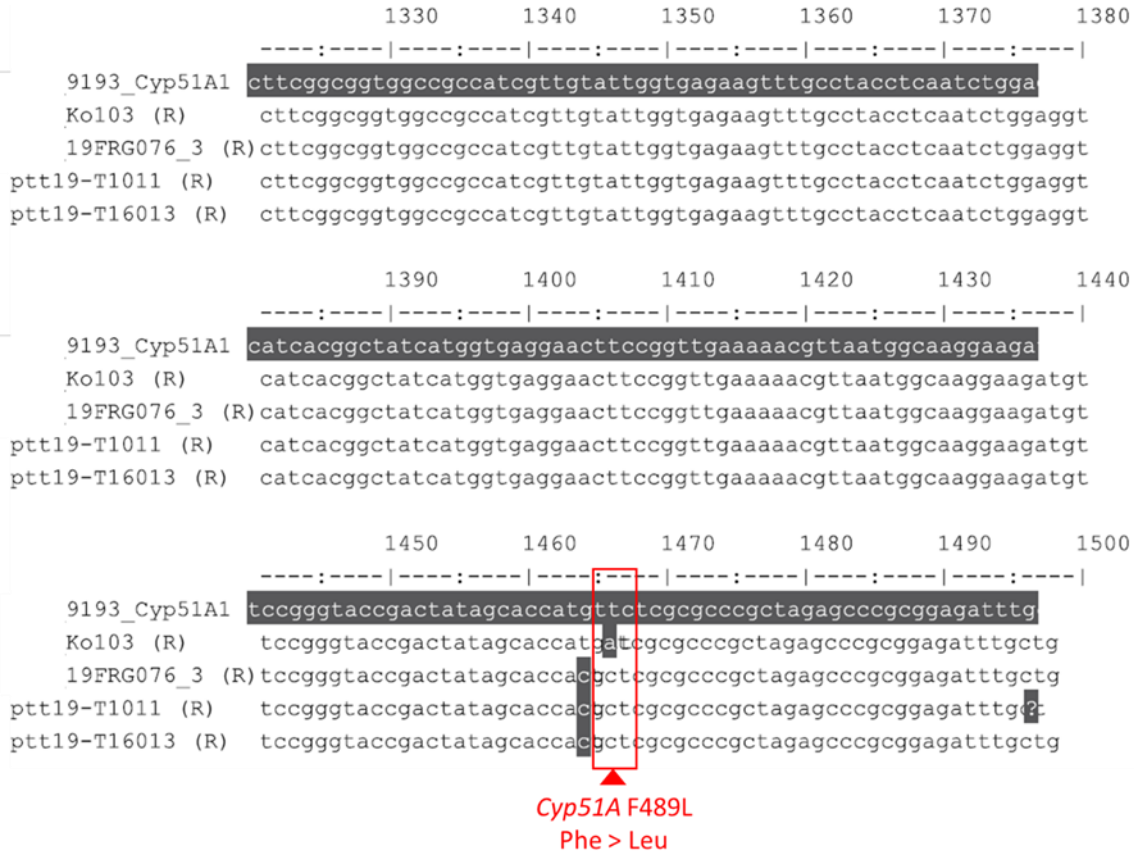


Appendix 10. Discriminatory dose assays and table showing Pyrenophora teres f. sp. teres *isolates ptt19-T1011, ptt19-T16013, Ko103 (Ptt), 9254 (Ptt)(source, Western Australian, Centre for Crop and Disease Management), ptt19-062, and ptt19-199 (source, Victoria) as sensitive to moderately resistant (5 µg mL^{-1}) and highly resistant (10 µg mL^{-1}) concentrations of fluxapyroxad. All isolates are compared with a negative control (0 µg mL^{-1}).*

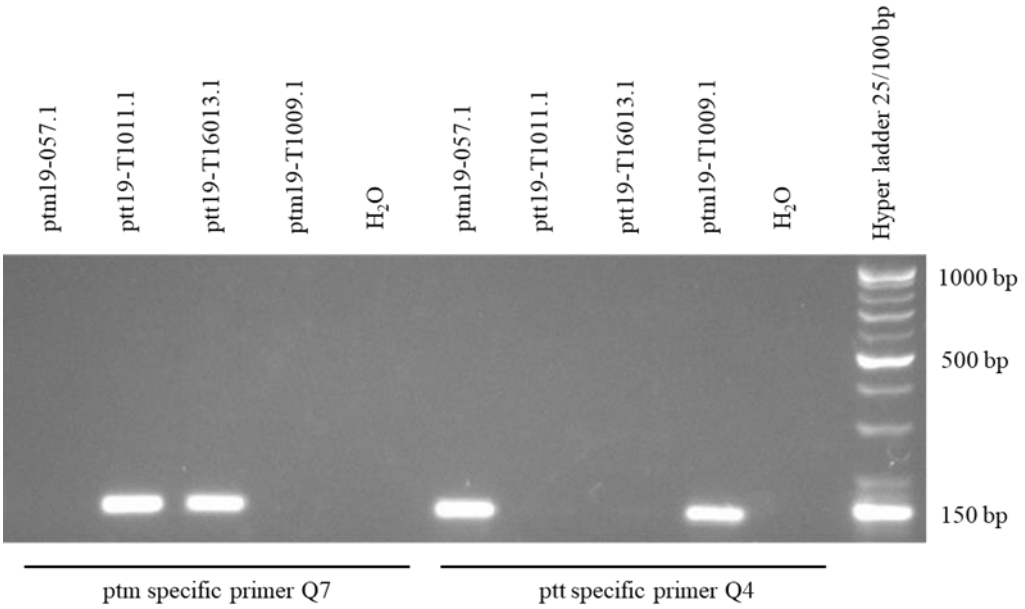


	Concentration (µg mL^{-1})		
Ptt isolate	0 µg mL^{-1}	5 µg mL^{-1}	10 µg mL^{-1}
ptt19-199	+	-	-
ptt19-062	+	-	-
ptt19-T16013	+	-	-
ptt19-T1011	+	-	-
9254	+	-	-
Ko103	+	-	-

Appendix 11. Extraction of Sequence alignment comparing West Australian Pyrenophora teres f. sp. teres (Ptt) *wild-type isolate 9193* Cyp51A1 *region compared to Western Australian* Ptt *mutant isolate Ko103, South Australian (19FRG076_3) and Victorian mutants (ptt19-T1011, ptt19-T16013) sourced from Geneious Prime version 2020.1 created by Biomatters.*



Appendix 12. Gel electrophoresis analysis of PCR product amplified from gDNA of Pyrenophora teres f. sp. maculata (ptm) *and* Pyrenophora teres f. sp. teres (ptt) *single spored from passaged (Koch's postulates) isolates by primers PtmQ7_F and PtmQ7_R and PttQ4_F and PttQ4_R respectively. PCR product appears at expected amplicon size 140 bp and 150 bp for PtmQ7 and Ptm Q4 respectively (Poudel et al. 2017). Compared against 25/100 bp Hyper Ladder (Bioline).*



Appendix 13. Gel electrophoresis analysis of PCR product amplified from gDNA of Pyrenophora teres f. sp. maculata *(*Ptm*) and* Pyrenophora teres f. sp. teres *(*Ptt*) to determine length polymorphisms that may influence DMI resistance in the Cyp51 promoter region.* Pyrenophora teres f. sp. maculata *length compared by Western Australian azole-resistant, promoter insertion* Ptm*-mutants (16FRG073, 19FRG001) predicted to be ~1143 bp and Victorian wild-type ptm18-134 at 1009 bp. Promoter length of* Ptt *was compared to Western Australia F489L mutant Ko103 (*Ptt*) and wild-type 9254 both at ~550 bp. Compared against 1 kb Hyper Ladder (Bioline).*