Resistance to fungicides in the plant pathogen *Microdochium nivale*

by
Ryan Allan Russell Gourlie

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ABSTRACT

RESISTANCE TO FUNGICIDES IN THE PLANT PATHOGEN *MICRODOCHIUM NIVALE*

Ryan Allan Russell Gourlie
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Advisor:
Professor Tom Hsiang

Samples of *Microdochium nivale* were collected from British Columbia and Ontario. The sampled isolates (161) and previously collected isolates (129) were tested for fungicide sensitivity by growing on amended media. Iprodione and propiconazole EC_{50} values ranged from 1 to 543 μg mL^{-1} and 0.001 to 0.87 μg mL^{-1} respectively. In field trials, resistance was significantly correlated with less disease. Draft genome assemblies of 13 isolates were searched for sequences associated with resistance. Alignments of the *mnos-1* gene revealed a mutation (A3503G) and a deletion (Δ430-462) in iprodione-resistant isolates. Mutations in *mnos-4* (G49A and C396T), and mutations in *skn7* (214 SNPs, six deletions, two insertions) were associated with a subset of iprodione-resistant isolates. Mutations in the *mrr1* gene (G2237A and G2392A) were associated with resistance to both fungicides in some isolates. No mutations in the propiconazole target site (*cyp51*) were found to be associated with propiconazole-resistant isolates of *M. nivale*.

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LIST OF ABBREVIATIONS AND ACRONYMNS

ABC – ATP-binding cassette

ANOVA – analysis of variance

AUDPC – area under disease progress curve

BC – British Columbia

BLAST – basic local alignment search tool

DMI – demethylation inhibitors

FRAC – Fungicide Resistance Action Committee

LSD –least significant difference

MOA – mode of action

NGS – next-generation sequencing

PCR – polymerase chain reaction

PDA – potato dextrose agar

PMRA – Pest Management Regulatory Agency

RF – resistance factor

SOA – site of action

Chapter 1 General Introduction & Literature Review

1.1 Introduction

Amenity turfgrass can be considered a high value crop, although its value is derived through the functions and services it provides rather than as a harvestable merchantable commodity. A recent assessment of the Ontario turfgrass industry calculated the total revenue as $2.61 billion in 2007 (Tsiplova et al., 2008). In commercial applications such as golf courses, there is often a low tolerance for aesthetic imperfections and high demand for functionality. Fungal diseases are a significant source of damage which can affect both the playability and attractiveness of turfgrass. Turfgrasses in this context are specific species of plants from the family Poaceae which are mowed to a short height and managed to meet aesthetic or recreational purposes. A great deal of effort goes towards managing the spread of fungal diseases and mitigating the damage they can cause. In North America, turfgrass managers generally rely heavily on preventative and curative fungicide applications to maintain a desired level of control (Vincelli and Munshaw, 2015). Per hectare, the golf course industry has the highest intensity of fungicide use in any agricultural sector (Anonymous, 1998). In addition, the snow moulds are among the most important group of fungal turfgrass disease in Canada and their control accounts for the single largest use of fungicides in the country (Hsiang et al., 1999). Two major groups of snow moulds are the gray snow moulds *Typhula ishikariensis* and *T. incarnata*, and pink snow mould, *Microdochium nivale. M. nivale* can also cause a severe disease on turfgrasses commonly referred to as Fusarium patch or Microdochium patch in the absence of snow cover under cool, wet conditions (Hsiang, 2009).

1.2 General Information about *Microdochium nivale*

1.2.1 Origin, distribution, and host range

The fungus *Microdochium nivale* was first reported in 1825 and described as *Lanosa nivalis* Fries. After a number of reclassifications (e.g *Monographella nivalis*, *Gerlachia nivalis*, *Fusarium nivale*) it is now most commonly referred to as *Microdochium nivale* (Fr.) Samuels & Hallett (Samuels & Hallett 1983; Tronsmo et al. 2001). *Microdochium nivale* var. *nivale* and *M. nivale* var. *majus* were, until recently, considered the same species. These are now considered distinct species, *M. nivale* and *M. majus* based on molecular markers and host specificity (Jewell and Hsiang, 2013).

Although *M. nivale* can be found year round in most regions supporting the growth of grasses (Smiley et al., 2005), Microdochium patch is only considered severe on turfgrass in cool, wet, temperate areas such as Scandinavia and the Pacific Northwest (Smiley et al., 2005). *Microdochium nivale* can attack a variety of hosts in the family Poaceae (grasses) such as commercial wheat and barley varieties (*Triticum* sp. and *Hordeum* sp.), as well as a number of economically important turfgrasses (*Agrostis* sp., *Lolium* sp., *Poa* sp.) (Simpson et al., 2000; Smiley et al., 2005). Among turfgrasses, *M. nivale* is able to infect all the cool season grass species, but some are more susceptible than others, namely creeping bentgrass (*Agrostis stolonifera*), annual bluegrass (*Poa annua*), and the perennial ryegrasses (*Lolium* sp.) (Hsiang, 2009).

1.2.2 Disease cycle and disease descriptions

The pathogenic fungus *M. nivale* is the cause of two distinct diseases on turfgrasses: Microdochium patch and pink snow mould (Hsiang, 2009). Symptoms of Microdochium patch

can develop after prolonged periods of cool (0-15 °C), wet weather (leaf wetness period >10 hr per day for several days) (Tronsmo et al., 2001). The disease progresses slowly, with the onset of symptoms possibly taking weeks to develop (or to appear) after initial infection (Smiley et al., 2005). Initial symptoms are small patches of water-soaked leaves, less than 5 cm in diameter. Over time these patches may coalesce or continue to grow, but usually individual patches do not exceed 20 cm in diameter (Figure 1.1). The patches also have a distinct colour progression, often beginning as orange/brown and transitioning to a darker reddish/brown before ending in gray or tan. White or white-pink, fluffy mycelia may be visible around the edge of patches depending on conditions (Figure 1.2) (Smiley et al., 2005).

When snow cover recedes in spring, bleached circular patches of damaged grass can be seen, and if caused by *M. nivale*, the disease is called pink snow mould (Hsiang, 2009). A disease cycle of this pathogen is presented in Figure 1.3 (Tronsmo et al., 2001). This pathogen also attacks other grasses such as winter wheat and overwintering perennial forage grasses (Wiese, 1987) and the disease is called pink snow mould. Often pinkish mycelia and sporodochia are visible, and the pinkness intensifies with exposure to sunlight and with persisting wet-cool conditions (Tronsmo et al., 2001). The colour of the mycelia gives this disease its name. Patches will often develop a bronze/brown/red edge which can be useful to differentiate it from other snow mould diseases such as Typhula blight (i.e. *T. ishikariensis* and *T. incarnata*) (Smiley et al., 2005; Hsiang, 2009).

1.3 Management of Diseases on Turfgrass

1.3.1 Cultural control

Cultural control includes methods and techniques for adapting the growing environment to create unfavorable conditions for disease growth. Certain variables such as temperature, humidity, and precipitation cannot be controlled but other variables can (Mohammad, 2007). The addition of fertilizers is a common practice which alters the soil environment to encourage healthy growth; healthy plants are better able to withstand disease (Mohammad, 2007). For turfgrass diseases, mowing can alter the density, texture, and quality of turfgrasses. Depending on the pathogen, regular mowing can contribute to spreading disease or may allow the grass to outgrow the disease symptoms (Smiley et al., 2005).

Moisture is an essential component of fungal spore germination and as a consequence, leaf wetness period is an important factor for controlling and monitoring diseases. Irrigation should be applied in early morning as grass is often already wet with dew. In addition, this gives plenty of time for water to evaporate off leaf surfaces. Deep infrequent watering is preferable to shallow frequent watering as a disease management practice (Smiley et al., 2005). Pruning of surrounding shrubs and trees, as well as removing other physical barriers will help with air circulation and reduce leaf wetness (Smiley et al., 2005). These practices are generally implemented for non-disease related reasons or as a means of disease prevention, since healthier plants are less susceptible to disease pressures. In addition to proper management and care of turfgrass, selection of appropriate grass species and cultivars can aid with disease control through breed resistance.

1.3.2 Chemical control

Many turfgrass managers do employ cultural practices to improve grass health and as a means of disease prevention or mitigation. However, most managers still need to contend with a large number of damaging fungal diseases which are not affected by such practices. As such, chemical control is often utilized to manage those diseases due to its application simplicity and its reliability to achieve expected levels of control.

The story of modern fungicides (compounds which inhibit or kill fungi) starts with copper sulphate which was noted to reduce the damage caused by bunt of wheat (caused by the pathogen *Tilletia caries*) in 1761. However, at the time scientists did not understand why or how the protection worked (Schulthess, 1761 as referenced by Russell, 2005). At that time, the presence of fungal growth was considered a symptom of disease rather than being recognized as the cause of the disease (Russell, 2005). In 1807, copper sulphate was again used to control *T. caries*, this time by Prevost, but his work was not recognized by the scientific community for many years (Russell, 2005; Taylor et al., 2007). It was not until after the Irish Potato Famine in the 1840s, that interest in methods of disease control were heightened, and researchers worked harder to obtain a better understanding of plant diseases. In 1882, Millardet observed that a combination of copper(II) sulfate ($CuSO_4$) and slaked lime ($Ca(OH)_2$) on grape vines reduced the amount of downy mildew present. He published his findings in 1885, introducing the Bordeaux mixture, the first widely used chemical control for plant diseases in Europe (Russell, 2005; Taylor et al., 2007). The Bordeaux mixture was first used on turfgrass in 1917 for the control of brown patch (caused by the pathogen *Rhizoctonia solani*) (Monteith and Dahl, 1932 as referenced by Russell, 2005).

Throughout the 1900s, many new chemicals were brought to market to control various plant diseases. As fungi comprise the largest group of causal agents of plant diseases, a considerable proportion of the new chemicals were fungicides. Mercury-based compounds like mercuric chloride were some of the earliest, but heavy metal fungicides have now largely been removed as plant disease control agents due to environmental and human health concerns. The introduction of thiram (a dithiocarbamate containing zinc) in 1942 in the U.S.A. (Russell 2005) started a rapid expansion into fungicide discovery and registration throughout the following decades. Thiram was followed by several major fungicides including captan (1952), chlorothalonil (1964), and benomyl (1968) all of which are still in use today.

Currently there are many fungicides available, although only a relatively small number are registered for use against diseases of turfgrasses in Canada. Fungicides can be grouped in a number of ways, but the most relevant grouping in terms of resistance management is by their mode of action (MOA). MOA broadly describes the effects on a pathogen to exposure of a chemical at the cellular level (e.g. respiration inhibition). Mode of action is sub-categorized by the more specific site of action (SOA) (e.g. complex II: succinate-dehydrogenase). Sites of action are the targets (e.g. enzymes) of specific chemical groups (e.g. succinate-dehydrogenase inhibitors) although sometimes more than one group will target the same site of action but in a different manner (FRAC, 2015).

The Pest Management Regulatory Agency (PMRA) is the governing body which regulates the use of all pesticides in Canada. When a fungicide is being registered it must be registered for specific uses (e.g. turfgrass), as well as for specific pathogens (e.g. *M. nivale*). The PMRA keeps historical and current data on the registration of fungicides online. The Fungicide Resistance Action Committee (FRAC), which is an international body composed of industry

representatives, government scientists, and university researchers, produces a classification of fungicides based on their risk of developing resistance. A compiled list of current and past fungicides registered for use on turfgrass in Canada is in Table 1.1.

1.3.2.1 Dicarboximides

The dicarboximide group of fungicides includes chlozinolate, iprodione, procymidone, and vinclozilin. Dicarboximides are locally systemic, meaning they are taken up by the plant, but are not circulated throughout the plant like a truly systemic fungicide (Vincelli and Munshaw, 2015). Iprodione (3-(3,5-Dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide) the first dicarboximide used in North America, was registered in the U.S in 1974 by Rhône-Poulenc. In 1987 iprodione was registered for use on turfgrass in Canada (Health Canada 2016a). Since iprodione is currently the only dicarboximide registered for use on turfgrass in Canada, it was selected as a representative for the dicarboximide group during fungicide sensitivity testing.

The MOA of the dicarboximides, including iprodione, is still not completely understood (FRAC, 2016). A number of experiments with *Botrytis cinerea*, *Alternaria alternata*, and *Neurospora crassa* have suggested that iprodione resistance and increased osmosensitivity are both phenotypes controlled by the same gene or genes (ie. pleiotropic phenotypes) (Yamaguchi and Fujimura, 2005; Tanaka and Izumitsu, 2010; Grabke et al., 2014). Further research suggests the *os-1* gene, which encodes a group III histidine kinase, may be the site of action for iprodione (Cui et al., 2002; Grabke et al., 2014). Histidine kinases are involved with signal transduction and responses to the environment, and the authors suggest that iprodione (and other dicarboximides) interrupt osmotic signaling in the fungi (Cui et al., 2002; Tanaka and Izumitsu, 2010; Grabke et al., 2014).

1.3.2.2 Demethylation inhibitors

The demethylation inhibitors (DMI) are a sub-group of the sterol biosynthesis inhibitors (SBI) composed of dozens of structurally diverse compounds. DMIs have been registered for use on turfgrass in Canada since 1994, with Banner containing propiconazole as the first representative (Health Canada, 2016b; Hsiang et al., 1997). For this study, propiconazole (1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole) was selected as a representative of the DMI group of fungicides.

Sterols play a vital role in maintaining properly functioning cell membranes. Membrane fluidity, permeability, phospholipid movement, and activity mediation of some membrane-bound enzymes are some of the most important roles of sterols (Hollomon et al. 1990; Weete et al. 2010). Ergosterol is an essential component of fungal cell membranes and is the equivalent of cholesterol in mammals (Hollomon et al. 1990; Weete et al. 2010). The DMI fungicides inhibit oxidative sterol 14α-demethylation, which is a step in the biosynthesis pathway of ergosterol (Siegel, 1981; Köller, 1988). The specific site of action is the enzyme C14-demethylase which is encoded by the *cyp51* (syn. *erg11*) gene (Délye et al., 1998; Hamamoto et al., 2000; Wyand and Brown, 2005; Angelini et al., 2015).

1.4 Fungicide Resistance

Antibiotic resistance in human and livestock diseases is a popular example of antimicrobial resistance (Costelloe et al., 2010) usually referring to bacterial pathogens. Plants, however, are more commonly infected with fungal diseases, which are controlled with fungicides (Agrios, 2005). Analogous to bacterial antibiotic resistance, fungicide resistance can develop over time in continually exposed populations (Ishii and Hollomon, 2015). Resistance begins as a

random mutation in a given population, often present prior to any application of fungicides. These types of mutations are commonly located in the site of action and prevent proper binding of the fungicide upon exposure. Mutations may also prevent the chemical from reaching a site of action, or may be involved in increased ability to remove or metabolize the fungicide (Angelini et al., 2015). In each case, the fungicide is less effective. After a fungicide is applied, the mutant resistant phenotype is able to grow and reproduce with reduced competition, and this shifts the population towards a higher proportion of individuals which are resistant (Figure 1.4). Over time, and with repeated fungicide applications, the population shifts towards predominantly resistant individuals (Angelini et al., 2015). It is somewhere in this process that noticeable product control failure may begin to occur.

1.4.1 Dicarboximide resistance

Resistance to the dicarboximides has been reported for several important species. *Botrytis cinerea* is one of the most studied plant pathogens due to its broad host range and the significant economic damage it causes (Williamson et al., 2007). *B. cinerea* also has an exceptional ability to develop fungicide resistance (FRAC, 2017), and one of the first uses for iprodione was for the control of *B. cinerea* (Morton and Staub, 2008). Over the decades since its introduction, *B. cinerea* has developed resistance to various dicarboximides in a large number of agricultural systems (Raposo et al., 2000; Ochiai et al., 2001; Oshima et al., 2002; Myresiotis and Agriculture, 2007; Kretschmer et al., 2009; Weber, 2011; Grabke et al., 2014). Other *Botrytis* species have also been reported as developing resistance to the dicarboximides: *B. elliptica* (Hsiang and Chastagner, 1991), *B. squamosa* (Tremblay and Talbot, 2003), and *B. tulipae* (Chastagner and Riley, 1987; Hsiang and Chastagner, 1991). Economically important species

such as *Alternaria alternata* (McPhee, 1980; Solel et al., 1996; Dry et al., 2004; Ma and Michailides, 2004), *Monilinia fructicola* (Elmer & Gaunt 1994; Sztejnberg & Jones 1978), *N. crassa* (Grindle and Temple, 1985; Hollomon et al., 1997; Fujimura et al., 2000; Ochiai et al., 2001; Zhang et al., 2002) and a number of others (FRAC, 2017) have also developed field resistance to various dicarboximides (Table 1.2).

Sclerotinia homoeocarpa (recently reclassified as *Clarireedia jacksonii* (Salgado-Salazar et al., 2018)) is the causal agent of dollar spot, and one of the most widespread and damaging pathogens of turfgrass. The first instance of *S. homoeocarpa* dicarboximide resistance was in an isolate from a Michigan golf course which was observed to have intolerance to iprodione (Detweiler et al., 1983). Since the first report, other instances of iprodione insensitivity in *S. homoeocarpa* have been reported across the U.S.A. Many of these report failure of the fungicide to provide adequate disease control (Brownback, 2002; Devries et al., 2008).

Reports of field resistance to iprodione (or other dicarboximides) in *M. nivale* have been far less frequent. The first report was on golf greens used by Rhône-Poulenc to perform iprodione field trials by Chastagner & Vassey (1982). A second report of field resistance came when Pennucci et al. (1990) surveyed several golf courses in New Zealand. Chastagner and Vassey (1982) and Pennuci et al. (1990) both reported failure of iprodione to control the disease in the field. To date, these are the only reports of field resistance to dicarboximides in *M. nivale* populations. Resseler & Buchenauer (1988a, 1988b) generated iprodione-resistant isolates in vivo, and these isolates showed much higher resistance factors compared to field generated isolates, but their ability to survive in the field is not known. Leroux et al. (1992) were also able to easily produce resistant isolates in the lab again without information on field viability.

Early experiments investigating dicarboximide fungicides' mode of action suggested a link between dicarboximide insensitivity and osmotic sensitivity (Pappas and Fisher, 1979). Shortly after this connection, Perkins et al. (1982) categorized six chromosomal loci associated with osmotic sensitivity in *Neurospora crassa*. Due to the phenotypic link between osmotic sensitivity and dicarboximide insensitivity, these loci were obvious candidates for further research. *Neurospora crassa* isolates with mutations in these six loci were then tested for their sensitivity to dicarboximides. These experiments showed that mutations in four of the six loci (*os-1*, *os-2*, *os-4*, and *os-5*) gave decreased sensitivity to dicarboximide fungicides while two out of six (*cut* and *gla*) did not lead to any change in sensitivity (Grindle and Temple, 1982).

Grabke et al. (2014) recently showed that *bos-1* mutations (homolog to *N. crassa os-1*) were present in all their iprodione-resistant *B. cinerea* isolates. However, these changes were associated only with "distinctly" low and medium levels of resistance. The authors also investigated a major facilitator transporter, *mfsM2*, whose over-expression had previously been shown to induce low levels of iprodione resistance (Kretschmer et al., 2009) as well as *bos-1* expression levels to account for the high level resistance group. Neither *mfsM2* nor *bos-1* expression differences could account for the high level resistance group. This and previous research, namely Dry et al. (2004), Avenot et al. (2005), Ma et al. (2007), and Tanaka and Izumitsu (2010), indicate that moderate levels of resistance can be conferred via a single point mutation in *os-1*, but the source of high levels of resistance still requires further investigation.

1.4.2 Demethylation inhibitor resistance

Resistance to the DMI fungicides can be conferred in a number of ways: a mutation in the target site *cyp51* gene or the *cyp51* promoter (Délye et al., 1998; Hamamoto et al., 2000; Wyand

and Brown, 2005; Angelini et al., 2015), over-expression of the *cyp51* gene (Ma et al., 2006), or through other mechanisms such as mutations in ABC (ATP-binding cassette) transporters (Nakaune et al., 1998). Resistance to DMIs can also be polygenic, meaning it is a result of multiple genes which individually have a small effect, but their added effects result in high levels of resistance (Leroux and Walker, 2011; Angelini et al., 2015). Cross-resistance to different DMI fungicides and other groups such as the dicarboximides are also commonly reported (Hsiang et al. 1997).

The first report of DMI resistance occurred in powdery mildew of barley (*Erysiphe graminis* f. sp. *hordei*) in 1981, only 3 years after introduction of DMIs on barley (Fletcher and Wolfe, 1981; Russell, 2005). Since then, DMI resistance has become relatively common, occurring in many important plant pathogens including: *Monilia fructicola* (Nuninger-Ney et al., 1989; Luo and Schnabel, 2008), *Mycosphaerella graminicola* (Leroux and Walker, 2011), *Blumeriella jaapii* (Ma et al., 2006), *B. cinerea* (Stehmann and De Waard, 1996), and many others (FRAC, 2017) (Table 1.3). Additionally, there are reported instances of DMI resistance in the turfgrass pathogen *S. homoeocarpa* (Golembiewski et al., 1995; Miller et al., 2002; Hsiang et al., 2007; Bishop et al., 2008; Van Den Nieuwelaar and Hsiang, 2014). There have been no laboratory confirmed reports of field resistance to DMIs in *M. nivale* populations. Cristani and Gambogi (1993) were able to generate resistant mutants in the lab, but nothing else could be found in the literature regarding *M. nivale* and DMI resistance.

1.4.3 Resistance-associated fitness costs

Any given protein may play several roles in an organism, and as a result, mutations in a single gene may have multiple effects. Fungicide resistance mutations can take several forms, the

best-characterized being protein modification which leads to reduced or eliminated fungicide binding (Zhan and McDonald, 2013). Mutations may also alter other biosynthetic pathways (Angelini et al., 2015), increase toxin efflux through ABC transporters (Anderson, 2005), or increased expression of the target site (Angelini et al., 2015). When a mutation confers resistance to a fungicide, it is possible that the mutation will have some debilitating pleiotropic effect on other processes. These effects may become more prominent and noticeable when fungicide applications are ceased, and because of the deleterious secondary effects of the mutation the wild-type individuals are able to out-compete resistant strains which are less fit in a fungicide-free environment. This dynamic plays an essential role in resistance management practices as the effects on fitness levels will determine whether resistant genotypes persist within a population (Zhan and McDonald, 2013).

Despite the importance of resistance-associated fitness costs to resistance management, it is not wholly confirmed with evidence that fitness costs may be absent, low, or high depending on the particular fungus and fungicide involved (Mikaberidze et al., 2015). If no or a low fitness cost is associated with resistance to a fungicide, then that fungicide (or group if there is cross-resistance) may become permanently ineffective (Mikaberidze et al., 2014). The differences seen in past studies may also be explained by the evolution of compensatory mutations that alleviate the fitness costs associated with fungicide resistance mutations (Anderson, 2005). These types of compensatory mutations have been documented in human pathogens which have developed antibiotic or antifungal resistance (Cowen et al., 2001; Anderson, 2005).

Potential effects on fitness caused by fungicide resistance mutations which could be tested include decreased growth rate, decreased disease symptoms or ability to cause disease, reduced reproductive capacity or reduced ability to survive stressful and dynamic environments

(Mikaberidze et al., 2015). The controlled environment of the lab may be suitable for initial detection of fitness costs; however the controlled lab environment is not a full substitute for assessing fitness costs associated resistance in outside environments. The trade-offs associated with resistance may be absent or relatively weak in the highly constrained lab environment. Therefore it is necessary to perform field trials where biotic and abiotic factors constantly fluctuate and trade-offs can become apparent (Zhan and McDonald, 2013).

Isolates of *Erysiphe graminis* f.sp. *tritici* with reduced sensitivity to fenpropimorph (SBI group of fungicides, of which DMI is a subgroup) were less competitive than sensitive isolates (Engels and de Waard, 1996). Propiconazole-insensitive isolates of *S. homoeocarpa* were associated with reduced virulence (Hsiang et al., 1998). However, an experiment with propiconazole-resistant *Pyrenophora teres* demonstrated no correlation between resistance and fitness (Peever and Milgroom, 1994). Isolates of *B. cinerea* resistant to dicarboximides were associated with lower mycelial growth, decreased sporulation, lower virulence, and decreased sclerotia survival (Hsiang and Chastagner, 1991; Pollastro et al., 1996; Raposo et al., 2000). Isolates of dicarboximide-resistant *N. crassa* (Grindle and Temple, 1985; Hollomon et al., 1997; Cui et al., 2002) and *Monilinia laxa* (Katan and Shabi, 1982) showed reduced fitness at a similar level as corresponding *B. cinerea* isolates. Fitness costs in SDHI-resistant *B. cinerea* (Lalève et al., 2014; Veloukas et al., 2014) and *Penicillium expansum* (Karaoglanidis et al., 2011) have also been reported.

1.5 High-throughput Sequencing and Analysis

Advances in sequencing technology and increases in computational power now allow many research labs to sequence, assemble, and analyze small genomes such as those found in fungi

(whose genome size can vary from 9 Mb to 175 Mb) (Haridas et al., 2011; Mohanta and Bae, 2015). Next-generation sequencers are high-throughput, returning large amounts of data, and providing increasingly longer read lengths. Sequenced reads can then be assembled using different methods such as overlapping reads in de Brujin graphs in programs such as Velvet (Zerbino and Birney, 2008), Abyss (Simpson et al., 2009), and SOAPdenovo (Li et al., 2008). The assembled genomes can be evaluated for fragmentation using the weighted statistic N50 (where 50% of the assembly is contained in contigs greater than that value), and for completeness by searching for a set of conserved set of single-copy orthologs such as implements in BUSCO (Simao et al., 2015).

Intrinsic gene prediction methods search for characteristic gene signals such as open and closed reading frames. However, this approach can be complicated by errors in the raw DNA data, frameshift mutations, and overlapping or spliced genes (Sleator, 2010). In addition, experimental data is usually needed to verify that a putative gene is expressed. Despite these challenges, intrinsic gene prediction is commonly used and has high sensitivity for gene detection (Sleator, 2010). However, high sensitivity is coupled with decreased accuracy since this sensitivity may increase false positives; as well, large introns in some eukaryotic organisms increases the complexity of gene prediction (Stanke et al., 2004). Gene prediction programs can utilize a hidden Markov chain statistical model. A Markov chain predicts the future state of a system given its current state, whereas a hidden Markov chain predicts the initial state based on the known outcome (Sleator, 2010; Stamp, 2012). AUGUSTUS is an open source program which detects potential genes in complex eukaryotic organisms using a generalized hidden Markov chain, and uses coding regions of related species to guide predictions (Stanke et al., 2004). Predicted gene sets can then be annotated for putative functionality using curated

reference sequences from NCBI or other online databases (O'leary et al., 2016). The annotated predicted gene sets can then be queried using BLAST (Basic Local Alignment Search Tool) for genes of interest (Altschul et al., 1990), and comparisons can be done between different isolates or species. Sequencing entire genomes of isolates of *M. nivale* either sensitive or resistant to different fungicides allows for direct comparisons of genes potentially associated with resistance.

1.6 Hypotheses and Objectives

1.6.1 Hypotheses

1) The biology of populations of *M. nivale* will reflect their fungicide exposure history, and where particular fungicides have been commonly used (e.g. dicarboximides or DMIs), the isolates will show decreased sensitivity compared to baseline non-exposed populations.

2) Isolates of *M. nivale* with decreased sensitivity to iprodione and/or propiconazole will grow more slowly than fully sensitive isolates.

3) Isolates of *M. nivale* which show reduced sensitivity to iprodione and/or propiconazole in lab tests will also show reduced levels of control in the field, reflected by decreased efficacy or duration of control.

4) Differences in gene sequence are responsible for decreased sensitivity to iprodione and propiconazole in *M. nivale.*

4a) Sequence differences (mutation/indel) in the *os-1* gene are responsible for iprodione insensitivity in *M. nivale.*

4b) Sequence differences (mutation/indel) in the *cyp51* gene or gene promoter are responsible for propiconazole insensitivity in *M. nivale*.

1.6.2 Objectives

The overarching objective of this work was to characterize the state of fungicide resistance in *M. nivale* for parts of Canada in order to provide a knowledge base which researchers and turfgrass managers may use to develop appropriate management practices. The following objectives were determined to be an effective way of testing the hypotheses noted above and to meet this overall objective. The first objective was to collect isolates of *M. nivale* from multiple turfgrass populations in Ontario and British Columbia with varying amounts of exposure to the widely used fungicides iprodione and propiconazole and to assess their sensitivity to those fungicides with both multiple concentration tests and discriminatory concentration tests. This objective allows for the identification of isolates with decreased sensitivity and also provides an indication of how resistant individual isolates are and what proportion of the population is insensitive to a particular fungicide. Although isolates with decreased sensitivity may be indentified with in vitro testing, those results may not reflect the effects of formulated control products in the variable field environment. The second objective of this work was to select representative groups of *M. nivale* isolates, with varying degrees of sensitivity to iprodione and propiconazole, and assess their ability to cause disease in the field under different fungicide selection pressures (with or without propiconazole or iprodione). The third objective was to determine if fitness costs such as slower growth or reduced virulence are associated with resistant isolates. The presence of fitness costs is of particular importance for selecting appropriate disease management practices when turf managers are faced with fungal populations exhibiting reduced fungicide sensitivity. The final objective was to sequence genomes of representative fully sensitive and reduced sensitive isolates to look for possible genetic differences which may underlie the observed insensitivity.

Table 1.1 Current and past fungicides registered for use in Canada (PMRA website) against the turfgrass pathogen *Microdochium nivale*. Similar FRAC codes indicate possibility of cross-resistance; table adapted from Vincelli & Munshaw (2015).

Fungicide	**Group**	**Registration for use on Turfgrass in Canada**	**Resistance Risk**
Azoxystrobin	QoI	Registered	High
Benzovindiflupyr	SDHI	Registered	Medium to High
Chlorothalonil	CN	Registered	Low
Difenoconazole	DMI	Registered	Medium
Fludioxonil	PP	Registered	Low to Medium
Fluoxastrobin	QoI	Registered	High
Iprodione	DC	Registered*	Medium to High
Metconazole	DMI	Registered	Medium
Mineral oil	NC	Registered	Unrated
Penthiopyrad	SDHI	Registered	Medium to High
Propiconazole	DMI	Registered	Medium
Pyraclostrobin	QoI	Registered	High
Thiophanate-methyl	MBC	Registered	High
Trifloxystrobin	QoI	Registered	High
Triticonazole	DMI	Registered	Medium
Benomyl	MBC	Deregistered	High
Oxycarboxin	SDHI	Deregistered	Medium to High
Phenylmercuric acetate	OM	Deregistered	High
Quintozene	PCNB	Deregistered	Low to Medium
Thiram	DTC	Deregistered	Low

* deregistered as of June, 2018 PMRA Re-evaluation Decision PRVD2016-09

Table 1.2 First instances of field resistance to dicarboximide fungicides, adapted from FRAC (2017).

Pathogen	**Host**	**Reference**
Botrytis cinerea	Strawberry	(Dennis and Davis, 1979)
Sclerotinia homeocarpa	Turfgrass	(Detweiler et al., 1983)
Monilinia fructicola	Stone fruit	(Penrose et al., 1985)
Botrytis tulipae	Tulip	(Chastagner and Riley, 1987)
Didymella bryoniae	Cucumber	(Van Steekelenburg, 1987)
Botrytis elliptica	Bulbs	(Hsiang and Chastagner, 1991)
Microdochium nivale	Turfgrass	(Pennucci et al., 1990)
Sclerotinia minor	Lettuce	(Hubbard et al., 1997)
Alternaria alternata	Pistachio	(Ma and Michailides, 2004)
Alternaria brassicicola	Brassicas	(Avenot et al., 2005)
Stemphylium vesicarium	Pear	(Alberoni et al., 2005)
Sclerotinia sclerotiorum	Oilseed rape & soybeans	(Zhou et al., 2014)

Table 1.3 First documented instances of field resistance to demethylation inhibitor class of fungicides, adapted from FRAC (2017).

Pathogen	Host	Reference
Erysiphe graminis f .sp. hordei	Barley	(Fletcher and Wolfe, 1981)
Sphaerotheca fuligenea	Cucumber	(Schepers, 1983)
Pyrenophora teres	Barley	(Sheridan et al., 1985)
Venturia inaequalis	Apple	(Stanis and Jones, 1985)
Erysiphe graminis f .sp. tritici	Wheat	(De Waard et al., 1986)
Rhynchosporium secalis	Barley	(Hunter et al., 1986)
Sphaerotheca mors-uvae	Blackcurrant	(Goszczynski et al., 1988)
Uncinula necator	Grapevine	(Steva et al., 1990)
Pseudocercosporella herpotrichoides	Wheat	(Leroux and Marchegay, 1991)
Botrytis cinerea	Various	(Elad, 1992)
Monilinia fructicola	Stone fruit	(Elmer et al., 1992)
Puccinia horiana	Chrysanthemum	(Cevat, 1992)
Cercospora beticola	Sugar beets	(Karaoglanidis et al., 2000)
Mycosphaerella graminicola	Wheat	(Metcalfe et al., 2000)
Mycovellosiella nattrassii	Eggplant	(Yamaguchi et al., 2000)
Sphaerotheca pannosa	Nectarine	(Reuveni, 2001)
Pyrenophora tritici-repentis	Wheat	(Reimann and Deising, 2005)
Blumeriella jaapii	Cherry	(Proffer et al., 2006)
Fusarium monilifore	Rice	(Yang et al., 2013)
Rhizoctonia solani	Rice	(Agrawal and Sunder, 2014)
Sclerotinia homoeocarpa	Turfgrass	(Vargas et al., 1992)
Calonectria pauciramosa	Nurseries	(Guarnaccia et al., 2014)
Calonectria polizzii	Nurseries	(Guarnaccia et al., 2014)
Fusarium graminearum	Wheat	(Spolti et al., 2014b)
Phakopsora pachyrhizi	Soybeans	(Schmitz et al., 2014)
Pyrenopeziza brassicae	Oilseed Rape	(Carter et al., 2014)

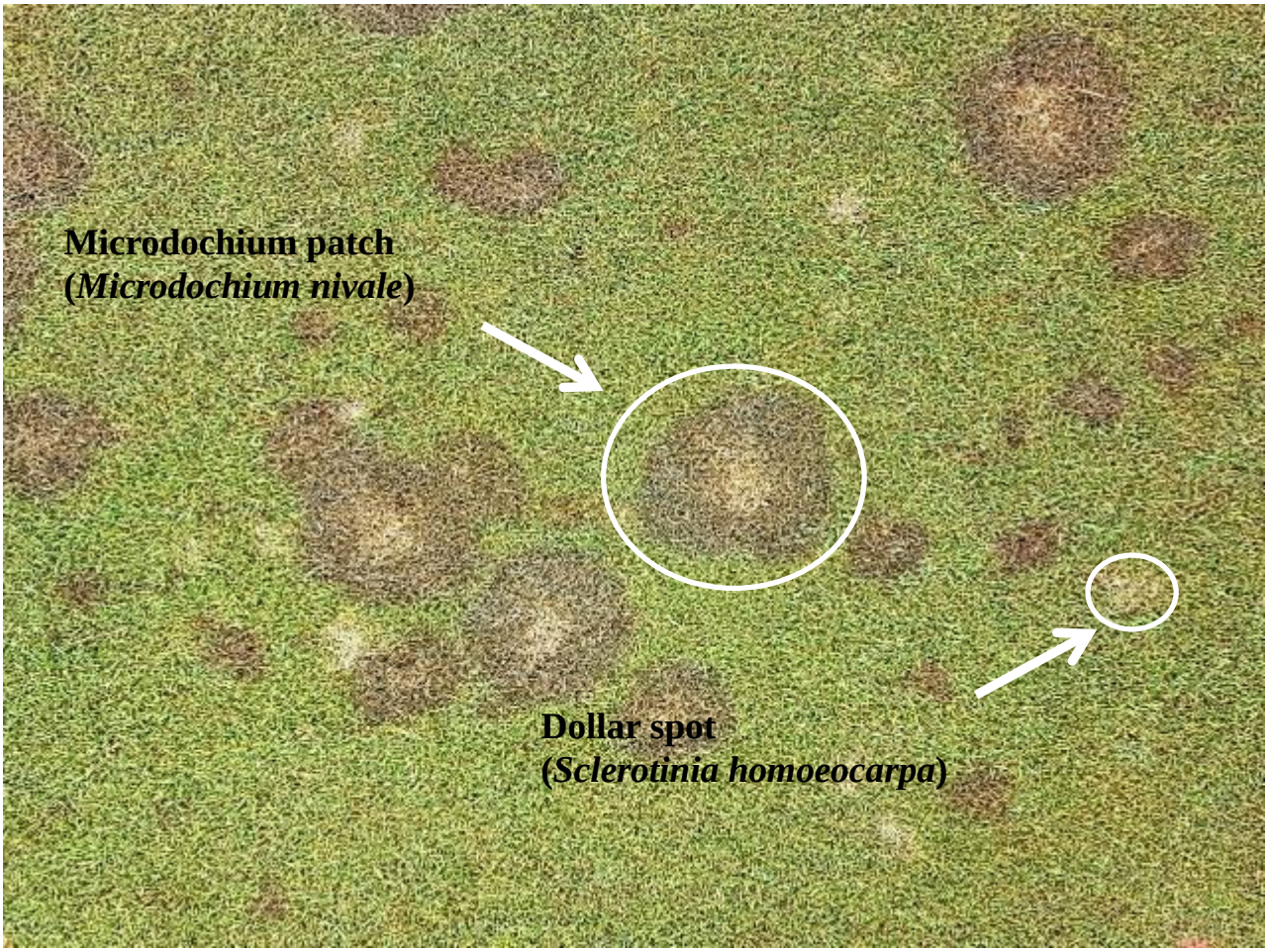


Figure 1.1 Symptoms of Microdochium patch (caused by *M. nivale*) on *Agrostis stolonifera* at the Guelph Turfgrass Institute, Guelph, Ontario, Canada. Symptoms of dollar spot (caused by *S. homoeocarpa*, recently renamed *Clarireedia jacksonii* (Salgado-Salazar et al., 2018)) are also visible for comparison. Photographed October 2016.

Figure 1.2 Damage on creeping bentgrass (*A. stolonifera*) caused by pink snow mould (*M. nivale*). After snow cover receded, pink-white mycelia is visible. Photo taken March 2017, shortly after snow cover receded at the Guelph Turfgrass Institute.

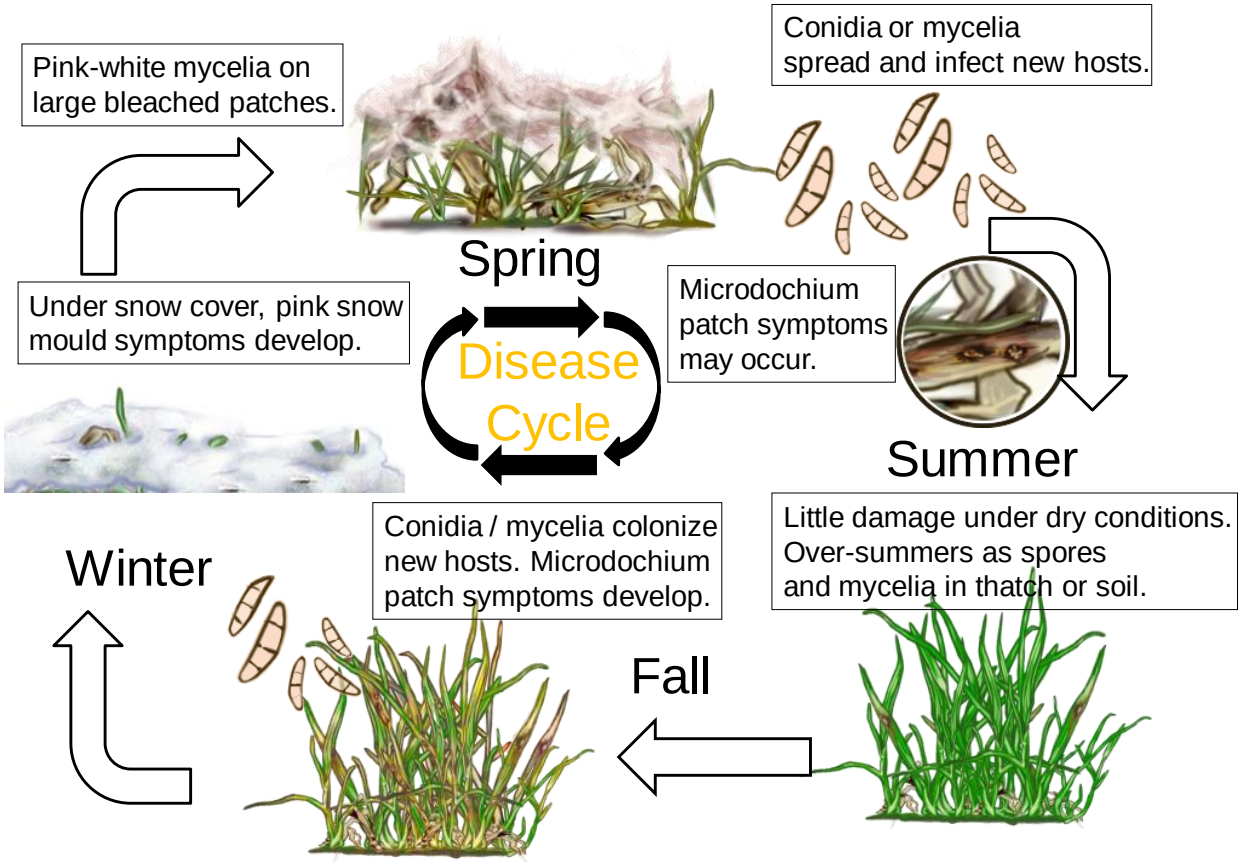


Figure 1.3 The disease cycle of *Microdochium nivale* on turfgrasses in typical temperate zones with wet spring and fall, snow-filled winters and warm dry summers (adapted from Tronsmo et al., 2001; illustrated by Kathryn Wilde).

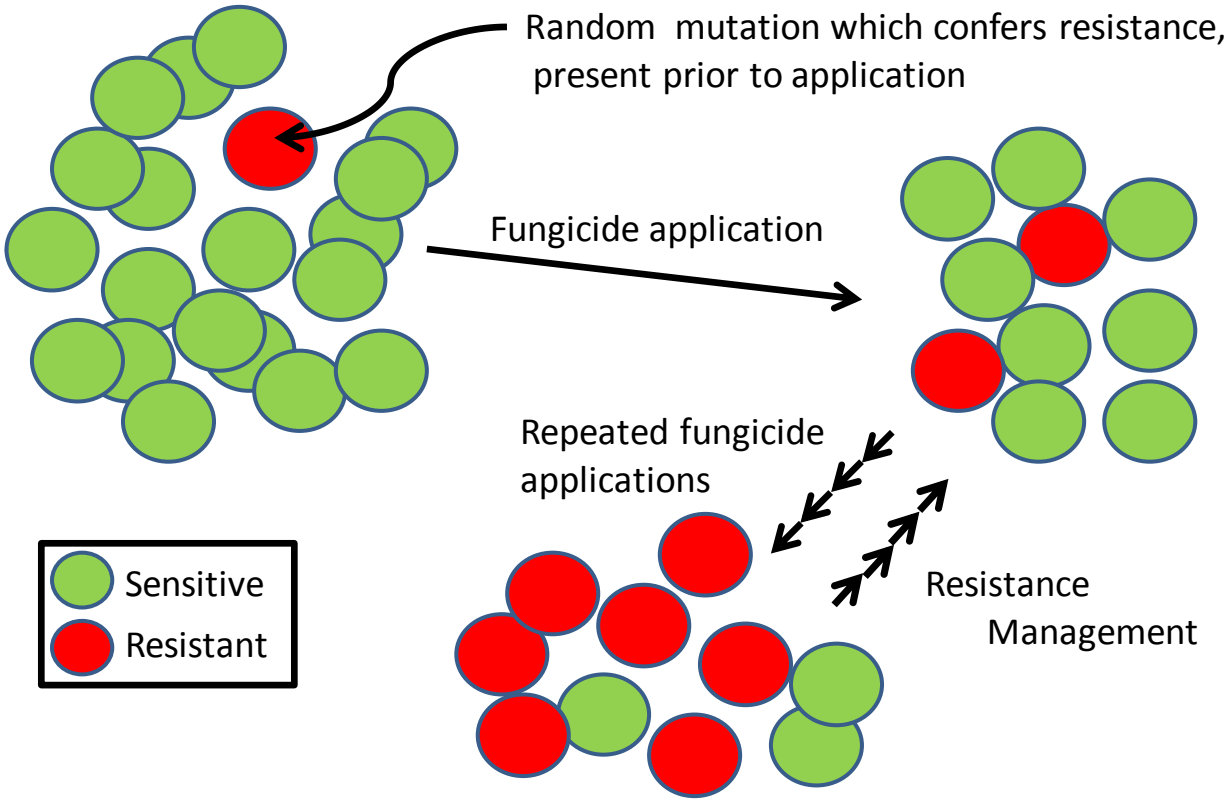


Figure 1.4 Generalized process of how fungicide resistance may develop in a given population of fungi. Green circles represent sensitive individuals and red represent isolates with decreased sensitivity.

Chapter 2 Turfgrass sampling, fungal isolation, and fungicide sensitivity testing

2.1 Introduction

Pink snow mould and Microdochium patch are common diseases of turfgrass caused by the fungal plant pathogen, *Microdochium nivale* (Smiley et al., 2005). Damage caused by these diseases can be extensive if left untreated (Figure 1.1 and Figure 1.2). Many turfgrass managers, particularly golf course superintendants, spray fungicides as both curative and preventative measures to control *M. nivale* and other fungal diseases (Anonymous, 2017). The cool-wet climate of coastal British Columbia is extremely conducive to Microdochium patch development. This higher disease pressure often translates to higher degrees of damage (i.e. large areas of damage) and as a result, turfgrass managers in these areas apply greater volumes of fungicides (Smiley et al., 2005).

Fungicides comprise a diverse set of chemicals which are characterized by their ability to inhibit growth or kill fungi by targeting and disrupting essential metabolic pathways. In a given population of fungi, individuals (i.e. unique genotypes) will exhibit a range of sensitivities to a particular fungicide mode of action, with some being generally more or less sensitive than others. When a fungicide is applied, it inhibits or kills the sensitive individuals in the population. However, there may be mutations present prior to the fungicide applications which reduce sensitivity. The individuals with reduced sensitivity have a distinct fitness advantage in the presence of the fungicide and are able to out-compete the inhibited sensitive individuals. Thus the development of resistance is a shift in the dominant genotype over time within the population, caused by the strong selection pressure exerted by the application of fungicides (Angelini et al., 2015).

2.1.1 Fungicide resistance

Iprodione, a dicarboximide, and propiconazole, a demethylation inhibitor (DMI), are registered in Canada for the control of *M. nivale* (Table 1.1) and are both widely used (Hsiang, 2005). Registration of iprodione was recently reviewed by the PMRA and has been deregistered, and after 2021 iprodione will not be available to turfgrass managers in Canada. Experiments with the fungi *Botrytis cinerea*, *Alternaria alternata*, and *Neurospora crassa* have shown that dicarboximide fungicides affect osmo-regulation, and that resistance can be conferred by mutation at a single locus (*os1*) (Beever, 1983; Cui et al., 2002; Oshima et al., 2002; Dry et al., 2004; Tanaka and Izumitsu, 2010). The mode of action for the DMI fungicides is well established; it acts by disrupting fungal growth via inhibition of 14-α demethylase, an essential enzyme in the synthesis of ergosterol (Ziogas and Malandrakis, 2015). Resistance to DMI fungicides can be conferred through mutations in target-site, *cyp51* (Albertini et al., 2003) and increased expression of *cyp51* (Ma et al., 2006). Both iprodione and propiconazole resistance can be conferred through increased expression of efflux transporters (Sang et al., 2015).

Field resistance to dicarboximides has developed in a number of important fungal species. Notably, *B. cinerea* (Oshima et al., 2002), *Alternaria alternata* (Solel et al., 1996; Dry et al., 2004), *N. crassa* (Grindle and Temple, 1985) and a number of others have also developed field resistance to various dicarboximides (Table 1.2)(FRAC, 2017). The development of field resistance to the DMI fungicides has been frequently reported in a wide variety of fungal pathogens such as *B. cinerea* (Stehmann and De Waard, 1996), *Fusarium graminearum* (Yin et al., 2009), and the turfgrass pathogen *Sclerotinia homeocarpa* (Hsiang et al., 1998) (Table 1.3).

2.1.2 Fungicide resistance in *Microdochium nivale*

The development of resistance in *M. nivale* is less well documented than for other turfgrass pathogens such as *S. homoeocarpa*, the cause of dollar spot disease. The first report of fungicide resistance in *M. nivale* was to the fungicide benzimidazole, a member of the MBC class of fungicides (Tanaka et al., 1983). In 2009, isolates from French wheat were found to be resistant to the QoI fungicide strobilurin (Walker et al., 2009). Cristani and Gambogi (1993) were able to induce mutations in *M. nivale* using ultraviolet radiation, creating DMI-resistant isolates, but lab generated mutants often have low fitness in field tests. Lab confirmed cases of field resistance to iprodione in *M. nivale* have only been reported twice. The first report by Chastagner and Vassey (1982) was from a location in Seattle, Washington, where iprodione had first been tested. The second report was by Pennucci et al., (1990) who performed a fungicide resistance survey of New Zealand golf courses. There have not been any lab confirmed cases of DMI resistance in *M. nivale*. There are, however, anecdotal reports from turfgrass managers in British Columbia about decreased efficacy of some fungicides (T. Hsiang, personal communication).

The objectives of this chapter were:

(1) to collect samples of *Microdochium nivale* from several locations in British Columbia and Ontario, Canada;

(2) to test sensitivity of these isolates and others in the lab stock collection to the dicarboximide fungicide, iprodione, and the DMI fungicide, propiconazole; and

(3) to explore the link between fungicide use and fungicide sensitivity in lab and field tests.

2.2 Methods and Materials

2.2.1 Sample collection

In April of 2016, turfgrass samples exhibiting symptoms of Microdochium patch were collected from three locations in Victoria, British Columbia, BC-1, BC-3, and BC-C. BC-C is a luxury condominium development facing Victoria Harbour, which includes small simulated putting greens for use by the residents. This location had limited exposure to fungicides as the condo owners were concerned about potential exposure. In late November of 2016, samples were collected from BC-2 (Vancouver, British Columbia). Two more sets of samples were collected from this site in May and December of 2017. In late March and early April of 2017, samples of *Poa annua* or *Agrostis stolonifera* with symptoms of Microdochium patch were taken from four golf courses in Guelph, Ontario, Canada: ON-1, ON-2, ON-3, and ON-4. Samples were taken from at least five locations (e.g. greens and fairways) at each golf course.

From each sample location, symptomatic leaf blades were taken from patches of damaged turfgrass with patches at least a meter apart, targeting at least 10 isolates per location. For the March and April 2017 collections from Guelph, the samples were placed into sterilized 1.5 mL microfuge tubes and stored at 4 ºC until isolation, which was performed within one week. For the samples from British Columbia, the grass was placed in envelopes and stored dry at 20 ºC for up to two weeks before isolation.

2.2.2 Fungal isolation and storage

Individual grass blades were surface sterilized by dipping in 75% ethanol for 5 to 10 seconds and then placed in a 1% sodium hypochlorite (NaClO) for 1 to 2 minutes. Sterilized leaves were placed in Petri plates containing 10 mL of autoclaved 2% potato dextrose agar

(PDA, Difco Laboratories) amended with the antibiotics streptomycin sulfate (Fisher BioReagents, Geel, Belgium) at 0.2 μg mL^{-1}, and tetracycline hydrochloride (Fisher Biotech, Fair Lawn, New Jersey) at 0.2 μg mL^{-1}, and then sealed with Parafilm. Plates were incubated at 20 ºC with 12 hr fluorescent lighting / 12 hr dark, and were checked daily for signs of mycelial growth. Specimens resembling *M. nivale* were isolated by removing 5-mm-diameter plugs from the growing edge of mycelia and placed on non-amended PDA and incubated for five to seven days (Figure 2.1). Isolates were then visually compared to known *M. nivale* cultures and later confirmed by the production of pink-orange sporodochia in culture which are enhanced by exposure to light. A single isolate was retained per patch. The isolates were maintained on 20 mL PDA plates, as well as stored at 4°C on agar slants and wheat seed tubes for future work. Select isolates from the Hsiang lab worldwide collection with incompletely documented fungicide exposure levels were included in tests as well. Isolates from the lab collection were revived from long-term storage (wheat seed tubes stored at -20°C).

2.2.3 Golf course fungicide application records

The records for Ontario locations were downloaded from the IPM Council of Canada website (http://public.ipmcouncilcanada.org). Several Ontario turfgrass managers provided additional records or anecdotal information upon request. British Columbia records are not available from government websites; however, some locations in British Columbia supplied partial records upon request. With incomplete records, total fungicide use was extrapolated from the available data. Location names were omitted and each location was given an identification code.

2.2.4 Fungicide sensitivity testing

2.2.4.1 Media preparation

2.2.4.1.1 Preparation of iprodione-amended media

Iprodione (23.3%; Bayer Crop Science Inc., Calgary, AB) in the form of Rovral Green GT Flowable Fungicide was dissolved in 100% acetone, 430 µL in 9.6 mL, for a stock concentration of 10,000 µg mL^{-1}. Iprodione stock solution was added to molten PDA cooled to 55°C, to obtain final iprodione concentrations of 0, 1, 10, and 100 µg mL^{-1} while maintaining an equal final concentration of acetone (0.10 % v/v). In preliminary tests, acetone at this concentration did not inhibit growth of *M. nivale*. Fungicide-amended PDA was pipetted into 9-cm-diameter plates in 12 mL aliquots and allowed to set.

2.2.4.1.2 Preparation of propiconazole-amended media

Propiconazole (14.3%; Syngenta) in the form of Banner Maxx was diluted in 100% acetone, 6.99 µL in 9.93 mL, for a stock concentration of 100 µg mL^{-1}. Propiconazole stock solution was added to molten PDA cooled to 55°C, to obtain final propiconazole concentrations of 0, 0.01, 0.1, and 1 µg mL^{-1} while maintaining an equal calculated final concentration of acetone (0.10 % v/v). Fungicide-amended PDA was added to 9-cm-diameter plates in 12 mL aliquots and allowed to set.

2.2.4.2 Full concentration range EC_{50} testing

A minimum of 50 isolates from BC and 50 isolates from ON were selected for full concentration range testing, with an additional five isolates from other countries. Fungicide-amended plates were prepared following protocol outlined above (Sections 2.2.4.1 and 2.2.4.2),

and the plates were further processed for a strip agar assay following Hsiang et al. (1997). The solid medium in the 9-cm-diameter plates was cut using six blades mounted on a 9-cm-diameter aluminum holder (Figure 2.2). Excess agar was then removed, leaving 1-cm-wide strips. Agar plugs 5-mm in diameter were taken from the growing edge of active mycelia and placed hyphae down on the centre of each strip. Three technical replicates were used per isolate per experiment with each replicate on a separate plate. Plates were grown at 20°C with 12 hour overhead fluorescent lighting and two growth measurements (180° from each other) were taken at 48 and 96 hours post plating.

Only the growth between 48 and 96 hours was used for analysis, since the first 48 hours could show variability as a result of establishment effects. Percentage of inhibition was calculated as [1 − (mean colony diameter on iprodione-amended medium/mean colony diameter on non-amended medium)] x 100%. SAS version 9.1 (SAS Institute, 2003) PROC PROBIT was used to calculate EC_{50} values (the concentration required to inhibit mycelium diameter growth by 50%). An example of the SAS command statement is given in Appendix 2.1. Probit transformation is used to straighten the dosage response curve and allows for more accurate estimations of EC_{50} values compared to then untransformed data (Sokal and Rohlf, 1981). Full concentration range experiments were conducted at least two times. Isolates with iprodione EC_{50} values exceeding 10 μg mL^{-1} were deemed resistant to iprodione, and isolates with propiconazole EC_{50} values exceeding 0.1 μg mL^{-1} were deemed resistant to propiconazole.

2.2.4.3 Discriminatory concentration testing

Based on the results of the full range tests and previous literature on insensitivity to the iprodione and propiconazole, the concentrations of 10 and 0.1 μg mL^{-1} respectively, were chosen

as discriminatory concentrations for further testing. This discriminatory concentration was chosen by creating response curves from the data to determine at which point presumed sensitive isolates would be fully inhibited (Section 2.3.2), and isolates showing reduced sensitivity would still show growth. In addition, bar graphs depicting EC_{50} values aided in identifying appropriate concentrations of iprodione and propiconazole (Section 2.3.2).

Fungicide-amended plates at the discriminatory concentration were prepared (Section 2.2.4.1 above) in addition to non-amended PDA plates. The plates were cut with the same method as in the full range testing to save on materials. Agar plugs 5-mm in diameter were taken from the growing edge of active mycelia and placed hyphae down on the centre of each strip. Three technical replicates were used per isolate per experiment. Plates were grown at 20°C with 12 hour overhead fluorescent lighting and two growth measurements (180° from each other) were taken at 48 and 96 hours post plating.

Means were calculated for growth on non-amended PDA for the various replicates. This mean was used as the denominator to calculate the percentage of growth on the discriminatory concentrations where individual measurements on amended media were used as the numerator. These data were subjected to ANOVA using SAS PROC GLM, with calculations of means and standard errors, and means were separated by Fisher's protected LSD ($p = 0.05$). An example of the SAS command statement is given in Appendix 2.2. Discriminatory tests were repeated two times per isolate per fungicide for a select number of isolates. The results of the two experiments were compared via linear relationship. The high R-squared values indicated that data from single experiments could be used (rather than two tests) in order to reduce labour and include a larger number of isolates. Large selections of isolates (Table 2.1) were then tested on these discriminatory concentrations. Isolates with a percentage growth ≤40% were deemed to be

sensitive, isolates >40% but ≤60% percentage growth were deemed moderately insensitive, and isolates ≥60% were deemed highly insensitive. Correlation analysis was used to assess the relationship between insensitivity to iprodione and insensitivity to propiconazole.

2.3 Results

2.3.1 Isolate collection

A total of 161 isolates of *M. nivale* were collected from various locations in British Columbia (BC-1, BC-2, and BC-3) and Ontario (ON-1, ON-2, ON-3, and ON-4) (Table 2.1). An additional 135 isolates previously collected from locations in Ontario (e.g. GTI) were also revived from stock and assessed for their sensitivity to iprodione and propiconazole (Table 2.1).

2.3.2 Full concentration range EC_{50} testing

The isolates used for testing of multiple concentrations of iprodione are shown in Appendix 2.3 along with their calculated EC_{50} averages which ranged from 1.2 μg mL^{-1} to 542.6 μg mL^{-1}. The EC_{50} for isolates from Ontario ranged from 1.2 μg mL^{-1} to 32 μg mL^{-1}. In comparison, the EC_{50} for isolates from British Columbia ranged between 1.5 μg mL^{-1} and 542.6 μg mL^{-1}. The B.C isolates as a group had statistically significant (p = 0.05) higher iprodione EC_{50} values than isolates from Ontario based on an ANOVA test. Within the Ontario collection, the resistant isolates (EC_{50} > 10 μg mL^{-1}) had a resistance factor (Average EC_{50} resistant / Average EC_{50} sensitive) of 13.6 / 5.3 = 2.6. Within the B.C population the resistant isolates had a resistance factor (RF) of 153.2 / 4.0 = 38.2.

The EC_{50} averages for the fungicide propiconazole ranged from <0.001 μg mL^{-1} to 8.7 μg mL^{-1} (Appendix 2.3). The values for isolates from Ontario ranged from <0.001 μg mL^{-1} to 0.89

μg mL^{-1}. In comparison, the values for isolates from British Columbia ranged between 0.02 μg mL^{-1} to 8.7 μg mL^{-1}. Again, the B.C isolates as a group had statistically significant (p = 0.05) higher propiconazole EC_{50} values than isolates from Ontario based on an ANOVA test. Within the Ontario collection, the resistant isolates had a resistance factor of 0.36 / 0.045 = 7.9. Within the B.C collection, the resistant isolates had a resistance factor of 0.79 / 0.04 = 20.4.

2.3.3 Discriminatory concentration testing

Response curves for iprodione and propiconazole were plotted to represent the growth of select isolates on the different concentrations of iprodione (Figure 2.3) and propiconazole (Figure 2.4). These curves, bar graphs depicting number of isolates per EC_{50} value (Figures 2.5 and 2.6), and previous literature on the sensitivity of other species to these fungicides (Detweiler et al., 1983; Pennucci et al., 1990) aided in selection of 10 μg mL^{-1} as the discriminatory concentration for iprodione, and 0.1 μg mL^{-1} for propiconazole. The linear relationship between the two tests yielded high R^2 values, 0.88 and 0.80 ($p < 0.005$), for iprodione and propiconazole respectively. The consistency between those tests allowed for the use of single test results in the large scale screening.

A distribution graph of percentage growth on iprodione discriminatory concentration was created (Figure 2.7). The Ontario set of samples had 144 of 193 isolates which were found to be sensitive (≤40%), 41 of 193 were moderately insensitive (>40% but ≤60%), and 8 of 193 were found to be highly insensitive (≤60%). Previous literature separated sensitive and resistant isolates at 50% growth. In this case 170 of 193 isolates were sensitive and 23 isolates were resistant in Ontario. In contrast, of the 93 B.C isolates tested on iprodione, 14 tested as sensitive, 12 as moderately insensitive, and 67 as highly insensitive. Separation at 50% growth resulted in

17 of 93 isolates being sensitive and 76 being resistant. British Columbia had a statistically significant ($p < 0.05$) higher proportion of iprodione insensitive isolates compared to Ontario based on a chi-squared test.

A distribution graph of percentage growth on propiconazole discriminatory concentration was created (Figure 2.8). Of the 181 Ontario isolates tested on propiconazole, 88 were found to be sensitive (≤40%), 72 moderately insensitive (>40% but ≤60%), and 21 highly insensitive (≤60%). Separation of Ontario isolates at the 50% growth mark resulted in 43 of 181 being classified as resistant. In contrast, of the 71 B.C isolates tested on propiconazole, 13 tested as sensitive, five as moderately insensitive, and 53 as highly insensitive. Separation at 50% resulted in 55 of 71 being classified as resistant. British Columbia had a statistically significant ($p < 0.05$) higher proportion of propiconazole insensitive isolates compared to Ontario based on a chi-squared test.

A scatter plot was created for isolates which were tested on the discriminatory concentrations of both iprodione and propiconazole (Figure 2.9). Using the 50% growth cutoff for insensitivity, 61 isolates which were insensitive to iprodione were also insensitive to propiconazole. There were 7 iprodione-insensitive propiconazole-sensitive isolates, and 39 isolates which were iprodione-sensitive propiconazole-insensitive. Finally, 160 isolates were sensitive to both iprodione and propiconazole. These groups of isolates comprise different sensitivity categories, double-resistant isolates, double-sensitive, only iprodione-resistant and only propiconazole-resistant.

2.3.4 Golf course fungicide applications

For the Ontario locations, records from six years were used (2010 to 2015). For one BC location (BC-1), only a single year's records (2017) was provided, however the turfgrass manager indicated it was “typical” of past year application rates and it would be accurate to use the same values for each year. Another BC location (BC-3) only provided anecdotal information indicating they applied iprodione and propiconazole approximately five times each per year. For this location it was assumed label rates were used for those five applications.

Most locations gave no indication over what area (e.g hectares) the fungicides were applied. In these cases, it was assumed the same rates were applied to greens, tees, and fairways. The areas of individual greens, tees, and fairways were calculated using the Google Maps measure function and then summed for the total location area (Figure 2.10). Additionally, as records did not indicate which applications were intended to treat *M. nivale*, the total annual application was used.

The fungicide records provided by four locations in Ontario and three locations in British Columbia were used to calculate average use of each fungicide per year. The calculated areas for each location and total fungicide use can be found in Table 2.2. The three BC locations (BC-1, BC-2, BC-3) used an average of 134.8 g 100 m^{-2}, 276.8 g 100 m^{-2} and 221.8 g 100 m^{-2} of iprodione per year, respectively. In contrast, the Ontario locations (ON-1, ON-2, ON-3, ON-4) used 8.6, 22.5, 7.3, and 67.2 g 100 m^{-2} of iprodione per year respectively. When graphed with the percentage of resistant isolates at each location, a significant polynomial relationship is apparent ($R^2 = 0.78$, $p < 0.05$) between fungicide use and the development of iprodione resistance (Figure 2.11).

For propiconazole, the three BC locations (BC-1, BC-2, BC-3) used an average of 34.7 g 100 m^{-2}, 24.3 g 100 m^{-2}, and 81.0 g 100 m^{-2} of propiconazole per year respectively. In contrast, the four Ontario locations (ON-1, ON-2, ON-3, ON-4) used 6.4, 17.2, 11.5, and 15.9 g 100 m^{-2} of iprodione per year respectively. When graphed with percentage of resistant isolates, a significant polynomial relationship is apparent ($R^2 = 0.86$, $p < 0.05$) between fungicide use and the development of propiconazole resistance (Figure 2.12).

Locations from both ON and BC were grouped into high and low fungicide use groups based on their fungicide records or anecdotal reports. Locations with annual applications exceeding 50 g 100 m^{-2} and 20 g 100 m^{-2} from iprodione and propiconazole were considered high use locations for the respective active ingredients. High fungicide use locations had a significantly higher proportion of insensitive isolates (discriminatory growth >50%) relative to low use locations based on a chi-square test ($p = 0.05$).

2.4 Discussion

In an effort to curb pesticide use, the Ontario government amended the Pesticides Act with the Cosmetic Pesticides Ban Act in 2008 (Bill 64) which restricts the use of pesticides for non-essential purposes. Ontario Regulation 63/09 from the Pesticide Act mandates that golf courses who use class 9 fungicides must be accredited by an approved integrated pest management (IPM) body and create annual reports justifying their applications. Accredited golf courses submit their yearly records to the IPM Council of Canada where they become publicly accessible (IPM Council of Canada, 2017).

The development of fungicide resistance is a well documented phenomenon in many fungal plant pathogens (Angelini et al., 2015; FRAC, 2017). Frequent and improper use of a

fungicide (or group with similar mode of action) increases the risks for the development of resistance to that group (Ishii and Hollomon, 2015). To maintain a target playing surface and create an aesthetically pleasing environment, golf courses are among the highest users of fungicides in Canada. We sampled turfgrass exhibiting Microdochium patch symptoms from golf courses in British Columbia and Ontario. Turfgrass managers in coastal British Columbia are more likely to use fungicides for the control of *M. nivale* relative to Ontario for a number of reasons. Firstly, the climate of coastal British Columbia is classified as Oceanic (Cfb) bordering on Mediterranean (Csb) according to the Köppen climate classification (McKnight, 2000). The climate is characterized by extended periods of cool temperatures with frequent rain and high humidity. This type of climate creates strong Microdochium patch disease pressure for more than half the year. Other areas of the world with high Microdochium patch incidence (British Isles, Scandanavia) have a similar (Cfb) climate. In comparison, southern Ontario is classified as Hot-Summer Continental (Dfa) bordering on Warm-Summer Continental (Dfb) (McKnight, 2000), having high humidity but also higher temperatures which is not favourable for Microdochium patch development (Snider et al., 2000). Another important climatic variable is the duration of snow cover. Southern Ontario winters allow for greater periods of snow cover and lower temperatures, while the warmer winters of south coastal B.C have low or absent periods of snow cover. Southern Ontario turf managers may generally use a single preventative application prior to snow cover to control pink snow mould, while south coastal B.C turf managers often make numerous applications through the winter months as well as spring and fall to control Microdochium patch (T. Hsiang, Personal Communication).

The increased Microdochium patch disease pressure in B.C translates directly into increased fungicide applications throughout the year (Section 2.2.4). In recent years, some B.C

turfgrass managers have suspected they are dealing with fungicide resistance issues (T. Hsiang, Personal Communication). Several managers have anecdotally reported that fungicide applications appear to provide shorter intervals of control (or none at all) relative to previous years, and the results of the fungicide testing here support this observation, and also reflect the fungicide use pattern in south coastal B.C.

Some isolates of *M. nivale* which exhibited decreased sensitivity to iprodione in our tests had EC_{50} values an order of magnitude higher (EC_{50} ranged between 10 and 542.6 μg mL^{-1}) than those reported by Pennucci et al., 1990 (14 to 54 μg mL^{-1}). Pennucci et al., 1990 also reported an overall incidence of iprodione resistance of 19% (categorization based on >50% growth on 10 μg mL^{-1} discriminatory concentration). Comparatively, the Ontario isolates had a low resistance frequency of 11.9% (23 of 193) and B.C had a much higher frequency of 81.7% (76 of 93). Chastagner and Vassey (1982) did not report EC_{50} values however they did note that Microdochium patch (referred to as Fusarium patch) was "common [at their sample location] despite regular and repeated use of iprodione". Additionally, the isolates collected by Pennucci et al. (1990) in New Zealand had an average resistance factor of 12 where the resistant isolates tested here from B.C had an average resistance factor of 35. Resseler and Buchenauer (1988) were able to generate lab resistant isolates of *M. nivale* with resistance factors to iprodione between 20 and 200, however their isolates showed high osmotic insensitivity indicating potential fitness costs associated with resistance (Resseler and Buchenauer, 1988a).

As early as 1979, only 5 years after the registration of iprodione in the U.S., iprodione tolerant *B. cinerea* was detected on strawberry (Dennis and Davis, 1979). Since 1979 iprodione-resistant *B. cinerea* have been report many times with EC_{50} values calculated in several cases: 4.3 to 13.9 mg mL^{-1} (RF = 7) (Lamondia and Douglas, 1997), 1.58 to 3.01 mg mL^{-1} (RF = 5)

(Raposo et al., 2000), and 16.1 to 96.1 mg mL^{-1} (RF = 101) (Weber, 2011). Another widespread pathogen, *Alternaria alternata*, has had EC_{50} values of iprodione-field-resistant isolates reported at 290 (RF = 145) (Hutton, 1988) and 280 mg mL^{-1} (Solel et al., 1996). The common turfgrass pathogen *Sclerotinia homoeocarpa* (causing dollar spot) has also developed field resistance with EC_{50} of 381 (Detweiler et al., 1983) and 360 mg mL^{-1} (Bishop et al., 2008). As of December 2017, field resistance to the dicarboximides has been reported in at least 12 species, including *M. nivale* (Table 1.2).

Field resistance to the demethylation inhibitors has been even more widespread than the dicarboximides, with documented field resistance in 26 different species (Table 1.3). However there are no previously reported cases of field DMI-resistant *M. nivale* in the literature for direct comparison. Cristani and Gambogi (1993) were able generate DMI tolerant *M. nivale* in the lab using UV radiation; however they did not check sensitivity to propiconazole. The small number of DMI insensitive isolates they were able to create showed low to moderate insensitivity to fenarimol, flutriafol, imazalil, penconazole, and tetraconazole with resistance factors ranging from 1.1 to 12.5. Propiconazole insensitive isolates from B.C had an average EC_{50} of 0.79 (RF = 20.4) and insensitive isolates from ON had average EC_{50} of 0.36 (RF = 7.9) which are comparable to DMI EC_{50} values produced by Cristani and Gambogi (RF = 1.2 to 12.5) (1993).

Even though there is little literature on the sensitivity of *M. nivale* to DMI fungicides, there is a large body of research on the sensitivity of other plant pathogen species. Due to the size of the DMI group research has been divided among each member fungicide, so although there is significant work on the DMI resistance as a whole there is relatively limited work specifically for propiconazole. One of the first papers investigating propiconazole resistance used UV irradiation to generate resistant isolates of *Ustilago avenae* (causing semi-loose smut of oats)

with ED_{50} ranges of 0.7 to 0.119 µg mL^{-1} (RF = 10 to 17) (Köller and Wubben, 1988). Propiconazole resistance in *S. homoeocarpa* has occurred many times at different locations. Golembiewski et al., (1995) identified propiconazole insensitive isolates on golf courses in Michigan and Ohio with an average EC_{50} value of 0.103 µg mL^{-1}(RF = 51.5). Hsiang et al., (1997) reported resistant isolates in Ontario with an average EC_{50} of 0.026 µg mL^{-1}(RF = 3.7), and Bishop et al., (2008) identified a single isolate with an EC_{50} of 8 µg mL^{-1}.

Propiconazole resistance in *Monilinia fructicola* (brown rot of stone fruits) has been reported twice with EC_{50} of 0.22 and 0.42 µg mL^{-1} (Schnabel et al., 2004; Chen et al., 2013). Resistant *Mycosphaerella fijiensis* was found to have an average EC_{50} between 0.225 to >1 µg mL^{-1} (RF = 9.4 to 27.2) (Cañas-Gutiérrez et al., 2009) and resistant *Blumeria graminis* had an average EC_{50} of 1.7 µg mL^{-1} (Wyand and Brown, 2005). Finally, a survey of *Fusarium graminearum* propiconazole sensitivity revealed a wide range of EC_{50} from 5.4 to 62.2 µg mL^{-1} (Talas and Mcdonald, 2015). However the authors did not indicate if the higher EC_{50} value indicated resistance or were part of the natural variability of *F. graminearum*.

A strong correlation between the average yearly application rate of fungicide and the percentage of resistant isolate found at a particular location is not surprising. Low use locations in Ontario had significantly lower incidence of insensitive isolates for both iprodione and propiconazole than the higher use locations in British Columbia. Similar results were found for DMI sensitivity in Ontario isolates of *S. homoeocarpa*, where high use locations had higher incidences of DMI insensitivity (Van Den Nieuwelaar and Hsiang, 2014). In *M. fructicola*, it has been suggested that as few as 12 applications of DMI fungicides can begin to widen the sensitivity range and isolates with reduced sensitivity can develop in a relatively short period (three years) (Zehr et al., 1999). Based on partial records and correspondence with the BC

turfgrass managers, the amount and the number of applications at those locations apparently exceeded the threshold for resistance development in *M. nivale*. The records for all BC locations indicated that they all exceed the recommend number of annual applications for both iprodione (two) and propiconazole (two) (OMAFRA, 2017). Based on the application records from the four Ontario locations and three BC locations, it is estimated that exceeding 75 g 100 m^{-2} $year^{-1}$ of iprodione may increase the risk of resistance development. Additionally, exceeding 35 g 100 m^{-2} $year^{-1}$ of propiconazole may increase the risk of resistance development in *M. nivale*. Although isolates with decreased sensitivity were identified in vitro, the results may be due to the constraints of the lab environment. Field trials are therefore necessary to determine whether insensitivity in laboratory experiments are correlated with a decrease in efficacy of formulated control products. Field trials with representative isolates are discussed in Chapter 3.

Tables

Table 2.1 Origin, host and number of isolates of *Microdochium nivale* used in fungicide sensitivity testing. Except for the six isolates labeled *various*, all isolates were from Canada.

Location	Host	Number of isolates
Along Highway 86 near Listowel, ON	*Triticum sp.*	6
Graham Hall, Guelph, ON	*Poa pratensis*	4
GTI, Guelph, ON	*P. pratensis*	7
GTI, between green and road, Guelph, ON	*P. pratensis*	14
GTI, hillside of upper green, Guelph, ON	*P. pratensis*	3
GTI, native green, Guelph, ON	*Agrostis stolonifera / P. annua*	15
GTI, pathology green fringe, Guelph, ON	*P. pratensis*	10
GTI, pathology green, Guelph, ON	*A. stolonifera*	7
GTI, roadside of upper green, Guelph, ON	*P. pratensis*	7
GTI, roadside, Guelph, ON	*P. pratensis*	9
GTI, roadway, Guelph, ON	*Lolium perenne*	26
Highway 131, near Atwood, ON	*Triticum sp.*	4
ON-1, Guelph, ON	*A. stolonifera / P. annua*	17
ON-2, Guelph, ON	*A. stolonifera / P. annua*	15
ON-3, Guelph, ON	*A. stolonifera / P. annua*	14
ON-4, Guelph, ON	*A. stolonifera / P. annua*	22
Ottawa Experimental Farm, Ottawa ON	*Triticum sp.*	11
BC-1, Victoria, BC	*P. annua*	9
BC-2, North Vancouver, BC	*P. annua*	40
BC-3, Victoria, BC	*A. stolonifera / P. annua*	37
BC-C, Victoria, BC	*A. stolonifera / P. annua*	7
Various	*Various*	6

[¥]GTI – Guelph Turfgrass Institute

Table 2.2 Estimated area and total fungicide use for sample locations (golf courses) which provided fungicide application records. Area of golf courses (tees, fairways, and greens) estimated using Google Maps measure function (Section 2.2.4). Total annual applications for ON locations sourced from the IPM Council of Canada fungicide record search, and for BC sourced from application records supplied by turfgrass managers. Average per area = Total annual application (converted to grams) / Estimated area.

Sample location	Estimated area ($100\ m^2$)	Total annual application (kg)		Average per area (g $100\ m^{-2}$)	
		Iprodione	Propiconazole	Iprodione	Propiconazole
ON-1	740	6.5	4.8	8.7	6.4
ON-2	540	12.1	9.2	22.5	17.2
ON-3	935	6.9	10.7	7.3	11.5
ON-4	675	45.5	10.7	67.2	15.9
BC-1	1030	138.5	47.8	134.8	46.5
BC-2	1355	122.2	68.6	90.3	50.7
BC-3	1030	230	59.4	221.0	57.5

Figures

Figure 2.1 Isolate of *Microdochium nivale* growing on PDA after 7 days at 20 ºC with 12 hours of overhead fluorescent light alternated with 12 hours of darkness. Characteristic pinkish mycelia is shown.

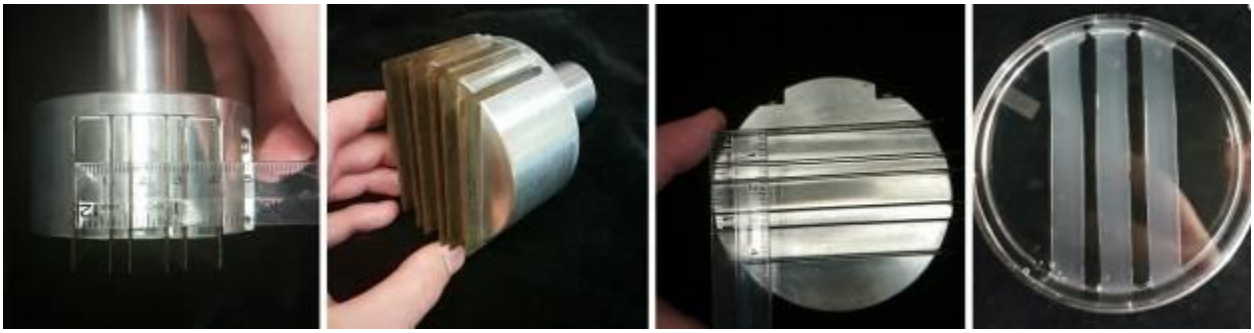


Figure 2.2 Custom agar cutter consisting of six blades mounted on a 9-cm-diameter aluminum holder and the resulting agar in a 9 cm diameter Petri plate after excess medium is removed.

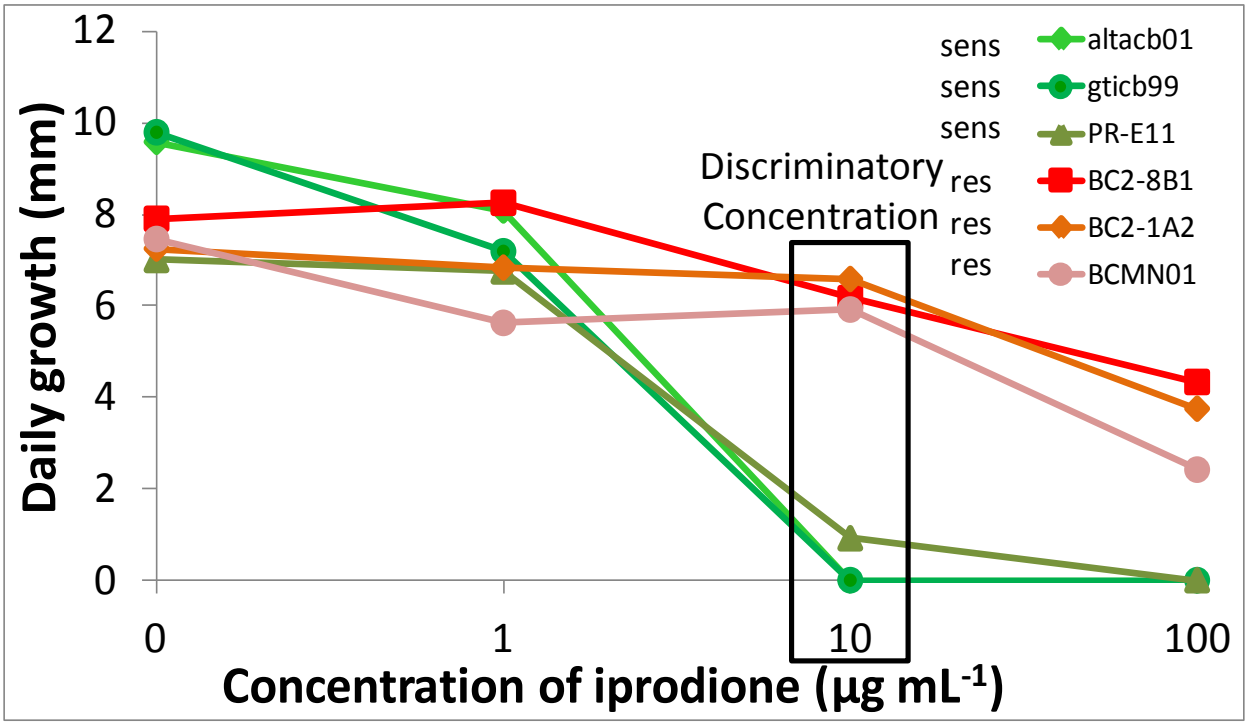


Figure 2.3 Effect of iprodione dose on hyphal growth of six *M. nivale* isolates grown on PDA containing 0, 1, 10, or 100 µg mL^{-1} of iprodione. Isolates are listed in Appendix 2.3.

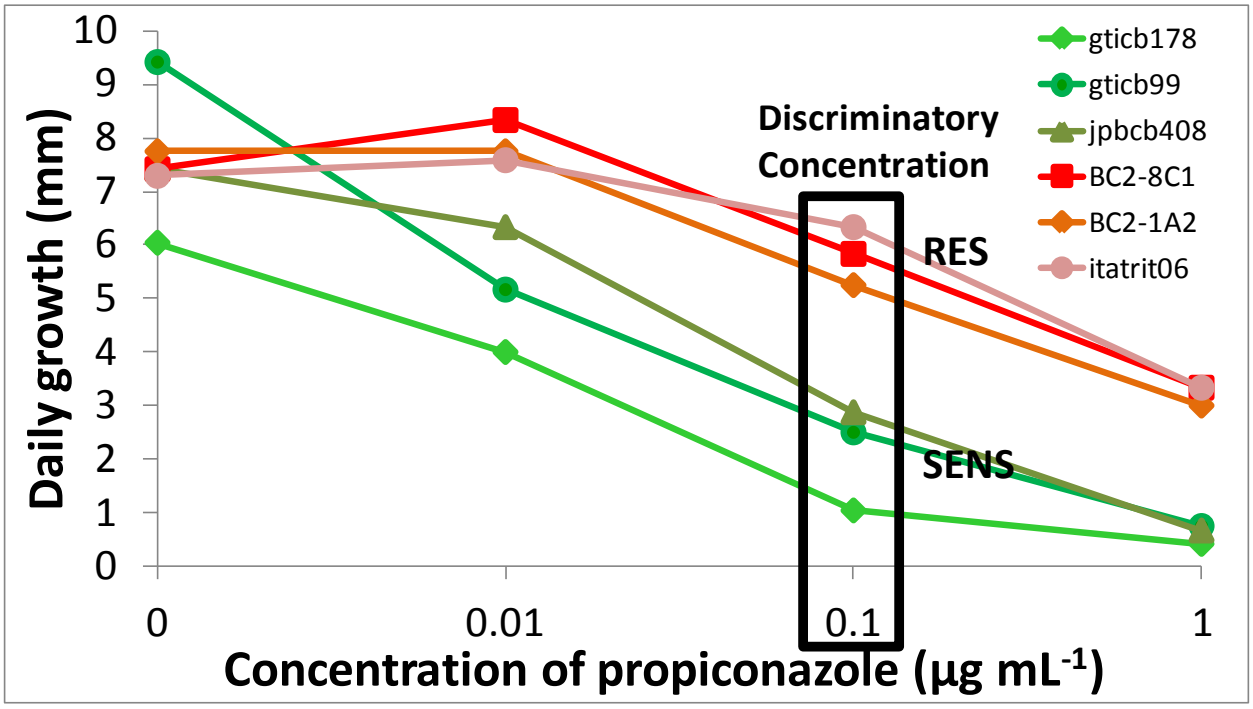


Figure 2.4 Effect of propiconazole dose on hyphal growth of six *M. nivale* isolates grown on PDA containing 0, 0.01, 0.1, or 1 µg mL^{-1} of propiconazole. Isolates listed in Appendix 2.3.

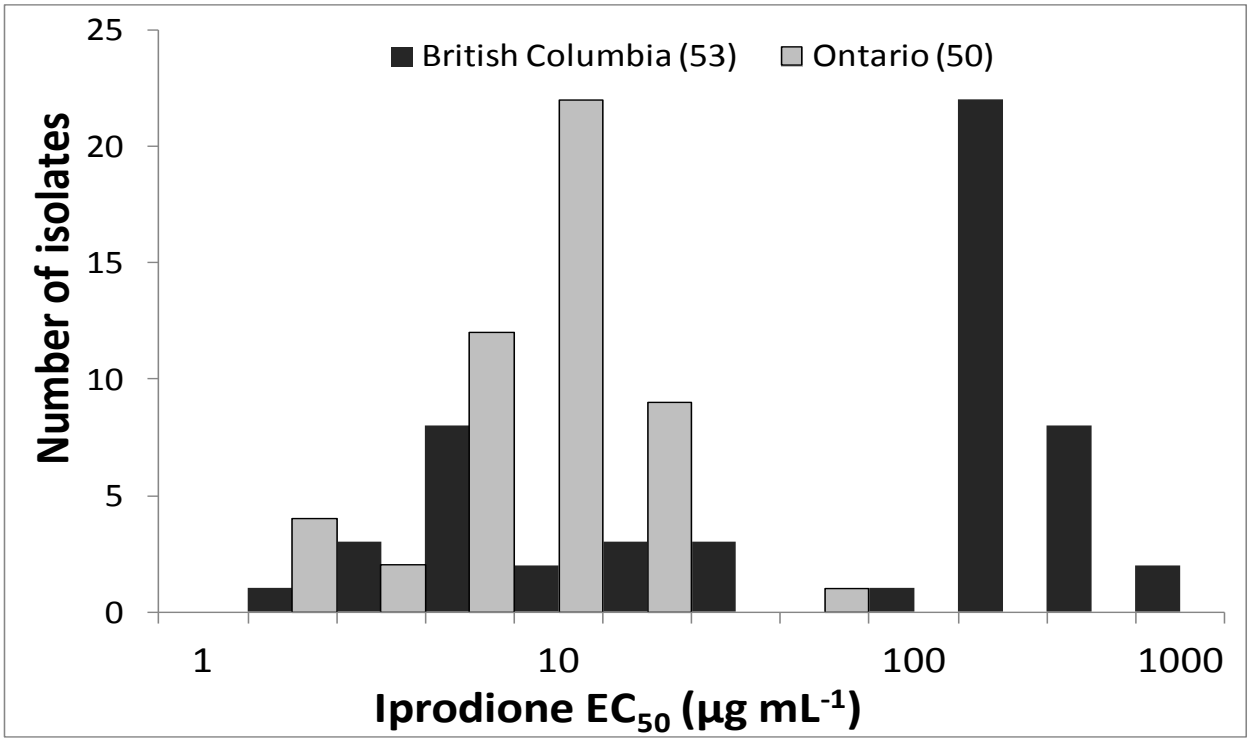


Figure 2.5 The number of isolates from Ontario and British Columbia grouped within iprodione EC_{50} bins. Total number of isolates included in EC_{50} tests are in parentheses.

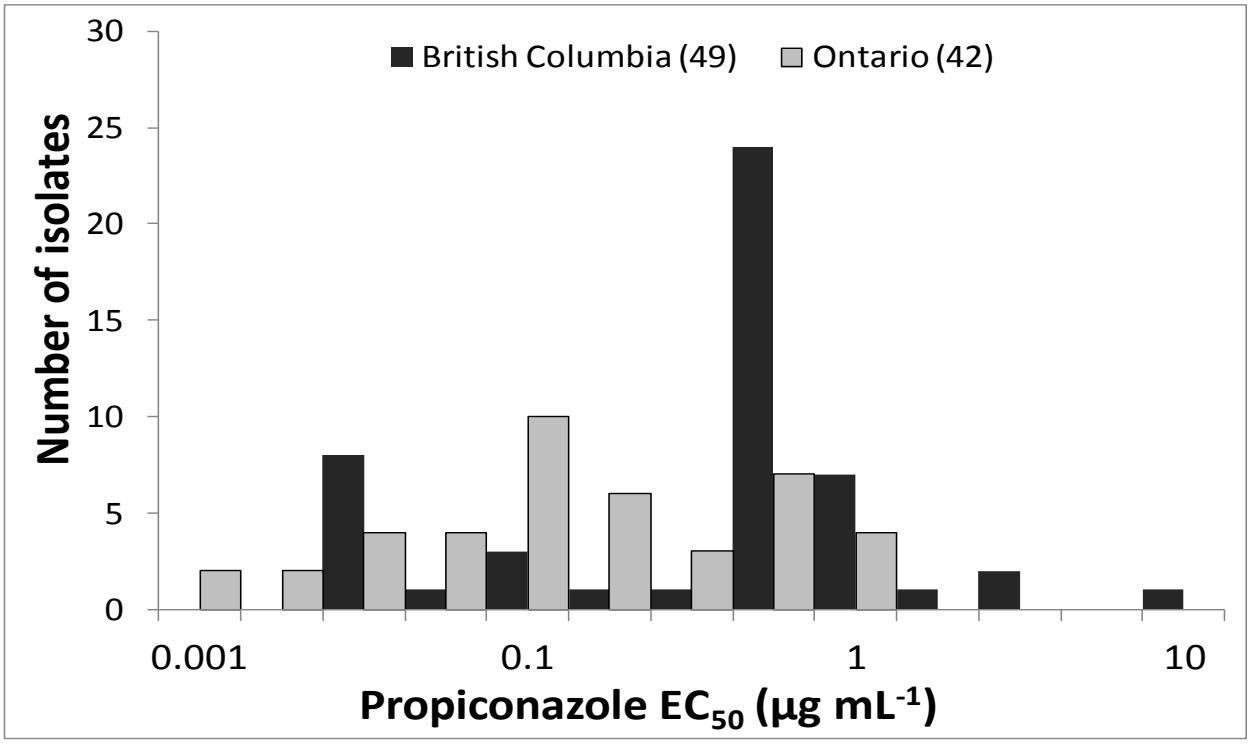


Figure 2.6 The number of isolates from Ontario and British Columbia grouped within propiconazole EC_{50} bins. Total number of isolates included in EC_{50} tests are in parentheses.

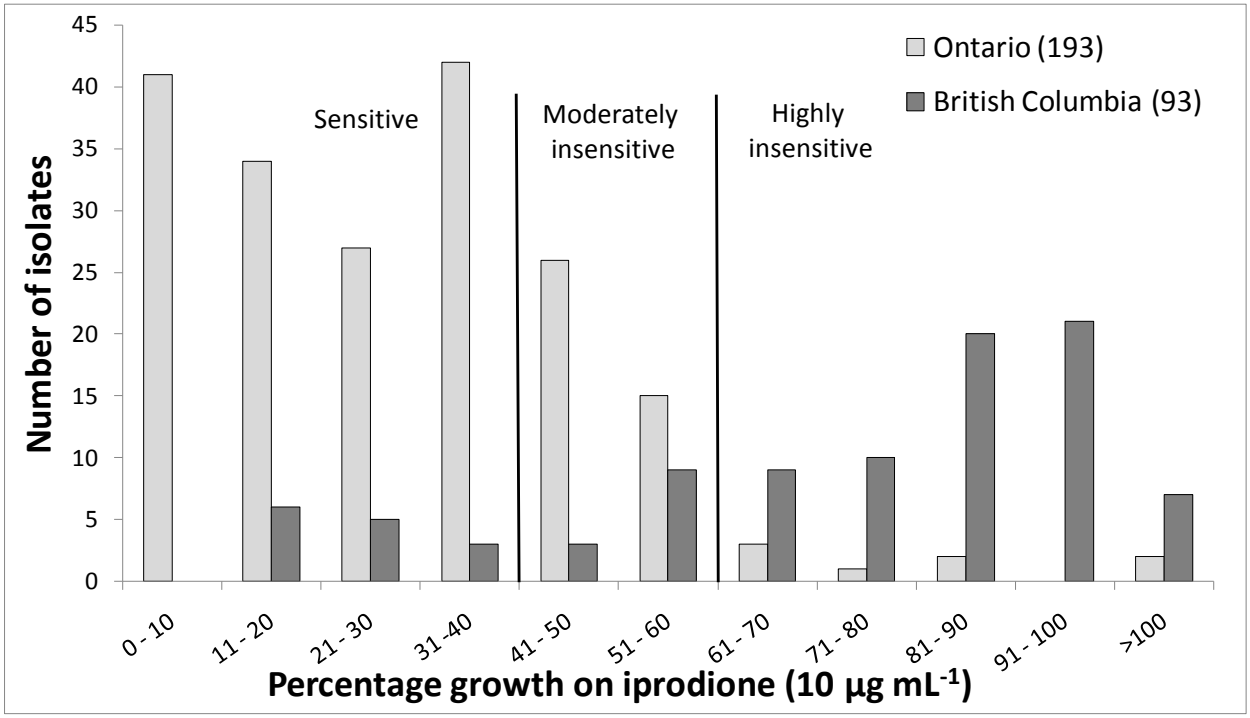


Figure 2.7 Sensitivity distribution depicting number of isolates from Ontario and British Columbia grouped by percentage growth on the discriminatory concentration of iprodione (10 µg mL^{-1}) where growth on non-amended PDA was used as the denominator. Total number of isolates included discriminatory tests are in parentheses.

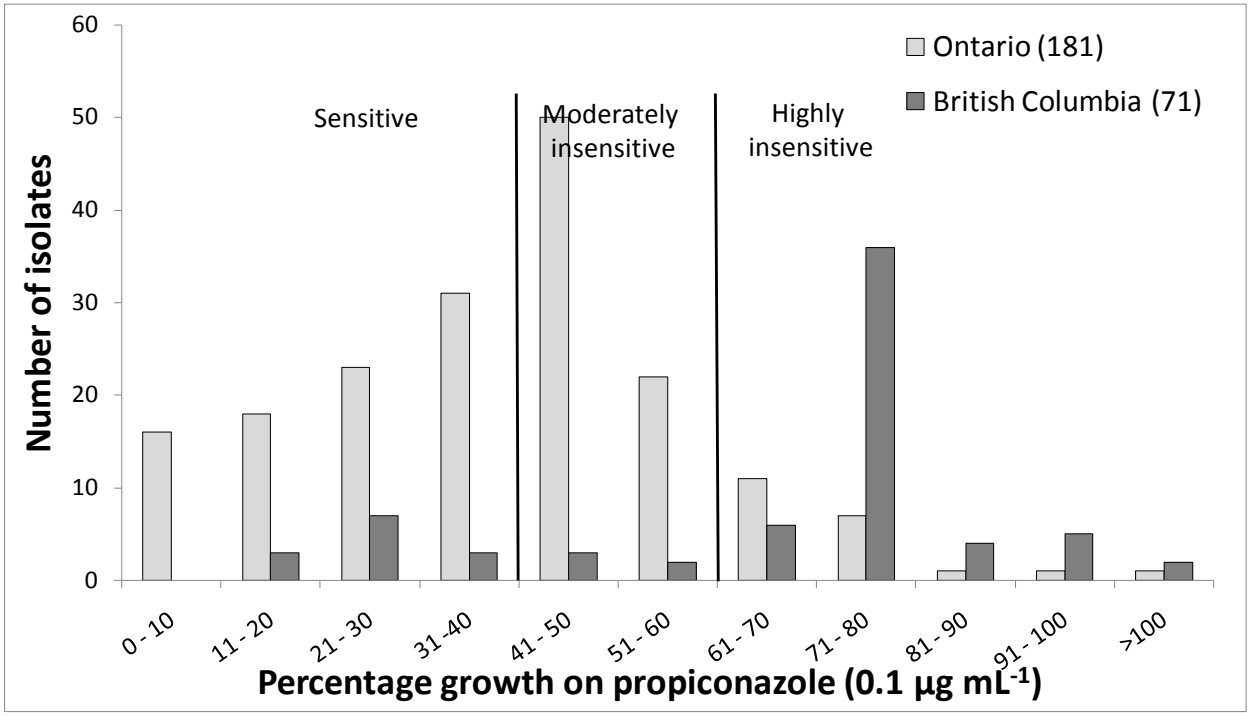


Figure 2.8 Distribution graph depicting number of isolates from Ontario and British Columbia grouped by percentage growth on the discriminatory concentration of propiconazole (0.1 µg mL^{-1}) where growth on non-amended PDA was used as the denominator. Total number of isolates included discriminatory tests are in parentheses.

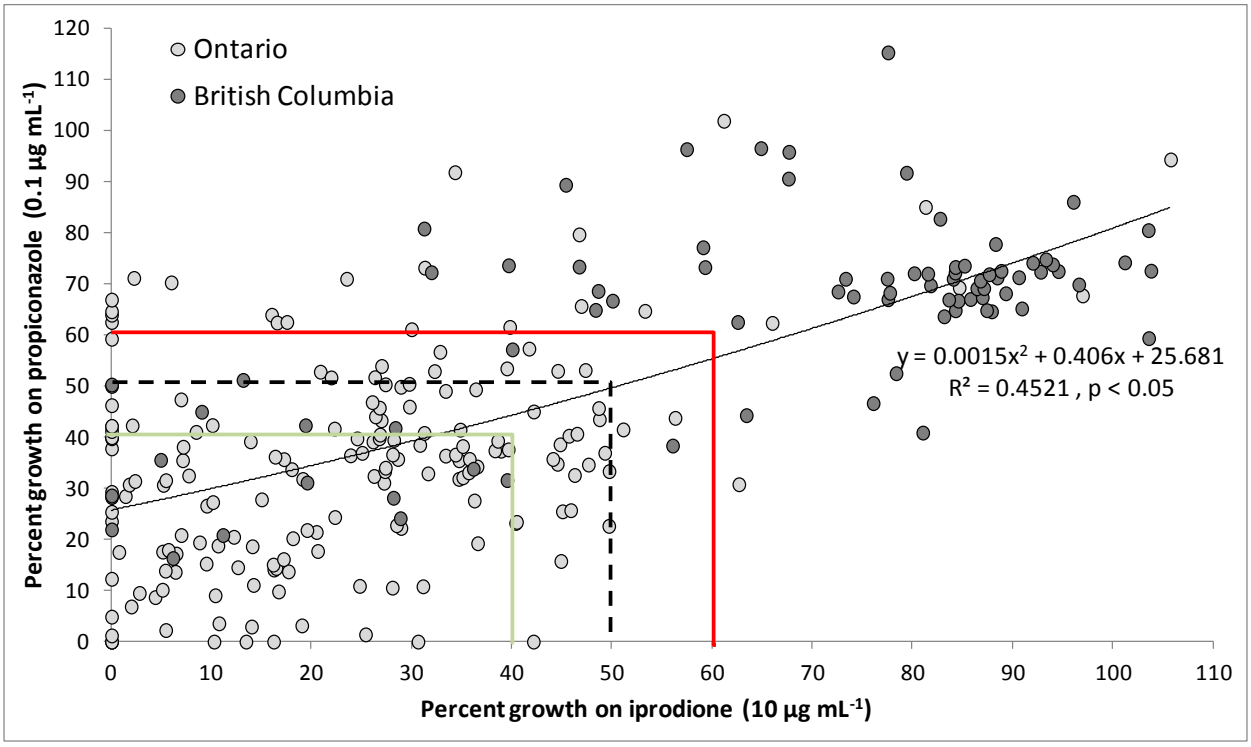


Figure 2.9 Scatter plot of sensitivity to iprodione against propiconazole based on percentage growth on iprodione (10 µg mL^{-1}) and propiconazole (0.1 µg mL^{-1}) where growth on non-amended PDA was used as the denominator. Isolates within the green lines (<40% growth) were considered sensitive to both fungicides. Isolates between the green and red lines (>40% to <60% growth) were deemed moderately insensitive, and isolates beyond the red lines (>60% growth) were deemed resistant. The black dashed border is a separate classification where isolates with >50% growth were deemed resistant to a particular fungicide.

Figure 2.10 Example of fairway and green area determination using Google Maps measure function. Unaltered image on the left and the applied measure filter on the right.

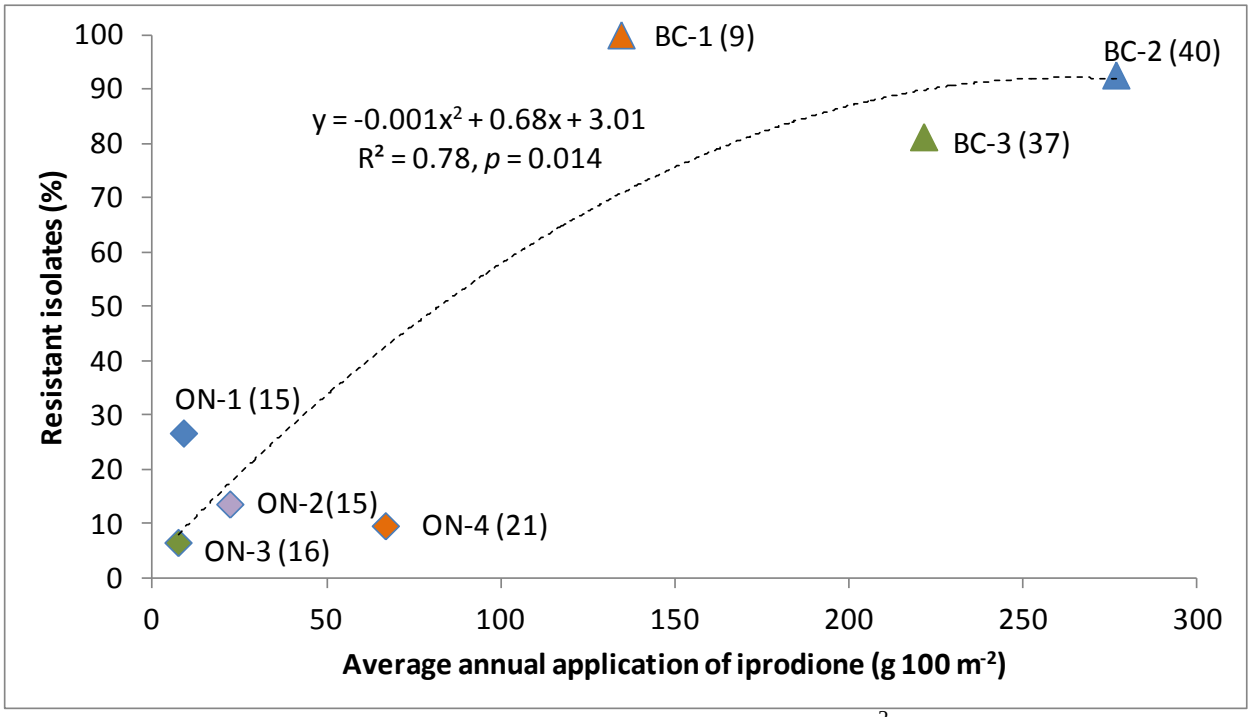


Figure 2.11 The average annual application of iprodione (g 100 m^{-2}) from four locations in ON and three locations in BC with their percentage of resistant isolates. Numbers in brackets indicate the number of tested isolates. Values from ON locations are average from six years of data (2010 to 2015), values from BC locations are extrapolated from one to six years of data and anecdotal notes from turfgrass managers.

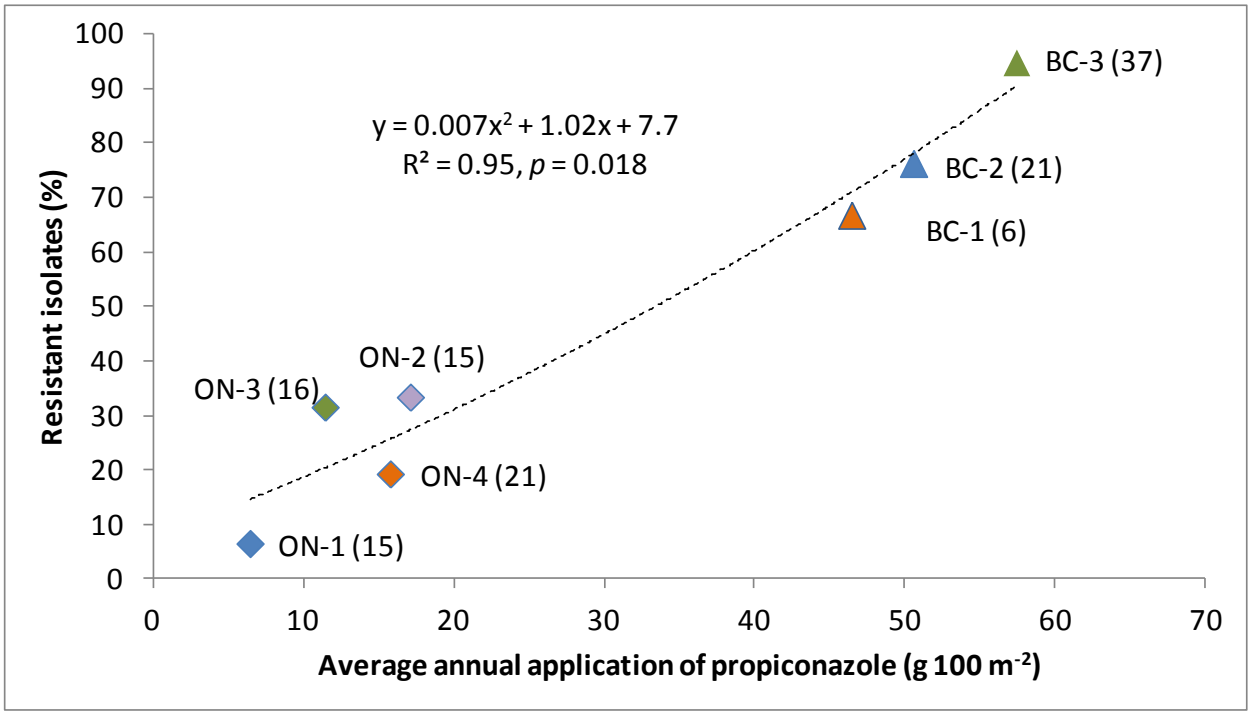


Figure 2.12 The average yearly application of propiconazole (g 100 m^{-2}) from four locations in ON and three locations in BC with their percentage of resistant isolates. Numbers in parentheses indicate the number of tested isolates. Values from ON locations are average from six years of data (2010 to 2015), values from BC locations are extrapolated from one, two, and six years of data and anecdotal notes from turfgrass managers.

Appendices

Appendix 2.1 Example of full concentration range (EC_{50}) SAS PROC PROBIT command statement.

```
data temp;
options pagesize=80 linesize=70;
infile cards expandtabs;
  input isolate$ number conc rep1 rep2 rep3 rep4 rep5 rep6;
    radmean = rep1; output;
    radmean = rep2; output;
    radmean = rep3; output;
    radmean = rep4; output;
    radmean = rep5; output;
    radmean = rep6; output;
cards;
BCMN01 15109 0 12 9 10 12 12 10
BCMN01 15109 1 10 8 7 10 9 8
BCMN01 15109 10 8 8 9 8 7 5
BCMN01 15109 100 1 1 1 1 1 1.5
;
run;
data temp;
set;
number = 1;
if conc = 0 then delete;
lconc=log10(conc);
if isolate = "BCMN01" then response = 1-(radmean/10.83);
if response <= 0 then response = 0;
run;
proc sort; by isolate;
run;
proc probit log10; by isolate;
model response/number=conc /lackfit inversecl itprint;
run;
```

Appendix 2.2 Example of discriminatory concentration SAS PROC GLM command statement.

```
data temp;
options pagesize=80 linesize=70;
infile cards expandtabs;
  input isolate$ PDA rep1 rep2 rep3 rep4 rep5 rep6;
    percgrowth = rep1/PDA; output;
    percgrowth = rep2/PDA; output;
    percgrowth = rep3/PDA; output;
    percgrowth = rep4/PDA; output;
    percgrowth = rep5/PDA; output;
    percgrowth = rep6/PDA; output;
cards;
BCMN01 10.83 8 8 9 8 7 5
run;
proc glm;
class isolate;
model percgrowth = isolate;
means isolate / LSD lines;
run;
```

Appendix 2.3 EC_{50} values for iprodione and propiconazole for *M. nivale* isolates based on in vitro full concentration range sensitivity tests. Isolates were deemed resistant to iprodione when EC_{50} values were >10 µg mL^{-1}, and resistant to propiconazole when EC_{50} values were >0.1 µg mL^{-1}.

Location	Accession	EC_{50} (µg mL^{-1})	
		Iprodione	Propiconazole
UK	10082	1.42	0.31
GER	10101	1.26	0.26
ITL	10106	1.33	0.56
ON	10178	1.24	0.01
ON	10179	2.08	0.17
ON	11002	9.31	0.26
ON	11007	9.74	0.16
ON	11016	10.65	0.11
ON	11020	9.07	0.04
ON	11021	8.84	0.32
ON	11026	10.72	0.32
ON	11027	8.33	0.09
ON	11029	10.00	0.08
ON	11030	13.13	0.09
ON	11031	10.75	0.28
ON	11037	1.57	0.28
ON	11039	5.20	0.07
ON	11040	8.35	0.08
ON	11042	6.37	0.05
ON	11072	6.44	0.05
ON	11074	3.96	0.08
ON	12062	8.90	0.06
ON	12063	5.28	0.01
ON	12099	2.34	0.03
ON	12120	6.35	0.02
ON	12123	3.94	0.02
ON	12124	6.70	0.01
ON	12125	4.72	0.06
ON	12143	32.21	<0.01
ON	12147	4.87	0.41
ON	12151	6.08	0.10
ON	12153	7.25	0.44

ON	12257	6.39	0.02
ON	12262	1.9	0.16
ON	12279	7.17	<0.01
ON	13155	5.90	0.68
ON	13156	9.05	0.36
ON	13162	11.83	0.06
ON	13165	7.69	0.14
ON	13169	12.88	0.68
ON	13172	10.39	0.14
ON	13189	9.83	0.24
ON	13190	3.72	0.06
ON	13208	3.21	0.55
ON	13209	4.43	0.02
ON	13213	11.40	0.36
ON	13225	6.34	0.04
ON	13234	5.25	0.89
ON	13240	1.25	0.69
ON	13266	5.46	0.17
JPN	13407	4.28	0.06
JPN	13408	2.55	0.06
BC	15109	26.74	0.11
BC	15110	40.78	0.81
BC	15111	346.52	0.07
BC	15113	17.36	0.62
BC	15115	30.57	0.08
BC	15116	22.27	0.06
BC	15138	3.30	1.23
BC	15139	197.66	0.29
BC	15140	6.92	0.61
BC	15141	5.56	1.83
BC	15142	5.18	2.73
BC	15143	124.88	0.49
BC	15144	140.17	0.38
BC	15145	147.16	0.45
BC	15146	166.44	0.35
BC	15147	159.75	0.33
BC	15148	157.17	0.64
BC	15149	149.61	0.56
BC	15150	119.96	0.47
BC	15151	191.44	0.41
BC	15152	146.56	0.34

BC	15153	167.79	0.38
BC	15154	185.16	0.44
BC	15155	186.97	0.52
BC	15156	1.51	0.02
BC	15157	4.31	0.03
BC	15158	163.06	0.40
BC	15159	138.50	0.40
BC	15160	160.13	0.54
BC	15161	140.92	0.57
BC	15162	169.81	0.57
BC	15163	149.62	0.35
BC	15164	6.62	8.73
BC	15165	190.87	0.36
BC	15166	93.82	0.39
BC	15167	160.77	0.39
BC	15168	144.44	0.42
BC	15169	192.67	0.60
BC	15170	114.63	0.44
BC	15171	132.84	0.57
BC	15172	251.74	0.39
BC	15173	191.66	0.52
BC	15174	152.88	0.41
BC	15175	4.04	0.03
BC	15176	3.47	0.04
BC	15177	3.39	0.03
BC	15178	3.43	0.03
BC	15179	2.84	0.03
BC	15180	2.40	0.02
BC	15181	3.13	0.02

Chapter 3 Fungicide effects on insensitive isolates in the field

3.1 Introduction

3.1.1 General overview of field resistance

The turfgrass industry, in general, has a low tolerance for aesthetic damage. High level maintenance programs with low mowing heights and high traffic conditions increase plant stress and, as a consequence, reduce the ability of the plant to defend against diseases (Smiley et al., 2005). In addition to the few known bacterial diseases, there are over 40 distinct fungal diseases of turfgrasses (Smiley et al., 2005). Turfgrass managers will often contend with a variety of these diseases throughout the year, depending on specific locale and environmental pressures. The most prominent fungal disease in Canada during the summer and early fall is dollar spot, caused by *Sclerotinia homoeocarpa*. The late fall, winter, and early spring months are often subject to high-disease pressure for Microdochium patch and pink snow mould, both caused by *Microdochium nivale*, which thrive under cool, wet conditions (Smiley et al., 2005).

Growing and maintaining healthy turfgrass requires the management of many variables. Selecting appropriate grass species and varieties, optimizing watering and fertilizer application, and disease monitoring and control programs are all essential. Disease management has historically been dominated by the use of fungicides (Morton and Staub, 2008). On Canadian golf courses, the DMI group of fungicides has been widely used for many years for the control of Microdochium patch and pink snow mould. The dicarboximide iprodione has also been used extensively as a preventative measure, more often when disease pressure is expected to be high (OMAFRA, 2017). Prolonged use of the same mode of action (i.e. fungicide group) creates a selection pressure on fungal populations to shift towards decreased sensitivity. Fungicide resistance is often exhibited in field settings as decreased intervals of control, causing mangers to

apply more frequently to achieve the same level of control (Hsiang et al., 1997). Disease control by a fungicide below acceptable levels could be caused by a number of other factors such as improper application (e.g. product mixing), sub-optimal timing, poor conditions (e.g. heavy wind), and above average disease pressure (Köller and Scheinpflug, 1987). However, if the fungicide is initially efficacious, but then offers poor control under a variety of conditions and over subsequent multiple seasons, this implies that fungicide resistance has developed in a local population (Vincelli and Munshaw, 2015). As well, reduced duration of efficacious control often signals an increase in fungicide resistance (Miller et al., 2002).

Growth rate experiments in amended agar medium are useful for identifying fungal isolates which may have decreased sensitivity to a particular fungicide. However, due to the highly constrained lab environment, it is possible that isolates which display low or high sensitivity in vitro may not exhibit a similar response in the variable field environment (Pringle and Taylor, 2002). Furthermore, as in cases of antibiotic resistance in human pathogens, fitness costs in vitro may not translate to fitness costs in vivo, and vice versa (Andersson and Hughes, 2010). For these reasons, it is important that reports of field resistance development be validated not only in vitro, but in field experiments as well (Leroux, 2007).

The term fitness is used here to describe the survival and reproductive success of an individual, or the success of alleles and other genetic determinants (Pringle and Taylor, 2002). In the past, the fitness of fungal plant pathogens has been measured using mycelial growth, spore production, pathogen virulence, and mixed inoculum competition tests (Zhan and McDonald, 2013). In these terms, a fungicide-resistance fitness cost is the difference in fitness between a resistant strain and a representative sensitive strain in the absence of the fungicide (Mikaberidze et al., 2015). When sensitive and resistant strains compete in the field, fitness costs associated

with gene mutations may determine the outcome. If fitness costs are absent in fungicide-resistant strains, those isolates may be fully competitive with sensitive strains as has been reported in some cases of benzimidazole resistance (Myresiotis et al., 2007), SDHI resistance (Lucas et al., 2015), and pyrimidine resistance (Bardas et al., 2008).

3.1.2 Field resistance to dicarboximide and DMI fungicides in other species

Field resistance to both the dicarboximide and DMI fungicides has developed in many species (Appendices 2.5 and 2.6). However, reported instances of field resistance to these fungicide groups in *M. nivale* is rare, with no previously verified cases of insensitivity to DMI fungicides and only two lab-verified cases of dicarboximide resistance (Chastagner and Vassey, 1982; Pennucci et al., 1990). Neither Pennucci et al. (1990) nor Chastagner and Vassey (1982) conducted experimental field trials with the iprodione-insensitive isolates they collected, although both noted that anecdotal applications of iprodione failed to control Microdochium patch (Fusarium patch) symptoms in the field. There have been no reports of field work conducted on the efficacy of the DMIs and dicarboximides fungicides on isolates of *M. nivale* which exhibit insensitivity.

A limited amount of research has been conducted on the effectiveness of fungicides in controlling other pathogens with documented resistance. Of particular interest is another turfgrass pathogen, *S. homoeocarpa*, in which resistance has been documented to both iprodione and propiconazole (Detweiler et al., 1983; Hsiang et al., 1997). When label rates of propiconazole were applied, isolates of *S. homoeocarpa* which exhibited decreased sensitivity to propiconazole in lab tests were shown to have a reduced interval of control relative to sensitive isolates (Hsiang et al., 1998). Popko et al. (2012) found the field efficacy of propiconazole in

reducing dollar spot disease was lower at test sites previously exposed to DMI fungicides relative to their baseline tests. Miller et al. (2002) showed a strong correlation between higher incidence of disease, measured by area under disease progress curves (AUDPC), and higher propiconazole EC_{50} values (EC_{50} is the concentration required to inhibit growth by 50%) for plants treated with propiconazole in a greenhouse. Additionally, putting greens which were treated with iprodione failed to control inoculated isolates of *S. homoeocarpa* which were found to be insensitive to iprodione in the lab (Detweiler et al., 1983). In contrast, Jo et al. (2006) tested isolates of *S. homoeocarpa* insensitive to iprodione in the field and found no reduction in control relative to sensitive isolates. Jo et al. (2006) also found no reduction in control from propiconazole when applied to propiconazole-insensitive isolates of *S. homoeocarpa*.

Research on other diseases is even more limited. Epoxiconazole-resistant *Mycosphaerella graminicola* were not correlated with a reduction of epoxiconazole efficacy in the field (Stammler et al., 2008). In greenhouse experiments, disease incidence and levels of mycotoxin (deoxynivalenol) were not reduced in plants inoculated with tebuconazole-resistant *Fusarium graminearum* relative to those inoculated with sensitive strains (Spolti et al., 2014a). Below-label rates of propiconazole did not adequately control *Colletotrichum cereale* resistant to propiconazole in greenhouse tests while sensitive isolates were fully inhibited (Wong and Midland, 2007). Hubbard et al. (1997) reported control of *Sclerotinia minor* with reduced sensitivity to iprodione was lower than that of sensitive isolates on lettuce in the field. Similarly, Washington et al. (1992) reported iprodione-resistant *B. cinerea* was not controlled with iprodione in strawberry fields. Finally, *Pyrenophora teres* with mutations conferring insensitivity to pyraclostrobin, a QoI fungicide, were not controlled by label rates on barley (Semar et al., 2007).

Resistance to iprodione in other species has had resulted in different responses to field applications. Additionally, neither Chastagner and Vassey (1982) nor Pennucci et al. (1990), conducted iprodione efficacy trials in the field. As a result, it is not known whether the development of iprodione-resistance in *M. nivale* will result in control reduction with commercially available products. Similarly, DMI-resistance in other species has resulted in variable field responses to control products and there are no previous reports which could be found of DMI-resistant *M. nivale* isolates for which field trials could be conducted. Therefore, there is a knowledge gap regarding the effectiveness of field applications iprodione and propiconazole for the control of lab-resistant isolates of *M. nivale*. This information would be especially useful for turfgrass managers as it can help inform management practices.

The objectives of this chapter were:

(1) to assess Microdochium patch disease development in the field caused by isolates with different fungicide sensitivities; and

(2) to assess the amount of disease reduction caused by applications of iprodione and propiconazole in the field.

3.2 Methods

3.2.1 Isolate selection

Samples of *M. nivale* were collected from various locations in Ontario and British Columbia (fully described in Section 2.3.1). In brief, fungal isolations from the field samples were done by surface sterilization in 1% hypochlorite for 30 to 60 sec, and after tentative assessment of cultures as *M. nivale*, isolates were stored in wheat seed tubes at -20°C (Section

2.2.2) for fungicide sensitivity testing. Based on results from the iprodione and propiconazole discriminatory concentration testing, isolates were placed into one of four sensitivity groups for field tests (Section 2.3.3): sensitive to both iprodione and propiconazole, resistant to both iprodione and propiconazole, resistant to only iprodione, or resistant to only propiconazole. Isolates with greater than 50% growth on a discriminatory concentration (compared to non-amended media) were deemed insensitive or resistant to that fungicide.

3.2.2 Inoculum production

Inocula were prepared by first soaking dry wheat seeds overnight in deionized water. The water was drained, and soaked seeds were rinsed several more times to ensure all debris washed away. Autoclaved 250 ml mason jars were one quarter filled with soaked wheat seeds, approximately 50 mL of deionized water was added to help retain moisture, and lids were loosely screwed in place with gasket side up. Prepared jars were autoclaved at least twice prior to use and allowed to cool to room temperature before use. Selected isolates of *M. nivale* were grown on PDA, and a single, fully colonized 9-cm-diameter plate was cut into ~1 cm^2 squares were stirred into each jar of ensuring an even distribution. Inoculated jars were placed inside a plastic bin and then placed inside a growth chamber for two to six weeks (20ºC). When the wheat seed was fully colonized, the colonized seeds were spread across a plastic lid (0.25 m^2), and allowed to air dry in a fume hood for up to four days (until seeds were hard to the touch). Dried wheat seed was ground using a grain mill (Bosch motor powered Family Grain Mill, Cris Enterprises, Daytona Beach, FL, USA). Ground inoculum was divided into individual mini-manila envelopes in 2.5 g portions for application to 0.25 m^2 plots.

3.2.3 Field trials to assess reduced level of control of lab insensitive isolates

3.2.3.1 Fall 2016

Isolates representative of fungicide sensitivity categories were selected for field tests. Inoculum for each isolate was prepared following Section 3.2.2 with the exception that envelopes were prepared with five gram portions and diluted in another 15 g of autoclaved, ground, un-inoculated wheat seed. Plots were set up at the Guelph Turfgrass Institute (GTI) on the California Green (Figure 3.1) which is predominantly composed of creeping bentgrass (*A. stolonifera*) and maintained by dedicated staff using cultural treatments similar to those used for golf course putting greens in Ontario. Plots were irrigated as needed and grass was mowed to a height of 5 mm. Sulphur-coated urea (N-P-K: 25-4-10) was applied twice annually, once in the spring and once in the late summer at the recommended product rate of 2 kg 100 m^{-2}.

A complete randomized block design was used to set up 0.5 m by 0.5 m plots with four replicate treatment by isolate plots for each isolate. Each plot was inoculated with 5 g inoculum from a single isolate (October 7, 2016). The treatments consisted of an inoculated check where no fungicides were applied, half-label rate of iprodione (92.5 mL Rovral Green GT 100 m^{-2} (containing approximately 21.5 g of iprodione), half-label rate of propiconazole (25.5 mL Banner MAXX 100 m^{-2} (containing approximately 3.6 g of propiconazole), and quarter-rates of combined Rovral and Banner Maxx (46.25 mL and 12.75 mL 100 m^{-2} respectively). Treatments were applied five days post inoculation (October 12, 2016) with a CO_2 sprayer at 35 psi using XC08 nozzles. Two weeks after initial inoculation, a second round of inoculation was done as there was no significant disease progression on the plots. Treatments were applied again five days after the second inoculation. Field plots were assessed for the percent of yellowed or damaged grass per 0.25m^2 area on a weekly basis until the end of the season (December 01,

2016) (Cortes-Barco et al., 2010). A final rating was done after snow cover had receded (January 12, 2017). Weather data was retrieved from the Fergus, ON weather station which is the nearest station to the GTI (21 km) with precipitation data (http://climate.weather.gc.ca).

3.2.3.2 Fall 2017

The fall 2017 field trial was conducted similarly to the fall 2016 trial with a few exceptions. Firstly, different isolates were used because the 2016 trial lacked isolates from the iprodione-resistant propiconazole-sensitive group (resistant to only iprodione) and as each trial only included 12 isolates it was desirable to maximize the overall number of isolates tested. In order to maintain some consistency between the trials, two isolates from the 2016 trial were included in the 2017 trial, one from the double sensitive group (15181) and one from the double-resistant group (15165). Each plot was inoculated with 2.5 g of inoculum from a single isolate on October 5, 2017. Treatments were the same as fall 2016 but first applied four days post inoculation (October 9, 2017) and re-applied 16 days after the first application (October 25, 2017). Field plots were assessed for the percent of yellowed or damaged grass on a weekly basis from October 12, 2017 until November 11, 2017. Weather data retrieved from the Fergus, ON weather station which is the nearest station to the GTI (21 km) with precipitation data (http://climate.weather.gc.ca).

3.2.3.3 Spring 2018

The same isolates, setup, treatment applications, and analysis as the fall 2017 trials were used for spring 2018 with the exception that plots were set up on the ‘Range 10’ research plot area at the Guelph Turfgrass Institute which is composed of primarily *A. stolonifera* and

maintained at USGA standards. Inoculation of plots occurred April 24, 2018, and the first application of fungicide treatments was three days later on April 27, 2018. Damage rating (percent yellowing) occurred weekly beginning April 27, 2018 and ended May 18, 2018.

3.2.4 Statistical analysis

The percent yellowing of the plots was subjected to ANOVA using SAS PROC GLM, sorted by sensitivity group, with calculations of means and standard errors. Means were separated by Fisher's protected LSD (p = 0.05). An example of the SAS command statements is given in Appendix 3.1. Area under disease progress curves (AUDPC) were calculated for each sensitivity group and treatment using the formula: AUDPC = $\sum$ [(Yi+1 + Yi) x0.5][Ti+1 + Ti] (Sparks et al., 2008). AUDPC values were subjected to ANOVA using SAS PROC GLM, sorted by sensitivity group and treatment, with calculations of means and standard errors. Means were separated by Fisher's protected LSD (p = 0.05). Correlation analysis between growth on inhibitory concentrations of iprodione (10 µg mL^{-1}) and propiconazole (0.1 µg mL^{-1}) with the AUDPC were done for each treatment in each field trial.

3.3 Results

3.3.1 Isolate selection

Out of 296 isolates of *M. nivale* which were each assessed for sensitivity to propiconazole and iprodione, 12 isolates were selected for the 2016 field trial using representatives of established sensitivity categories (Section 2.3.2). For the 2017 field trial, 12 isolates were also used, however ten of the isolates differed from the 2016 trial and two isolates

were the same as the 2016 trial. The 2018 field trial utilized the same isolates as 2017. The lists of isolates selected for fall 2016, 2017 and 2018 trials can be found in Table 3.1.

3.3.2 Fall 2016

The temperature at the GTI during the fall of 2016 was typical, with an average maximum temperature of 12ºC and an average minimum temperature of 2ºC (Appendix 3.2). The total precipitation during the trial was approximately 110 mm with precipitation occurring on 31 days. Microdochium patch symptoms developed slowly, with little damage appearing during the first two weeks post-inoculation (October 12 to October 29, 2016). Symptoms (patches of yellowed / necrotic grass) grew larger every week beginning on the third rating date (November 11, 2016). Area under disease progress curves showed significant differences in the severity of disease caused by the different sensitivity groups in the absence of fungicides (Table 3.2). The fully sensitive group had an AUDPC of 1083 corresponding to a large amount of damage. At the end of the trial >60% of the plot area exhibited symptoms of Microdochium patch. The AUDPC of the propiconazole-resistant group was 418 and the double-resistant group had an AUDPC of 263 (LSD = 358, p = 0.05), which is significantly lower than the sensitive group. The propiconazole-resistant and double-resistant groups exhibited ~20% and ~10% damage to the plot areas by the end of the trial.

When the iprodione treatment was applied, the AUDPC of the fully sensitive group was significantly decreased relative to the no-fungicide treatment, with an AUDPC value of 363 (LSD = 452, p = 0.05); this corresponds to a damage reduction of 66%. The iprodione treatment also significantly decreased the incidence of disease for the propiconazole-resistant group with an AUDPC of 168 (LSD = 160, p = 0.05), with a comparable 60% reduction. Damage caused by

the double-resistant group was also reduced by the iprodione treatment with an AUDPC of 173 (LSD = 85, $p = 0.05$), a much lower damage reduction of 35%.

The propiconazole treatment also significantly reduced damage of the sensitive group with an AUDPC value of 238 (LSD = 452, $p = 0.05$), representing a reduction of 75%. Interestingly, damage from the propiconazole-resistant group was also significantly reduced with an AUDPC of 197 (LSD = 160, $p = 0.05$), with damage reduced by 53%. Alternatively, the propiconazole treatment did not significantly reduce damage from the double-resistant group with an AUDPC of 197 (LSD = 85, $p = 0.05$).

When both iprodione and propiconazole were applied, the results were similar to the propiconazole treatment. Damage from the fully sensitive group was reduced with an AUDPC of 335 (LSD = 452, $p = 0.05$), a 69% reduction. The propiconazole-resistant group was also reduced with an AUDPC of 229 (LSD = 160, $p = 0.05$), a 45% reduction. Finally, damage from the double-resistant group was not significantly decreased by the mixed fungicide treatment with an AUDPC of 245 (LSD = 85, $p = 0.05$). Appendix 3.3 contains AUDPC for individual isolates and replicates.

Correlation analysis between AUDPC and percent growth on the discriminatory concentration of iprodione (10 µg mL^{-1}) showed a significant correlation between damaged caused by isolates iprodione-sensitive and iprodione-resistant isolates in the absence of fungicides ($R^2 = 0.57$, $p = 0.04$). The AUDPC for the iprodione, propiconazole, and double-fungicide treatments showed little to no correlation with decreasing iprodione sensitivity with $R^2 = 0.24$ ($p = 0.21$), $R^2 = 0.06$ ($p = 0.5$), and $R^2 = 0.06$ ($p = 0.5$) respectively (Figure 3.2).

Correlation between AUDPC and percent growth on the discriminatory concentration of propiconazole (0.1 µg mL^{-1}) showed a significant correlation between damaged caused by

isolates propiconazole-sensitive and propiconazole-resistant isolates in the absence of fungicides (R^2 = 0.89, p = 0.0001). The correlation with AUDPC of the iprodione treatment was also significant with R^2 = 0.49 (p = 0.03). The AUDPC for the propiconazole and double-fungicide treatments showed lower correlation with decreasing propiconazole sensitivity with R^2 = 0.39 (p = 0.07) and R^2 = 0.30 (p = 0.13) respectively (Figure 3.3).

3.3.3 Fall 2017

The temperature during the fall of 2017 was similar to that of 2016 with an average maximum temperature of 10ºC and an average minimum of 1ºC (Appendix 3.4). The 2017 season experienced significantly more rainfall relative to the same time period in 2016. The total precipitation during the 2017 trial was approximately 191 mm over the course of 33 days. Microdochium patch symptoms progressed over the same time scale as the previous year. Symptoms developed slowly at first, with little damage appearing during the first two weeks post-inoculation. Symptoms (patches of yellowed or necrotic grass) became slightly larger every week beginning at three weeks post inoculation (October 17, 2017).

Area under disease progress curves showed significant differences in the severity of disease caused by the different sensitivity groups and fungicide treatments. In the absence of fungicides, the sensitive isolate group AUDPC was 600, while the AUDPC of the propiconazole-resistant, the iprodione-resistant, and double-resistant groups were 268, 368, and 364 respectively (LSD = 217, p = 0.05). All three treatments (iprodione, propiconazole, and iprodione with propiconazole) significantly reduced damage caused by the sensitive group of isolates with AUDPC values of 452, 389, and 421 respectively (LSD = 139, p = 0.05). The three

other sensitivity groups tested were not significantly affected by any of the three fungicide treatments. A summary of these results can be found in Table 3.3.

When analyzed separately, the double-resistant isolates behaved differently. In the absence of fungicides, two of three isolates, 15165 and 15170 (these two isolates may be the same genotype, Section 5.3.2) had very low damage with AUDPC values of 200 and 102 respectively. In contrast, isolate 15110 had a large AUDPC of 807 which was not significantly different from the sensitive isolates (LSD = 217, p = 0.05). None of the three fungicide treatments reduced the damage from any of the three double-resistant isolates. Appendix 3.5 contains AUDPC for individual isolates and replicates.

Correlation analysis between AUDPC and percent growth on the discriminatory concentration of iprodione (10 µg mL^{-1}) showed a significant correlation between damaged caused by isolates iprodione-sensitive and iprodione-resistant isolates in the absence of fungicides (R^2 = 0.43, p = 0.05). The correlation with AUDPC of the double-fungicide treatment was also significant with R^2 = 0.47 (p = 0.04). The AUDPC for the iprodione and propiconazole treatments showed low correlation with decreasing iprodione sensitivity with R^2 = 0.24 (p = 0.19) and R^2 = 0.21 (p = 0.22) respectively (Figure 3.2).

Correlation between AUDPC and percent growth on the discriminatory concentration of propiconazole (0.1 µg mL^{-1}) showed a significant correlation between damaged caused by propiconazole-sensitive and propiconazole-resistant isolates in the absence of fungicides (R^2 = 0.45, p = 0.05). The correlation with AUDPC of the iprodione and double-fungicide treatment were also significant with R^2 = 0.64 (p = 0.001) and R^2 = 0.68 (p = 0.04). The AUDPC for the propiconazole treatment showed lower correlation with decreasing propiconazole sensitivity with R^2 = 0.29 (p = 0.13) respectively (Figure 3.3).

3.3.4 Spring 2018

Disease pressure at the time of this trial was too low to produce significant amounts of disease from any of the isolates used. For this reason data from this trial was not useful and the trial was discarded from analysis.

3.4 Discussion

The results of the fall 2017 trial were inconclusive in terms of fungicide efficacy for the control of the lab-resistant isolates. In the absence of fungicides, the single-resistant and the double-resistant isolate groups caused significantly lower amounts of damage than the fully sensitive isolates. In fact, the resistant groups caused such low amounts of disease that detecting any fungicide treatment effects was difficult with the relatively high variability of the field environment. Although all three treatments effectively reduced the damage caused by the fully sensitive isolates, it is not certain whether the low amounts of damage in the fungicide treated lab-resistant plots was due to the treatments or the inherently low amounts of damage these isolates appear to cause.

There were a number of confounding factors with the fall 2016 trial. Firstly, the trial did not include isolates from the iprodione-resistant propiconazole-sensitive group. This was caused by contamination of the inoculum leading up to the trial; unfortunately there was not enough time to produce more inoculum. Additionally, when inoculum was applied to the plots, twice the standard amount of inoculum was used which caused larger amounts of disease than would be expected to occur naturally. Although there were some issues with the trial, the extra inoculum may have turned out to be a benefit. As the isolates from the fall 2017 trial did not produce enough damage to distinguish treatment effects, the larger amount applied during the 2016 trial

allowed distinctions in fungicide efficacy against lab insensitive isolates to be resolved. Again, all treatments effectively reduced damage caused by the fully sensitive isolates. However, in this experiment all three treatments significantly reduced the damage caused by the propiconazole-insensitive isolates. Considering the application rates were half the recommend concentration, it is extremely likely that full rates would fully control these lab-insensitive isolates. Additionally, isolates deemed insensitive to both iprodione and propiconazole in lab tests were effectively controlled with the iprodione treatment (half rate) but not the propiconazole (half rate) or the mixed treatment (quarter rates of both).

The results of the 2016 trial are somewhat inconsistent with those in previous literature. Generally, previous reports of fungicide efficacy for the control of lab-insensitive isolates showed inadequate or reduced disease suppression. Additionally, many of the previous experiments used full application rates, where half and quarter rates were used in this work. The 2016 trial suggests that in *M. nivale,* lab insensitivity may not correlate with field resistance in some cases. The different effects of propiconazole on the single-resistant and the double-resistant groups suggest that the mechanism of in vitro propiconazole-insensitivity is caused by different mutations, or that double-resistant isolates possess multiple additive mutations.

In both fall 2016 and 2017 trials, the fungicide treatments significantly reduced damage caused by the fully sensitive group of isolates. The very low amounts of damage caused by the resistant isolates were not significantly different from sensitive isolates treated with fungicides as shown by the correlation analysis (Figures 3.2 and 3.3). This suggests that fungicide-resistant *M. nivale* may be more prevalent than the literature suggests, but their inability to effectively cause disease masks their presence and could mitigate their importance in terms of disease

management programs. The results highlight the need to confirm insensitivity observed in lab tests with field experiments.

Ideally, resistance management strategies are implemented prior to resistance issues. These practices generally involve minimizing use to delay the onset of resistance. The Fungicide Resistance Action Committee (FRAC) makes a number of recommendations for use of dicarboximide and DMI fungicides in the field. Reducing total number of DMI applications is supported by several studies as an effective means of resistance management (Kuck, 1994; Steva, 1994; Engels and de Waard, 1996). In fact, FRAC suggests limiting DMI use to only the most critical times in a season. Similarly, they recommend dicarboximide use be restricted to only two or three applications per crop per season (Leroux, 2007). Most sampling locations from British Columbia appear to have exceeded FRAC recommendations for the use of dicarboximide and DMI fungicides (Table 2.2).

Resistance management strategies are designed to prevent the development of fungicide resistance rather than control a resistant population. For turfgrass managers dealing with an existing resistant population the control options are limited. By removing or limiting use of products containing iprodione or propiconazole sensitivity may gradually return in the population. This strategy is predicated on the hypothesis that fitness costs are associated with the mutations which confer resistance. When the selection pressure of the fungicide is removed, it is thought the resistant mutants will be debilitated allowing the sensitive isolates (no mutations) to outcompete the resistant mutants. The results from the field trials suggest that there may be a relationship between decreased sensitivity to iprodione and propiconazole with decreased virulence. Potential fitness costs associated with resistance to iprodione and propiconazole are explored in Chapter 4.

In the cases where fitness costs are not associated with resistance, products containing those fungicides may be permanently ineffective. In the analogous system of antibiotic-resistant bacteria, when fitness costs were found to be absent the management strategies were essentially limited to the use of medicines with different modes of action (Andersson and Hughes, 2010). Within a turfgrass system, if fitness costs are not present in fungicide-resistant isolates, the implementation of resistance-management strategies such as tank-mixing may provide increased control (Ishii and Hollomon, 2015). Additionally, cultural control methods such as lowering the soil pH and dew removal may be useful in reducing disease pressure, and as a consequence, reduce the need for fungicides (Smiley et al., 2005).

Table 3.1 *Microdochium nivale* isolates selected for fall 2016, 2017, and 2018 field trials and their respective sensitivities to iprodione and propiconazole. Isolates with >50% growth on percentage growth on a discriminatory concentration were deemed resistant.

Isolate	Trial Year		Sensitivity		Percentage growth on discriminatory concentration	
	2016	2017 and 2018	Iprodione	Propiconazole	Iprodione (10 µg mL^{-1})	Propiconazole (0.1 µg mL^{-1})
10179	Yes	No	Sens	Sens	0	23.4
11021	Yes	No	Sens	Sens	48.6	45.7
13265	Yes	No	Sens	Sens	7.1	35.5
15180	Yes	No	Sens	Sens	14	18.6
10082	Yes	No	Sens	Res	0	50.3
15138	Yes	No	Sens	Res	34.3	91.8
15139	Yes	No	Res	Res	87.9	64.6
15151	Yes	No	Res	Res	92.7	72.3
15171	Yes	No	Res	Res	92	74.1
15165	Yes	Yes	Res	Res	77.5	66.9
15181	Yes	Yes	Sens	Sens	9.5	15.3
12156	No	Yes	Sens	Sens	0	4.9
16169	No	Yes	Sens	Sens	0	0
10101	No	Yes	Sens	Res	0	64.6
12278	No	Yes	Sens	Res	0	59.2
15138	No	Yes	Sens	Res	34.3	91.8
11015	No	Yes	Res	Sens	56.3	43.8
12246	No	Yes	Res	Sens	63.4	44.3
15115	No	Yes	Res	Sens	81	40.9
15110	No	Yes	Res	Res	82.7	82.7
15170	No	Yes	Res	Res	93.9	73.7

Table 3.2 Area under disease progress curve (AUDPC) for each sensitivity group during fall 2016 field trial. Isolates which grew >50% on a discriminatory concentration in lab tests were deemed resistant and grouped together. Lower values indicate less damage over the whole season. Means were separated using Fisher's protected LSD ($p = 0.05$).

Treatment	**Disease severity (AUDPC) by sensitivity group**			**LSD for row ($p = 0.05$)**
	Ipro Sens Prop Sens	**Ipro Sens Prop Res**	**Ipro Res Prop Res**	
No fungicide	1083	418*	263*	358
Iprodione	363[¥]	168*[¥]	173*[¥]	103
Propiconazole	265[¥]	197[¥]	197	90
Ipro + Prop	335[¥]	229[¥]	245	185
LSD for Column ($p = 0.05$)	452	160	85	

AUDPC = $\sum [(Yi+1 + Yi) \times 0.5][Ti+1 + Ti]$ (Sparks et al., 2008)
*indicates significant difference from the full sensitive group within a treatment (row LSD)
[¥] indicates significant difference from the control treatment within a sensitivity group (column LSD)

Table 3.3 Area under disease progress curve (AUDPC) for each sensitivity group during fall 2017 field trial. Isolates which grew >50% on a discriminatory concentration in lab tests were deemed resistant and grouped together. Lower values indicate less damage over the whole season. Means were separated using Fisher's protected LSD ($p = 0.05$).

Treatment	**AUDPC in Fall 2017 by sensitivity group**				**LSD for row ($p = 0.05$)**
	Ipro Sens Prop Sens	**Ipro Sens Prop Res**	**Ipro Res Prop Sens**	**Ipro Res Prop Res**	
No fungicide	600	368*	268*	346*	217
Iprodione	452[¥]	192*	224*	303	177
Propiconazole	389[¥]	269	248	350	222
Ipro + Prop	421[¥]	308	228	357	207
LSD for Column ($p = 0.05$)	139	204	111	270	

AUDPC = $\sum$ [(Yi+1 + Yi) x0.5][Ti+1 + Ti] (Sparks et al., 2008)
*indicates significant difference from the full sensitive group within a treatment (row LSD)
[¥] indicates significant difference from the control treatment within a sensitivity group (column LSD)

Figures

Figure 3.1 Locations of field trials at the Guelph Turfgrass Institute.

Blue = Fall 2016, Orange = Fall 2017. (Source: Google Maps)

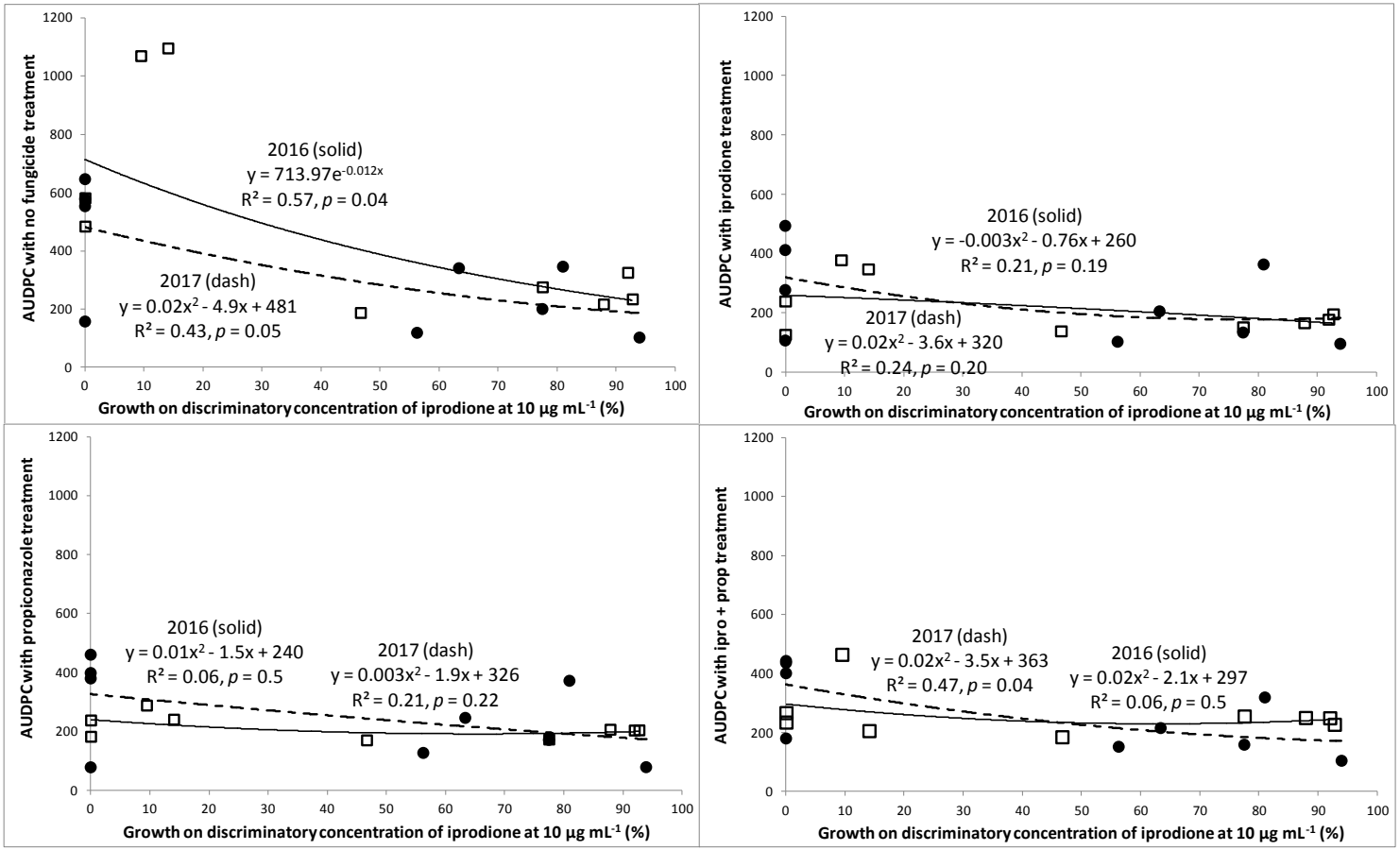


Figure 3.2 Correlation between percentage daily growth relative to non-amended PDA on discriminatory concentration of iprodione (10 µg mL^{-1}) and AUDPC for each of the four fungicide treatments (top left: no fungicides; top right: iprodione treatment 92.5 mL Rovral Green GT 100 m^{-2}; bottom left: propiconazole treatment 25.5 mL Banner MAXX 100 m^{-2}; bottom right: iprodione and propiconazole treatment: 46.25 mL Rovral Green GT and 12.75 mL Banner MAXX 100 m^{-2}) from fall 2016 (open squares) and fall 2017 (solid circles) field trials.

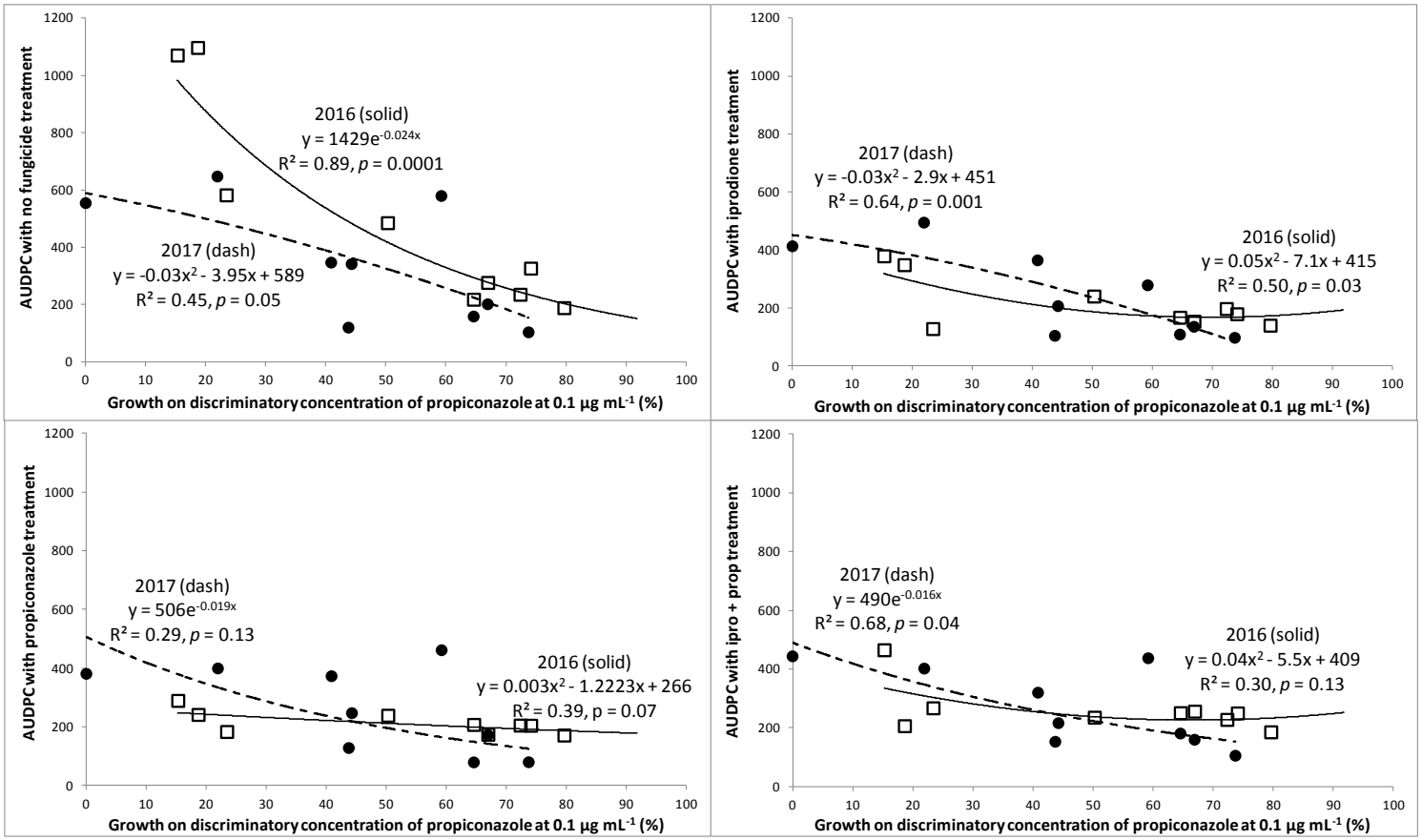


Figure 3.3 Correlation between percentage daily growth relative to non-amended PDA on discriminatory concentration of propiconazole (0.1 µg mL^{-1}) and AUDPC for each of the four fungicide treatments (top left: no fungicides; top right: iprodione treatment 92.5 mL Rovral Green GT 100 m^{-2}; bottom left: propiconazole treatment 25.5 mL Banner MAXX 100 m^{-2}; bottom right: iprodione and propiconazole treatment: 46.25 mL Rovral Green GT and 12.75 mL Banner MAXX 100 m^{-2}) from fall 2016 (open squares) and fall 2017 (solid circles) field trials.

Appendices

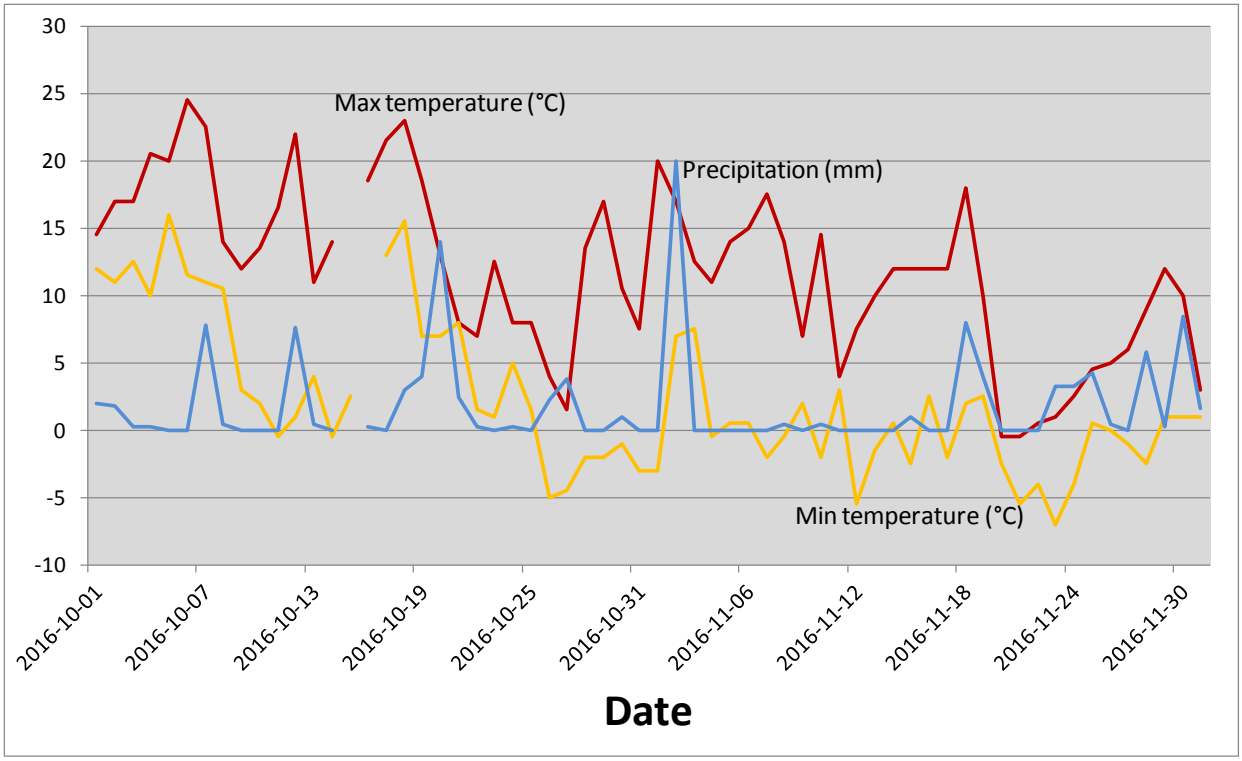
Appendix 3.1 Example of AUDPC SAS command statement.

```
data temp;
options pagesize=80 linesize=100;
infile cards;
input isolate TRT$ sens$ rep1 rep2 rep3 rep4;

  AUDPC=rep1; output;
  AUDPC=rep2; output;
  AUDPC=rep3; output;
  AUDPC=rep4; output;

cards;
10082 C ISPS 287.5 509.5 219.5 921.5
15139 C IRPR 238.5 331.5 131.5 167
15151 C IRPR 276 177.5 185 299.5
11021 P ISPR 257 101 236 89
15138 P ISPR 174.5 499.5 506 249
15139 P IRPR 195.5 139.5 299.5 193
15180 IP ISPS 153 381.5 122.5 165.5
15181 IP ISPS 119 1130.5 413.5 194
;
run;
data temp;
set;

run;
proc sort; by TRT;
proc glm;  by TRT;
class sens;
model AUDPC = sens;
lsmeans sens;
means sens / LSD lines;
output out=glmout;
run;
```



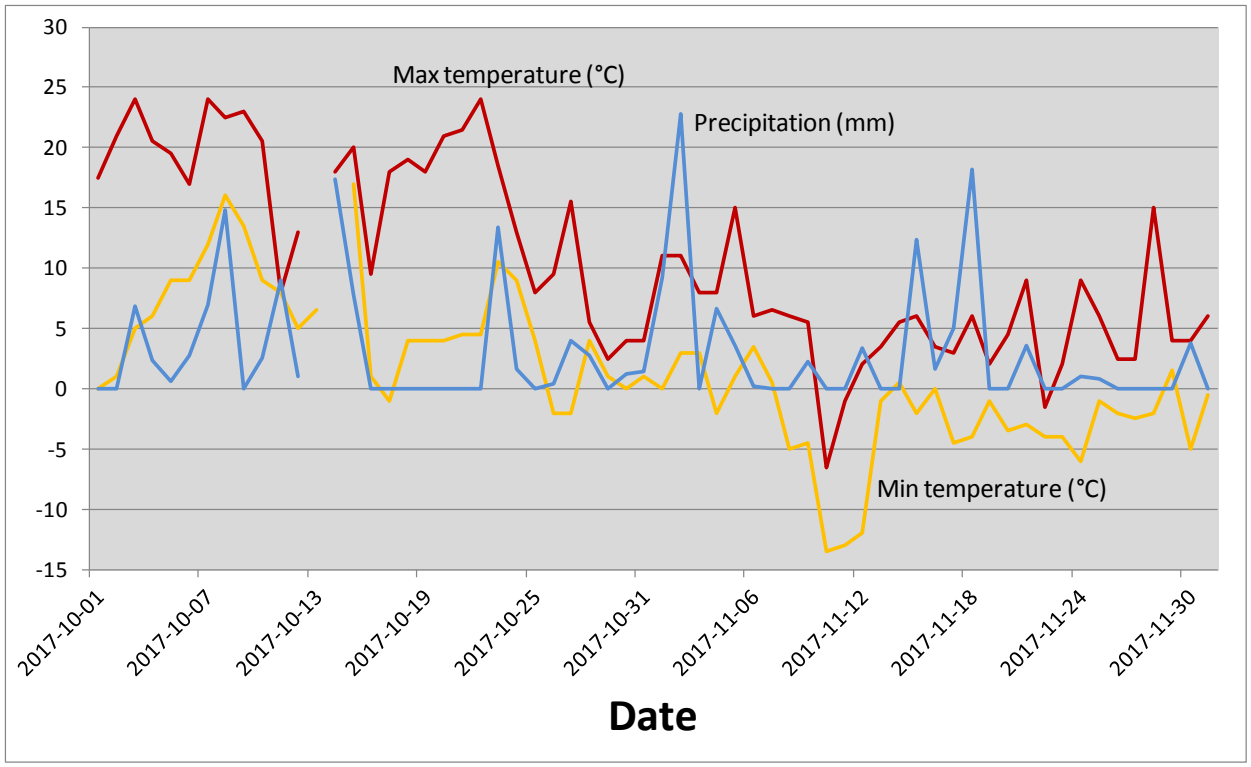
Appendix 3.2 Weather data for the fall 2016 trial (from www.climate.weather.gc.ca, Fergus MOE station, latitude = 43.7º, longitude = -80.38º).

Appendix 3.3 Area under disease progress curves for individual isolates during fall 2016 field trial. AUDPC = ∑ [(Yi+1 + Yi) x0.5][Ti+1 + Ti] (Sparks et al., 2008).

Isolate	Treatment	Sensitivity		Rep1	Rep2	Rep3	Rep4
		Iprodione	Propiconazole				
10082	No fungicide	Sens	Res	287.5	509.5	219.5	921.5
10179	No fungicide	Sens	Sens	954.5	241	201	930.5
11021	No fungicide	Sens	Sens	172	213.5	112	253.5
13265	No fungicide	Sens	Sens	199.5	327.5	126.5	155.5
15138	No fungicide	Sens	Res	1253	663.5	389	1989.5
15139	No fungicide	Res	Res	238.5	331.5	131.5	167
15151	No fungicide	Res	Res	276	177.5	185	299.5
15165	No fungicide	Res	Res	252.5	231.5	172	447.5
15171	No fungicide	Res	Res	261.5	348.5	147.5	545.5
15180	No fungicide	Sens	Sens	2184.5	1264.5	626.5	310.5
15181	No fungicide	Sens	Sens	1955	463.5	207.5	1654.5
10082	Iprodione	Sens	Res	133.5	481.5	238	104.5
10179	Iprodione	Sens	Sens	85	174.5	107	141.5
11021	Iprodione	Sens	Sens	216	122	111	106
13265	Iprodione	Sens	Sens	172	110.5	150	110
15138	Iprodione	Sens	Res	181.5	279	174.5	182
15139	Iprodione	Res	Res	145.5	211.5	181.5	126
15151	Iprodione	Res	Res	246.5	264	124	150
15165	Iprodione	Res	Res	125.5	250.5	130.5	103
15171	Iprodione	Res	Res	142.5	89.5	349.5	131
15180	Iprodione	Sens	Sens	394.5	698.5	150.5	147
15181	Iprodione	Sens	Sens	346.5	571.5	265.5	329.5
10082	Propiconazole	Sens	Res	526.5	93.5	178	155
10179	Propiconazole	Sens	Sens	147	230	223	131.5
11021	Propiconazole	Sens	Sens	257	101	236	89
13265	Propiconazole	Sens	Sens	207	100.5	361.5	114.5
15138	Propiconazole	Sens	Res	174.5	499.5	506	249
15139	Propiconazole	Res	Res	195.5	139.5	299.5	193
15151	Propiconazole	Res	Res	238	105.5	278	197
15165	Propiconazole	Res	Res	205	170.5	185	133.5
15171	Propiconazole	Res	Res	132.5	101.5	438.5	144
15180	Propiconazole	Sens	Sens	305	256.5	286.5	116
15181	Propiconazole	Sens	Sens	293	136	238	488
10082	Ipro + Prop	Sens	Res	116	151	486	185.5
10179	Ipro + Prop	Sens	Sens	201.5	515	169	181

11021	Ipro + Prop	Sens	Sens	131.5	320.5	127.5	160
13265	Ipro + Prop	Sens	Sens	261.5	341.5	134	289.5
15138	Ipro + Prop	Sens	Res	153	1320.5	183.5	117.5
15139	Ipro + Prop	Res	Res	162	620.5	120.5	97
15151	Ipro + Prop	Res	Res	110.5	586	122.5	89.5
15165	Ipro + Prop	Res	Res	203	498.5	158.5	160.5
15171	Ipro + Prop	Res	Res	189	226.5	156.5	424.5
15180	Ipro + Prop	Sens	Sens	153	381.5	122.5	165.5
15181	Ipro + Prop	Sens	Sens	119	1130.5	413.5	194

Treatment rates: no fungicide = 0 mL product 100 m^{-2}; iprodione = 92.5 mL Rovral Green GT 100 m^{-2}; propiconazole = 25.5 mL Banner MAXX 100 m^{-2}; ipro + prop = 46.25 mL Rovral Green GT and 12.75 mL Banner MAXX 100 m^{-2}.



Appendix 3.4 Weather data for the fall 2017 trial (from www.climate.weather.gc.ca, Fergus MOE station, latitude=43.7º, longitude=-80.38º).

Appendix 3.5 Area under disease progress curves for individual isolates during fall 2017 field trial. AUDPC = ∑ [(Yi+1 + Yi) x0.5][Ti+1 + Ti] (Sparks et al., 2008).

Isolate	Treatment	Sensitivity		Rep1	Rep2	Rep3	Rep4
		Iprodione	Propiconazole				
10101	No fungicide	Sens	Res	128.5	209	210	82
11015	No fungicide	Res	Sens	82	103	165	124
12156	No fungicide	Sens	Sens	837	750	332	382
12246	No fungicide	Res	Sens	389.5	392	385	195
12278	No fungicide	Sens	Res	585.5	792	552	384
15110	No fungicide	Res	Res	982	697	805	455
15115	No fungicide	Res	Sens	507	357	287	231
15165	No fungicide	Res	Res	128.5	205	237	230
15170	No fungicide	Res	Res	68	89	98.5	153
15181	No fungicide	Sens	Sens	937	497	407	744
16169	No fungicide	Sens	Sens	709.5	470	565	470
10101	Iprodione	Sens	Res	52	129	106	141
11015	Iprodione	Res	Sens	82	145	98.5	84.5
12156	Iprodione	Sens	Sens	514.5	975	597	230
12246	Iprodione	Res	Sens	197	387	154	82
12278	Iprodione	Sens	Res	264.5	388	307	148
15110	Iprodione	Res	Res	867	577	610	662
15115	Iprodione	Res	Sens	539.5	241	307	365
15165	Iprodione	Res	Res	112	167	120	136
15170	Iprodione	Res	Res	82	127	82	91
15181	Iprodione	Sens	Sens	657	460	362	495
16169	Iprodione	Sens	Sens	432	396	500	320
10101	Propiconazole	Sens	Res	66	82	82	82
11015	Propiconazole	Res	Sens	124	145	171	68
12156	Propiconazole	Sens	Sens	417	580	500	417
12246	Propiconazole	Res	Sens	264.5	442	133	144
12278	Propiconazole	Sens	Res	523	467	510	341
15110	Propiconazole	Res	Res	1070	812	697	627
15115	Propiconazole	Res	Sens	397	377	213	500
15165	Propiconazole	Res	Res	248	235	120	82
15170	Propiconazole	Res	Res	82	68	82	82
15181	Propiconazole	Sens	Sens	509.5	495	280	309
16169	Propiconazole	Sens	Sens	522	318	555	124
10101	Ipro + Prop	Sens	Res	205.5	186	245	82
11015	Ipro + Prop	Res	Sens	162.5	204	157	82
12156	Ipro + Prop	Sens	Sens	814.5	900	550	542

12246	Ipro + Prop	Res	Sens	289.5	235	171	165
12278	Ipro + Prop	Sens	Res	528	427	615	174
15110	Ipro + Prop	Res	Res	1052	605	885	690
15115	Ipro + Prop	Res	Sens	467	377	245	186
15165	Ipro + Prop	Res	Res	68	266	177	123
15170	Ipro + Prop	Res	Res	60	133	82	141
15181	Ipro + Prop	Sens	Sens	557	382	287	376
16169	Ipro + Prop	Sens	Sens	507	416	422	425

Treatment rates: no fungicide = 0 mL product 100 m^{-2}; iprodione = 92.5 mL Rovral Green GT 100 m^{-2}; propiconazole = 25.5 mL Banner MAXX 100 m^{-2}; ipro + prop = 46.25 mL Rovral Green GT and 12.75 mL Banner MAXX 100 m^{-2}.

Chapter 4 Fitness costs associated with insensitivity to iprodione or propiconazole

4.1 Introduction

4.1.1 Resistance and associated fitness costs

The term fitness is used to describe the survival and reproductive success of an individual, or the success of alleles and other genetic determinants (Pringle and Taylor, 2002). The incidence of drug-resistant bacteria in human pathogens has been steadily increasing as antibiotic use increases worldwide, often with unnecessary prescriptions and misuse by patients. As antibiotic resistance among bacteria is directly associated with human health, this area has been widely studied, including investigations of resistance-associated fitness costs (reviewed in Björkholm et al., 2001). Past experiments examining potential fitness costs in drug-resistant bacteria have measured exponential growth rates in the absence of drugs, for in vitro and in vivo competition (reviewed in Andersson and Hughes, 2010). When fitness costs are observed in laboratory experiments it was assumed that in clinical conditions, resistant strains would exhibit similar fitness reduction. However this has not been experimentally supported, there are studies indicating that in vitro fitness costs are not necessarily observed in vivo (reviewed in Sander et al., 2002), and fitness costs observed in clinical conditions may not be present under laboratory conditions (reviewed in Bjorkman et al., 2000). Additionally, multi-drug resistance often occurs to due changes in efflux transporters, and in competition experiments it has been noted that in vivo debilitation cannot necessarily be inferred from in vitro experiments (Sanchez, 2002).

Unlike in bacteria, cases of fungal resistance to fungicides have been found to involve fitness costs. Fungicides have a wide range of applications and are used extensively in many industries including human medicine and agriculture. Continuous applications of some fungicides which inhibit fungal populations may select for less sensitive individuals, and

eventually those chemicals could lose their efficacy (Ishii and Hollomon, 2015). Resistance can be conferred by genetic mutations which directly affect target sites (Délye et al., 1998), alter target-site gene expression (Ma et al., 2006), increase the expression of efflux transporters (Nakaune et al., 1998), or alter other biosynthetic pathways (Angelini et al., 2015). These mutations provide an advantage over fungicide-sensitive individuals in the presence of the chemicals, however, in the absence of fungicide, the respective mutations may reduce fitness (Pringle and Taylor, 2002), as has been found in a variety of studies (Appendix 4.1).

Despite the importance of fitness costs in resistance management, only a few fungal plant pathogens with fungicide-resistant populations have been investigated. There is evidence to suggest that fitness costs may be absent, low, or high depending on the particular fungus and fungicide involved (Mikaberidze et al., 2015). Differences in fitness costs could affect the selection of particular resistance management strategies, with absent and low fitness cost scenarios being more difficult to manage. Mutations which are harmful or deleterious in the absence of fungicides, may be maintained within a population if they are accompanied by other compensatory mutations, and management strategies may need to contend with these mutations as well (Cools et al., 2013). In human bacterial pathogens it has been suggested that compensatory mutations are much more likely to occur than sensitivity reversions (Andersson and Hughes, 2010). The Fungicide Resistance Action Committee (FRAC) has given each class of fungicides a rating associated with the risk of resistance development (Table 1.1).

In the past, the fitness of fungal plant pathogens has been measured using mycelial growth, spore production, pathogen virulence, and mixed inoculum competition tests (Zhan and McDonald, 2013). In these terms, a fungicide-resistance fitness cost is the difference in fitness values between a resistant strain and a representative sensitive strain in the absence of the

fungicide (Mikaberidze et al., 2015). Fitness costs associated with fungicide resistance are an important part of resistance management strategies (Zhan and McDonald, 2013). When sensitive and resistant strains compete in the field, fitness costs associated with gene mutations may determine the outcome. In this case, resistance genes could be maintained in the population indefinitely, permanently reducing the effectiveness of those fungicides. If fitness costs are present, however, then disease management strategies can be optimized to control the pathogen while minimizing the risk of future resistance development. As mentioned in the previous chapter, if fitness costs are absent in fungicide-resistant strains, those isolates may be fully competitive with sensitive strains as has been reported in some cases of benzimidazole resistance (Myresiotis et al., 2007), SDHI resistance (Lucas et al., 2015), or pyrimidine resistance (Bardas et al., 2008).

4.1.2 Fitness costs associated with dicarboximide resistance

There is strong evidence to support the existence of fitness costs with respect to dicarboximide resistance. It has long been established that iprodione-resistant fungi have increased osmotic sensitivity (Beever, 1983). *Botrytis cinerea* and *B. elliptica* with dicarboximide resistance have exhibited decreased mycelial growth, decreased sporulation, lower virulence, or lower rates of sclerotia survival (Katan, 1982; Hsiang and Chastagner, 1991; Pollastro et al., 1996; Raposo et al., 2000). Both *Neurospora crassa* and *Monilinia laxa* with dicarboximide resistance exhibited reduced sporulation, and such *M. laxa* isolates caused less damage to host fruit tissues (Katan and Shabi, 1982; Grindle and Temple, 1985). Some iprodione-resistant *Monilinia fructicola* isolates were found to have reduced virulence on peach fruit and produced fewer spores relative to sensitive strains (Elmer and Gaunt, 1994).

Dicarboximide-resistant *Sclerotinia minor* showed reduced virulence on lettuce (Hubbard et al., 1997). Similarly, dicarboximide-resistant *Rhizoctonia cerealis* was found to have reduced virulence on wheat (Hamada et al., 2011), and iprodione-resistant *Penicillium expansum* had reduced mycelial growth and reduced pathogenicity (Karaoglanidis et al., 2011). Previously identified iprodione-insensitive *M. nivale* were found to have decreased growth on non-amended PDA (Chastagner and Vassey, 1982).

Not all research has supported the hypothesis that dicarboximide resistance is necessarily associated with fitness costs. Although Raposo et al. (2000) reported decreased fitness in *B. cinerea* associated with dicarboximide resistance, other researchers have found no such link, with no observable fitness reduction observed in their iprodione-resistant isolates (e.g. Lamondia and Douglas, 1997; Cui et al., 2004; Oshima et al., 2006; Grabke et al., 2014). Different mutations may be responsible for resistance in different populations of *B. cinerea*, and thus different fitness costs could be present (Grabke et al., 2014). Brenneman et al., (1987) found no fitness costs associated with dicarboximide-resistant isolates of *S. minor*. However, Hubbard et al., (1997) did not support the findings of Brenneman et al., (1987) reporting a moderate virulence reduction in iprodione-insensitive *S. minor*. Finally, there were no fitness costs detected with iprodione-resistant *Alternaria alternata* (Hutton, 1988). Appendix 4.1 provides a summary on fitness costs that have been reported associated with dicarboximide resistance.

There is strong supporting data to suggest that the gene *os-1* is a major target of the dicarboximide fungicides (Dry et al., 2004; Avenot et al., 2005; Ma et al., 2007; Tanaka and Izumitsu, 2010). *Os-1* encodes a type III osmosensing-kinase which plays an important role for osmotic regulation in filamentous fungi (Schumacher et al., 1997; Wolanin et al., 2002).

Dicarboximide-resistant fungi which were found to have mutations in *os-1* homologs were shown to have increased sensitivity to osmotic stress (Pappas and Fisher, 1979; Beever, 1983; Grindle and Temple, 1985).The genes *os-2*, *os-4*,*os-5*, and *dic2* (*skn7*) are directly associated with *os-1* in the osmo-regulation pathway, and these genes have also been implicated in dicarboximide resistance and increased osmotic sensitivity (Fujimura et al., 2000; Zhang et al., 2002; Ochiai et al., 2007; Izumitsu et al., 2009).These findings suggest that in an osmotically-variable environment, such as in the field, fungi with mutations in these genes could be less fit than their sensitive counterparts.

4.1.3 Fitness costs associated with DMI resistance

There are many different mutations known to confer resistance to the various DMI fungicides, and any associated fitness costs are likely dependent on the particular mutations. Literature for propiconazole is similar to iprodione, in that there is support for both the existence of or the lack of fitness costs associated with DMI-resistant fungi. Fenpropimorph-insensitive isolates of the powdery mildew pathogen *Erysiphe gramini s*f.sp. *tritici* were less competitive then sensitive isolates in mixed inoculum tests, decreasing in population frequency over time (Engels and de Waard, 1996). Isolates of *S. homoeocarpa* with decreased sensitivity to propiconazole were associated with reduced virulence on creeping bentgrass at greens height (Hsiang et al., 1998). Tebuconazole-resistant *P. expansum* isolates were found to have reduced growth rates and reduced pathogenicity (Karaoglanidis et al., 2011). Thiabendazole-resistant *P. digitatum* produced smaller lesions than sensitive isolates on fruit (Kinay et al., 2007). The frequency of flutriafol-resistant *Cercospora beticola* isolates decreased while competing with sensitive strains, and the resistant isolates had reduced sporulation rates (Karaoglanidis et al.,

2001). Finally, epoxyconazole-resistant isolates of *Pyrenophora tritici-repentis* exhibited reduced growth rates on agar media (Reimann and Deising, 2005).

Research on propiconazole-resistant *Pyernophora teres* showed no correlation between resistance and various fitness metrics (Peever and Milgroom, 1994). Two separate groups showed propiconazole-resistant *M. fructicola* isolates had similar fitness to sensitive isolates in growth, sporulation, and ability to cause disease, although Cox et al. (2007) noted that the incubation period was delayed for their resistant isolates (Cox et al., 2007; Chen et al., 2013). Tebuconazole-resistant *Fusarium graminearum* isolates exhibited no competitive difference in mixed inoculum experiments (Spolti et al., 2014). Finally, tebuconazole and propiconazole-resistant *Rhynchosporium secalis* were found to cause the same amount of disease as sensitive isolates (Kendall et al., 1993). Appendix 4.1 provides a summary on fitness costs associated with DMI resistance.

*Cyp51*and its homologs such as *erg11* are the main target of DMI fungicides (Siegel, 1981; Köller, 1988). *Cyp51* encodes a cytochrome P450 enzyme, lanosterol-14α-demethylase, which is involved in ergosterol synthesis (Délye et al., 1998; Hamamoto et al., 2000; Wyand and Brown, 2005). Ergosterol is an essential component of fungal cell membranes, and many fungi cannot survive without it (Hollomon et al. 1990; Weete et al. 2010). Alteration of *cyp51* has been associated with DMI resistance many times in many species (Appendix 2.6).Target-site alteration could create a less efficient metabolic pathway for ergosterol production, increasing energy requirements. Alternatively, target site alterations may be combined with compensatory mutations involving alternate metabolic pathways, which may be less efficient, or which use resources normally dedicated to other processes.

Resistance to the dicarboximide and the DMI fungicides may be conferred through gene expression alteration. Increased expression of the target site (*cyp51*) has been documented for DMI-resistant fungi (Schnabel and Jones, 2001), and increased expression of efflux transporters has been reported as a cause of resistance to both fungicide groups (Sang et al., 2015). Higher levels of expression may require a significantly larger resource input, potentially lowering the fitness in other areas.

If fitness costs are absent in fungicide-resistant strains of *M. nivale*, those isolates maybe fully competitive with sensitive strains. This has important implications for disease management and resistance management practices implemented by turfgrass managers. Resistant and full competitive isolates, in theory, will persist in the population resulting in a permanent loss of control products.

The objectives of this chapter were:

(1) to assess if iprodione-resistant and propiconazole-resistant isolates differ from fully sensitive isolates in the following:

(a) growth rate and biomass production on PDA; and

(b) ability to cause disease in the field.

4.2 Methods

4.2.1 Isolate selection

Samples of *M. nivale* were collected from various locations in Ontario and British Columbia (Section 2.3.1). Fungal isolations from the raw samples were done using a solution of dilute hypochlorite, and isolates were stored in wheat seed tubes at -20°C (Section 2.2.2). Based

on results from the iprodione and propiconazole discriminatory concentration testing, isolates were placed into one of four sensitivity groups for field tests (Section 2.3.3): sensitive to both iprodione and propiconazole, resistant to both iprodione and propiconazole, resistant to only iprodione, or resistant to only propiconazole. Isolates with greater than 50% growth on a discriminatory concentration (compared to non-amended media) were deemed insensitive or resistant to that fungicide. All 262 isolates from sensitivity tests were included in growth rate analysis, 22 were selected for biomass testing using representatives of the four sensitivities categories. Finally, 12 isolates with representatives from each sensitivity group were chosen for each of the 2016 and 2017 field trials (Table 3.1).

4.2.2 In vitro growth rate in the absence of fungicides

Full details for the methods of the strip-agar assay used to collect growth rate data can be found in Section 2.2.3.2. Briefly, 5-mm plugs of mycelia were placed on 1-cm wide strips of PDA and left to growth for 48 hr. Radial growth was then marked and isolates were incubated for a further 48 hr at 20°C, and radial growth marked again. Daily growth rates was calculated by measuring the distance between radial marks representing the 48 hr growth, and dividing by two. Growth rates on non-amended PDA from both the full EC_{50} tests (Section 2.3.2) and the discriminatory concentration tests (Section 2.3.3) were compiled and treated as experimental replicates. Growth measurements from 246 isolates were used in the analysis. These data were subjected to ANOVA using SAS PROC GLM, sorted by sensitivity group, with calculations of means and standard errors. Means were separated by Fisher's protected LSD (p = 0.05). An example of the SAS command statement is given in Appendix 4.2. Regression analysis was used

to assess the correlation between the percentage growth on discriminatory fungicide concentration and the daily growth rate in the absence of fungicides.

4.2.3 Biomass in the absence of fungicides

Glass tubes (15-cm long with screw caps) containing 20 mL PDA were autoclaved. Lids were placed on tightly and tubes placed horizontally on a flat surface, allowing media to solidify while covering the full length of each tube. Tubes were lightly flamed at the cap-tube junction prior to opening, and excess agar was removed from the lip of tube. Plugs of mycelia 5-mm in diameter were taken from the actively growing edge of select isolates grown on PDA, and placed hyphae side down inside the tube opening. Caps were loosely screwed on and sealed with parafilm. Inoculated tubes were placed in plastic boxes and incubated at 20°C until harvesting.

Tubes were deemed ready to harvest when 90% of the tube length was covered in mycelium. Large batches of tubes were stored at -20°C until harvested. Water (10 mL) was added to tubes to help dissolve media, tubes were placed in a beaker filled with water to allow even heating, and microwaved in 60 second intervals until media was completely molten (bubbling). Filter paper was weighed prior to use (Whatman #1 5-cm-diameter) and a 125 mL vacuum flask with a ceramic filter (model 60239 from CoorsTek, Golden, CO) was lined with filter paper. Tubes were then poured through ceramic-filter and filter paper (attached to vacuum pump) leaving behind a mass of mycelia. Filter paper was folded over and carefully removed from ceramic filter dried in an oven for 24 hours at ~70°C. Paper containing mycelia was then weighed and the pre-use weight was subtracted to calculate a final mycelial dry weight. The final weight was divided by the number of growing days to calculate mass day^{-1}, with three replicates. The experiments were repeated twice. Significant differences between the means were checked

using analysis of variance (ANOVA), and means separated using Fisher's protected least significant difference (LSD).

4.2.4 Virulence in field tests

Details on field trial setup and execution can be found in Section 3.2.3. Briefly, experiments were conducted at the Guelph Turfgrass Institute (GTI) on greens maintained to USGA standards. Each experiment contained four blocks (replicates) composed of 0.25m^2 plots. Plots were inoculated with select isolates using wheat seed inoculum at a rate of 10 g m^{-2}. Damage was assessed by rating yellowed area of individual plots on a weekly basis. Data from the inoculated control plots (i.e. no fungicides) from field trials in fall 2016 (Appendix 3.1) and fall 2017 (Appendix 3.2), were compiled. The percent yellowing of the plots was subjected to ANOVA using SAS PROC GLM, sorted by sensitivity group, with calculations of means and standard errors. Means were separated by Fisher's protected LSD (p = 0.05). An example of the SAS command statement is given in Appendix 4.3. Area under disease progress curves (AUDPC) were calculated for each sensitivity group in the absence of fungicides using the formula: AUDPC = $\sum$ [(Yi+1 + Yi) x0.5][Ti+1 + Ti] (Sparks et al., 2008). AUDPC values were subjected to ANOVA using SAS PROC GLM, sorted by sensitivity group, with calculations of means and standard errors. Means were separated by Fisher's protected LSD (p = 0.05).

4.2.5 Principal component analysis

The AUDPC for each of the four treatments for both 2016 and 2017 isolates were compiled along with the percentage growth on the discriminatory tests of iprodione and propiconazole (Section 2.3.3), and growth on PDA in the absence of fungicides were compiled.

These data were subject to principal component analysis (PCA) using SAS PROC PRINCOMP and sorted by sensitivity group. The two principal components which accounted for the largest variation between isolates were plotted to determine if isolates with similar fungicide sensitivities clustered. The contribution of each variable (e.g. growth on PDA) to the variation of the largest principal components (PRIN1 and PRIN2) was calculated by squaring the eigenvector of each contributing variable and multiplying by 100 to achieve a percentage. An example of the SAS statements is given in Appendix 4.4.

4.3 Results

4.3.1 Isolate selection

All isolates used in the discriminatory sensitivity tests has been used in the analysis of growth rate (Section 2.2.3.3, Table 2.1), and those data from non-amended media were used in this test. The isolates selected for the 2016 and 2017 field trials can be found in Table 3.1. Finally, a list of isolates selected for inclusion in the biomass testing can be found in Table 4.1, as well as their biomass production rates.

4.3.2 Growth rate in the absence of fungicides

A distribution graph of daily growth on non-amended PDA for 149 fully sensitive isolates showed an average of 6.9 mm per day with the 8.5 mm bin (containing isolates ranging between 8.25 mm to 8.75 mm) containing the largest number of isolates (25) (Figure 4.1 and Table 4.2). The propiconazole-resistant group (40 isolates) had a significantly lower group average of 5.7 mm per day (p = 0.05) with the 6 mm bin (between 5.75 mm to 6.25 mm) containing the highest number of isolates (10) (Figure 4.1). The iprodione-resistant group had a

significantly lower average growth rate of 5.7 mm per day (p = 0.05), however the distribution graph for the iprodione-resistant group contained too few isolates (7) to make meaningful inferences (Figure 4.1).The double-resistant group (61 isolates) had an average of 7 mm per day (Figure 4.1), which was not significantly different from the sensitive group. The double-resistant group had the highest number of isolates (17) in the 7.5 mm bin (between 7.25 mm to 7.75 mm).

Regression analysis between growth on iprodione (10 µg mL^{-1}) and growth on non-amended PDA showed a low correlation (R^2 = 0.01, p = 0.07). When propiconazole-resistant isolates (including all double-resistant isolates) were excluded, only 2.7% (p = 0.05) of the variation in daily growth on non-amended PDA could be explained by decreased sensitivity to iprodione (Figure 4.2). Regression analysis between percentage growth on propiconazole (0.1 µg mL^{-1}) and daily growth on non-amended PDA showed a modest correlation (R^2 = 0.13, p = 0.05). When iprodione-resistant isolates (including double-resistant isolates) were excluded, 16.8% (p = 0.05) of the variation in daily growth on non-amended PDA could be explained by decreased sensitivity to propiconazole (Figure 4.3).

4.3.3 Biomass in the absence of fungicides

There were no significant differences between biomass productions for sensitive vs. resistant grouping of isolates (p > 0.5). On non-amended PDA, isolates which were sensitive to both iprodione and propiconazole produced an average 2.9 mg of mycelium per day (Table 4.2). Isolates which were resistant to only iprodione produced 3.0 mg of mycelium per day. Isolates resistant to only propiconazole grew 2.5 mg of mycelium per day. Isolates which were resistant to both iprodione and propiconazole grew 3.5 mg of mycelium per day. Growth rates varied

widely between replicates within experiments, as well as between experiments, and no significant differences were detected between the different sensitivity groups.

4.3.4 Virulence in field tests

4.3.4.1 Fall 2016

Isolates were previously grouped into fungicide sensitivity categories based on growth on discriminatory concentrations of iprodione and propiconazole with at least two isolates per group (Table 3.1). Area under disease progress curves showed significant differences in the severity of disease caused by the different sensitivity groups. In the absence of fungicides, the sensitive isolate group AUDPC was 1083, while the AUDPC of the propiconazole-resistant group was 418 and the double-resistant group both had AUDPCs of 263 (LSD = 358, p = 0.05). Regression analysis between increased growth on the discriminatory concentration of iprodione (10 µg mL^{-1}) and decreased damage in the field (AUDPC) yielded an R^2 value of 0.49 (p = 0.03) (Figure 3.2). The regression between increased growth on the discriminatory concentration of propiconazole (0.1 µg mL^{-1}) and decreased damage in the field yielded an R^2 value of 0.89 (p = 0.0001) (Figure 3.3).

The fully sensitive group caused ~49% and ~60% damage on the final two rating dates before snow cover (November 25 and December 01, 2016) (Figure 4.4) in the absence of fungicide treatments. The propiconazole-resistant isolates caused ~12% and ~17% damage on the same dates, which was significantly less than the fully sensitive isolates (p = 0.05). As a group, the double-resistant isolates caused an average of ~6% damage on the same dates which was significantly lower than the fully sensitivity group as well as the propiconazole-resistant group (p = 0.05).

4.3.4.2 Fall 2017

Isolates were grouped into sensitivity/resistance categories based on growth on discriminatory concentrations with at least two isolates per group (Table 3.1). Area under disease progress curves showed significant differences in the severity of disease caused by the different sensitivity groups. The sensitive isolate group AUDPC was 600, while the AUDPC of the propiconazole-resistant group and the iprodione-resistant group were 268 and 368 respectively. The double-resistant group had an AUDPC of 364 (LSD = 217, p = 0.05). Regression analysis between growth on the discriminatory concentration of iprodione (10 µg mL^{-1}) and damage in the field (AUDPC) yielded an R^2 value of 0.43 (p = 0.05) (Figure 3.2). Regression between growth on the discriminatory concentration of propiconazole (0.1 µg mL^{-1}) and damage in the field yielded an R^2 value of 0.45 (p = 0.05) (Figure 3.3).

The fully sensitive group caused ~24% damage on the final two rating dates before snow cover (November 15 and November 25, 2017) (Figure 4.5). The iprodione-resistant isolates caused ~12 % and 9% on the same dates, which was significantly lower than the sensitive isolates (p = 0.05). The propiconazole-resistant isolates caused ~15% and ~18% damage on those dates, which was significantly less than the fully sensitive isolates but not significantly different from the iprodione-resistant group. The double-resistant isolates caused ~15% and ~17% damage on the two dates, and were significantly lower than the sensitive isolates. When the three double-resistant isolates (15110, 15165, and 15170) were examined individually, they were observed to cause significantly different amounts of damage (Figure 4.6). Isolates 15165 and 15170, both from location BC-3, caused very low damage (~5% each) throughout the trial. Isolate 15110, from location BC-1, caused a large amount of damage, approaching 45% at the end of the season which was not significantly different from the sensitive isolates.

4.3.5 Principal component analysis of the variables associated with fungal fitness and fungicide resistance

Plotting the principal component analysis loadings PRIN1 (principal component 1) and PRIN2 (principal component 2) separated the isolates into two distinct clusters of fully sensitive isolates and isolates deemed resistant to both iprodione and propiconazole (Figure 4.7). Component 1 contributed ~42% to the overall variance between isolates based on the input variables (Section 4.2.5), and component 2 contributed ~25% to the variation between isolates. The variable which contributed the highest variance to PRIN1 was the AUDPC values from field plots treated with a combination of iprodione and propiconazole with a contribution of 25.4%. The iprodione treatment AUDPC contributed 25.1% to the variance of PRIN1, propiconazole AUDPC contributed 20.5%, and AUDPC untreated contributed 18%. The in vitro variables of percentage growth on the discriminatory concentrations of iprodione and propiconazole contributed 5.6% and 5.2% respectively. Finally, growth on PDA in the absence of fungicides only contributed 0.2% to the variance.

4.4 Discussion

Fitness testing of sensitive and fungicide resistant isolates has revealed costs associated with resistance to iprodione and propiconazole in *M. nivale*. The iprodione-resistant group of isolates had a growth reduction of 17% on PDA, which is in line with Chastagner and Vassey (1982), whose iprodione-resistant *M. nivale* grew 15% slower on PDA. Similar results were also reported for iprodione-resistant *B. elliptica*, which grew 20% slower than sensitive isolates in the absence of fungicides (Hsiang and Chastagner, 1991). One study which supported the presence of fitness costs in iprodione-resistant *B. cinerea* reported a growth rate reduction between 25%

and 30% (Katan and Shabi, 1982). The propiconazole-resistant group of isolates also showed a growth reduction of 17% relative to sensitive isolates. At the time of writing, only a single article has reported a significant decrease in vegetative for DMI-resistant isolates. Epoxyconazole-resistant *Erysiphe graminis* f.sp. *tritici* isolates characterized by Reimann and Deising (2005) grew 70% slower than their sensitive isolates, which is a much greater reduction than that seen in *M. nivale*. Considering both single-resistant groups had exhibited a reduction in vegetative growth, it was expected that the double-resistant group would be even more debilitated. However, this was not the case here. For example, DMI-resistant *P. expansum* was not significantly different from sensitive isolates, but isolates resistant to DMI, iprodione, and/or fludioxonil grew between 15% and 40% slower than sensitive isolates (Karaoglanidis et al., 2011). Isolates of *M. nivale* resistant to both iprodione and propiconazole showed no reduction in vegetative growth relative to the sensitive group.

The time consuming nature and poor sensitivity of the biomass tests made them difficult to repeat and there were no observable differences between sensitivity groups in the biomass tests. These results could be explained by the wide variations within experiments and the small weights involved, which are likely to have obscured any biomass differences present. As a result, these tests are unlikely to have yielded meaningful results.

Field tests revealed differences in the virulence of different sensitivity groups. The iprodione-resistant group was not included in the fall 2016 trials due to unsuccessful inoculum production. However, in the fall 2017 trial, iprodione-resistant isolates caused close to half the amount (~40%) of damage that the sensitive group in the absence of fungicides did. The propiconazole-resistant group had reduced virulence in field seasons, causing ~60% and ~55%

less damage than sensitive isolates. Similarly, isolates resistant to both iprodione and propiconazole in lab tests caused ~75% and ~40% less damage than sensitive isolates.

Unfortunately, there are few examples in the literature which include the testing of virulence under field conditions for direct comparisons. However, some comparisons can be made to *in vitro* pathogenicity tests. Hsiang et al. (1991) reported that dicarboximide-resistant *B. elliptica* exhibited a ~35% reduction in lesion size and colonization relative to sensitive strains. Dicarboximide-resistant *R. cerealis* reportedly lost their ability to infect their wheat hosts, which could be considered 100% damage reduction (Katan and Shabi, 1982). The iprodione-resistant *M. fructicola* of Elmer and Gaunt (1994) caused 15% less blight to peach flowers. Hsiang et al. (1998) reported a relationship between increased propiconazole resistance and reduced virulence in *S. homoeocarpa*. They showed that 20% (p = 0.05) in the variation of AUDPC could be explained by EC_{50}, with higher propiconazole EC_{50} values being correlated with decreased AUDPC. Regressions between AUDPC and growth on discriminatory concentrations of iprodione and propiconazole yielded higher associations in both F16 and F17 trials, all >40%, with higher insensitivity correlated with decreased AUDPC.

Although the double-resistant group was not significantly different from the sensitive isolates in terms of vegetative growth, they caused significantly less damage than the sensitive isolates in both field seasons. Additionally, they caused less damage than the propiconazole-resistant isolates in 2016, although not in 2017. Within the 2017 trial, when the isolates were looked at individually rather than as a group, a single isolate (15110) caused a large amount of damage while the other two caused very little damage. This supports the possibility that different mutations can cause fungicide resistance in *M. nivale* or indicates the presence of additional compensatory mutations. This hypothesis is explored in Chapter 5.

Tables

Table 4.1 Biomass production per day for isolates with different sensitivities to iprodione and propiconazole grown on PDA. Res = resistant to that fungicide, Sens = sensitive to that fungicide, based on percentage growth on discriminatory concentrations where isolates with >50% were deemed resistant.

Accession	Sensitivity		Dry weight (mg day^{-1})	Growth on discriminatory concentration	
	Iprodione	Propiconazole		Iprodione (10 µg mL^{-1})	Propiconazole (0.1 µg mL^{-1})
10082	Sens	Res	1.88	0.0	56.1
10106	Sens	Res	3.14	0.0	72.5
10178	Sens	Sens	1.75	0.0	7.4
10179	Sens	Sens	3.84	0.0	23.4
11015	Res	Sens	3.18	56.3	43.8
11021	Res	Sens	2.77	50.7	49.9
12063	Sens	Sens	2.98	5.8	2.1
12143	Sens	Res	2.25	35.7	65.9
13407	Sens	Sens	3.01	1.6	23.3
13408	Sens	Sens	2.70	20.8	22.0
15138	Sens	Res	2.83	36.2	80.9
15139	Res	Res	3.24	87.7	66.2
15151	Res	Res	3.78	92.6	72.2
15157	Sens	Sens	3.17	4.9	11.9
15165	Res	Res	3.09	77.7	67.1
15169	Res	Res	3.41	84.3	77.0
15171	Res	Res	3.50	93.2	73.5
15172	Res	Res	2.94	83.0	65.2
15173	Res	Res	3.88	87.7	71.9
15179	Sens	Sens	2.54	14.6	13.1
15180	Sens	Sens	3.16	14.5	18.7
15181	Sens	Sens	3.15	9.4	15.3

Table 4.2 Summary of data on fitness costs associated with resistance to the fungicides iprodione and propiconazole in *Microdochium nivale*. An asterisk (*) indicates significant difference from the fully sensitive group based on ANOVA tests (p = 0.05). AUDPC = $\sum$ [(Yi+1 + Yi) x0.5][Ti+1 + Ti] (Sparks et al., 2008).

Sensitivity	Growth (mm day^{-1})	Dry weight (mg day^{-1})	AUDPC	
			Field 2016	Field 2017
Fully sensitive	6.9	2.9	1083	600
Iprodione-resistant	5.7*	3.0	--	268*
Propiconazole-resistant	5.7*	2.5	418*	367*
Double-resistant	7.0	3.5	263*	346*

Figures

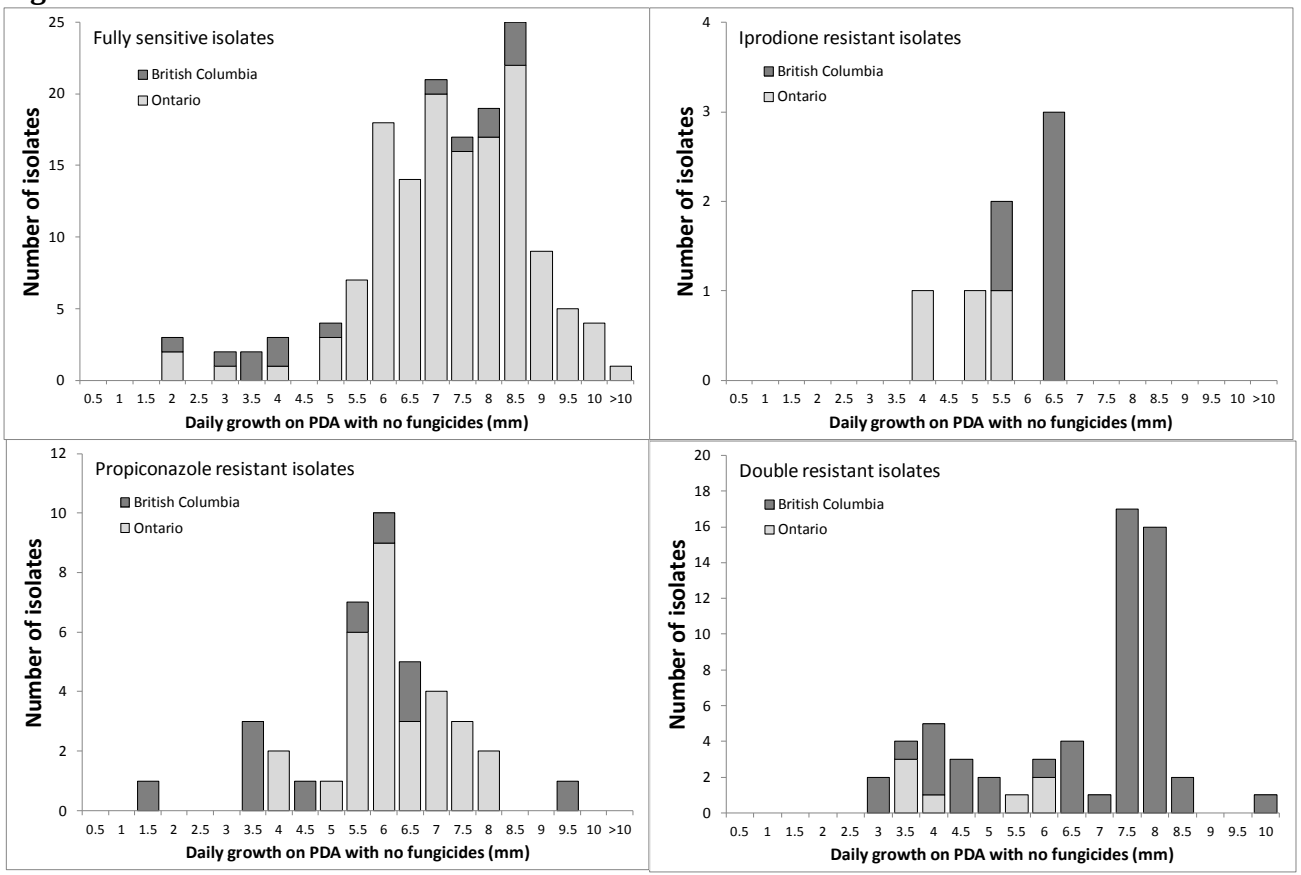


Figure 4.1 Distribution of daily growth rates (mm) on non-amended PDA of *M. nivale* isolates with different fungicide sensitivities. Sensitivity based on percentage growth on discriminatory concentrations where isolates with >50% were deemed resistant. (Top left: fully sensitive isolates; top right: iprodione-resistant; bottom left: propiconazole-resistant; bottom right: resistant to both iprodione and propiconazole)

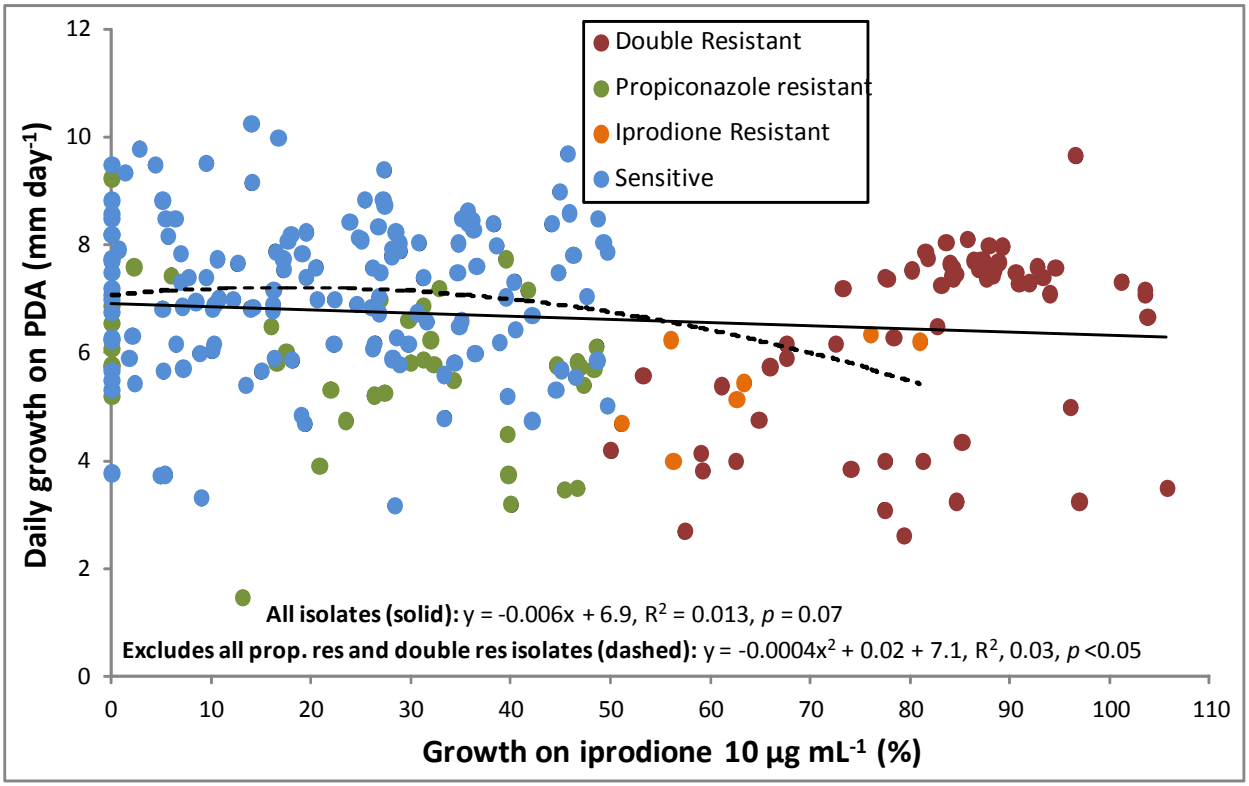


Figure 4.2 Daily growth in the absence of fungicides (non-amended PDA) as a function of percentage growth on a discriminatory concentration of iprodione (10 µg mL^{-1}) where resistant isolates grew >50% on PDA amended with the discriminatory concentration.

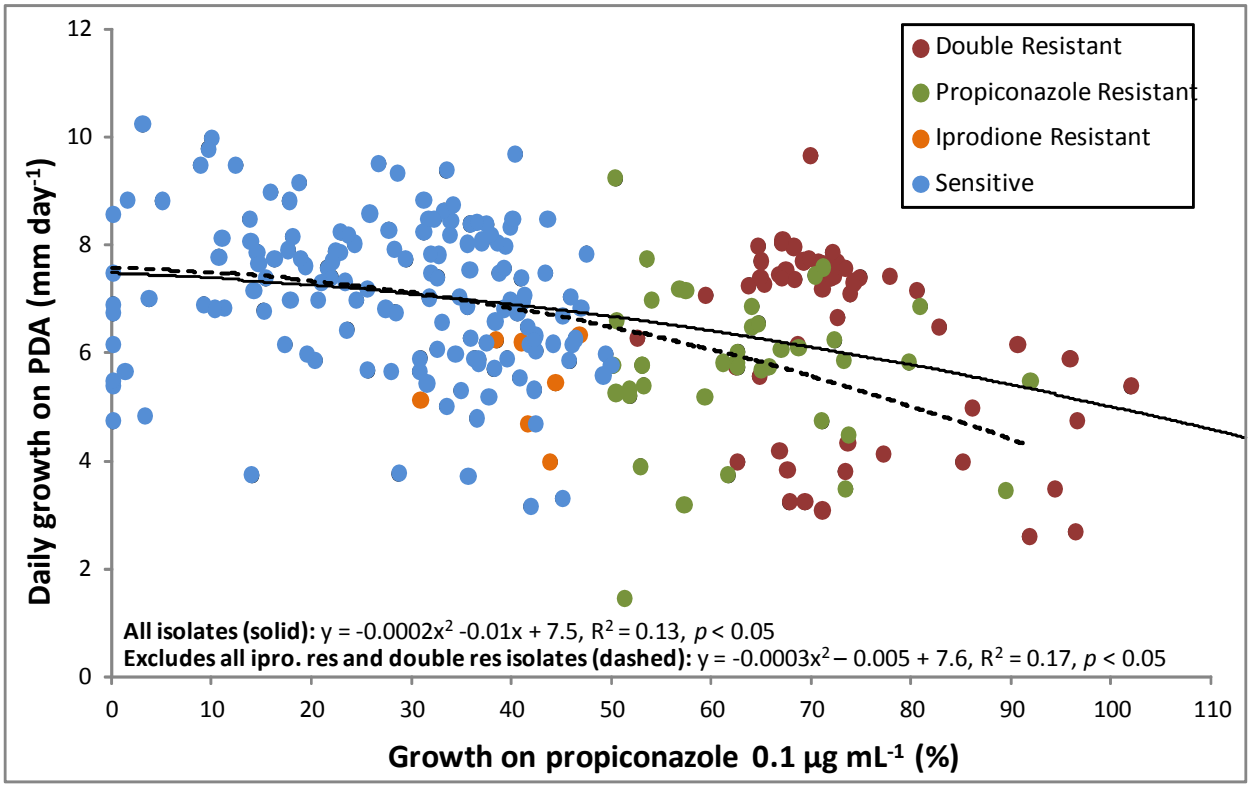


Figure 4.3 Daily growth in the absence of fungicides (non-amended PDA) as a function of percentage growth on a discriminatory concentration of propiconazole (0.1 µg mL^{-1}) where resistant isolates grew >50% on PDA amended with the discriminatory concentration.

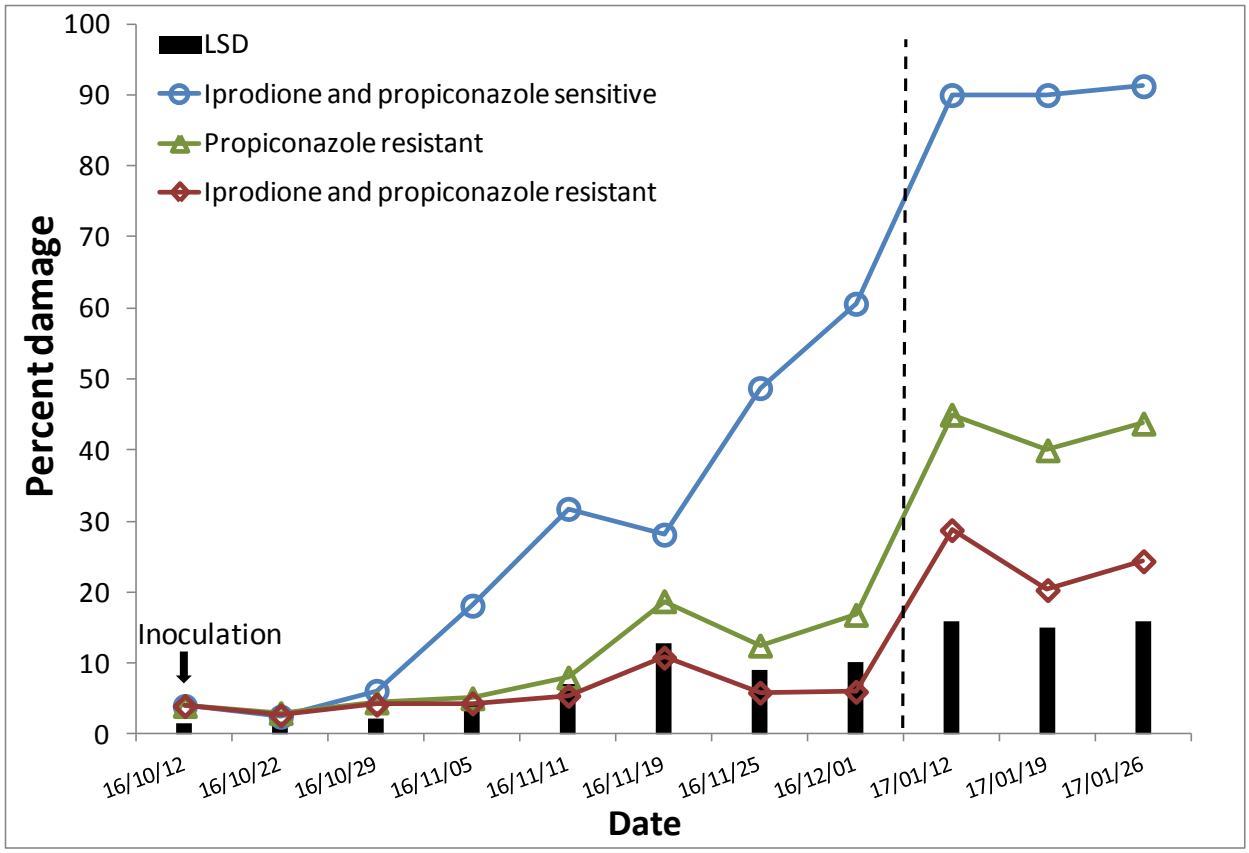


Figure 4.4 The average percent damage to *A. stolonifera* without any fungicide treatments when inoculated by *M. nivale* isolates having different sensitivities to iprodione and propiconazole during fall 2016 field trial. Least significant differences are shown for each date based on ANOVA (p = 0.05). Vertical dashed line indicates period of snow cover.

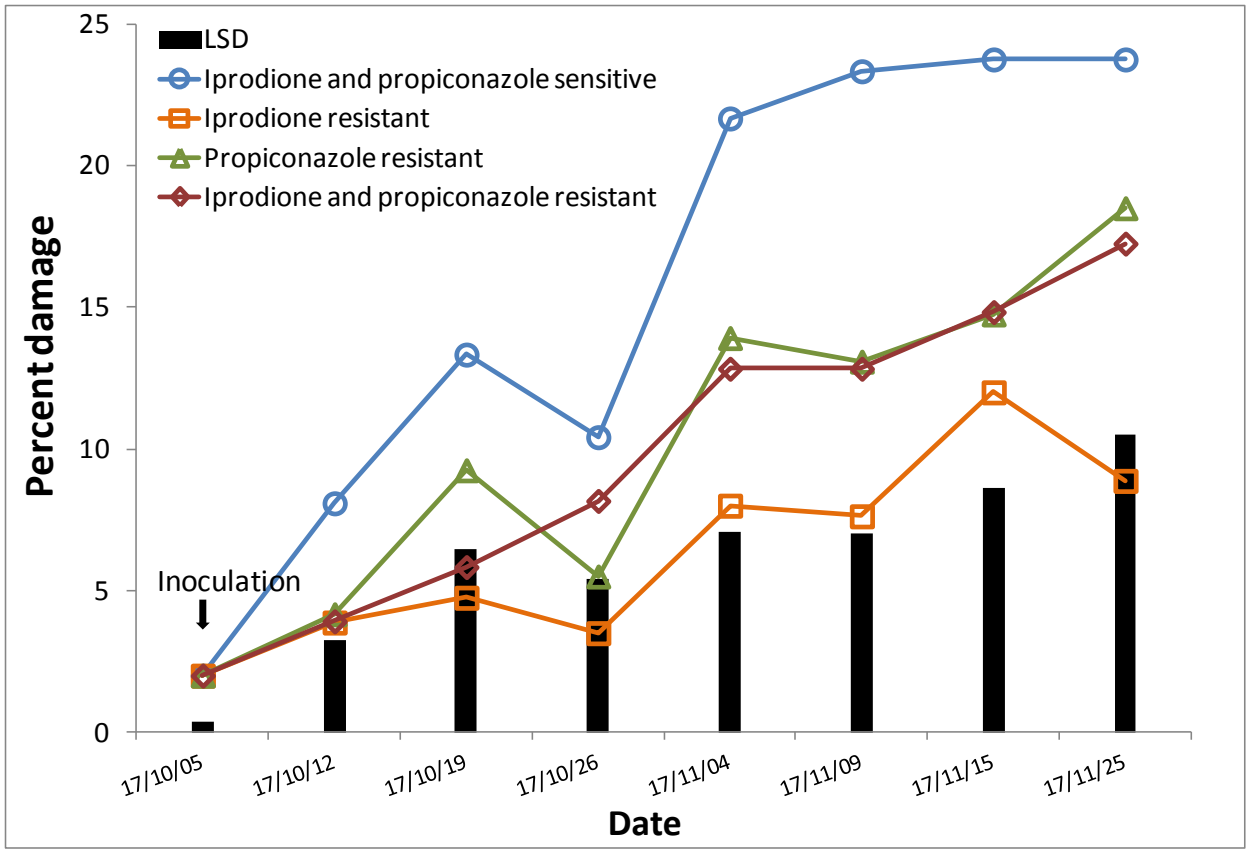


Figure 4.5 The average percent damage to *A. stolonifera* without any fungicide treatments when inoculated by *M. nivale* isolates having different sensitivities to iprodione and propiconazole during fall 2017 field trial. Least significant differences are shown for each date based on ANOVA (p = 0.05).

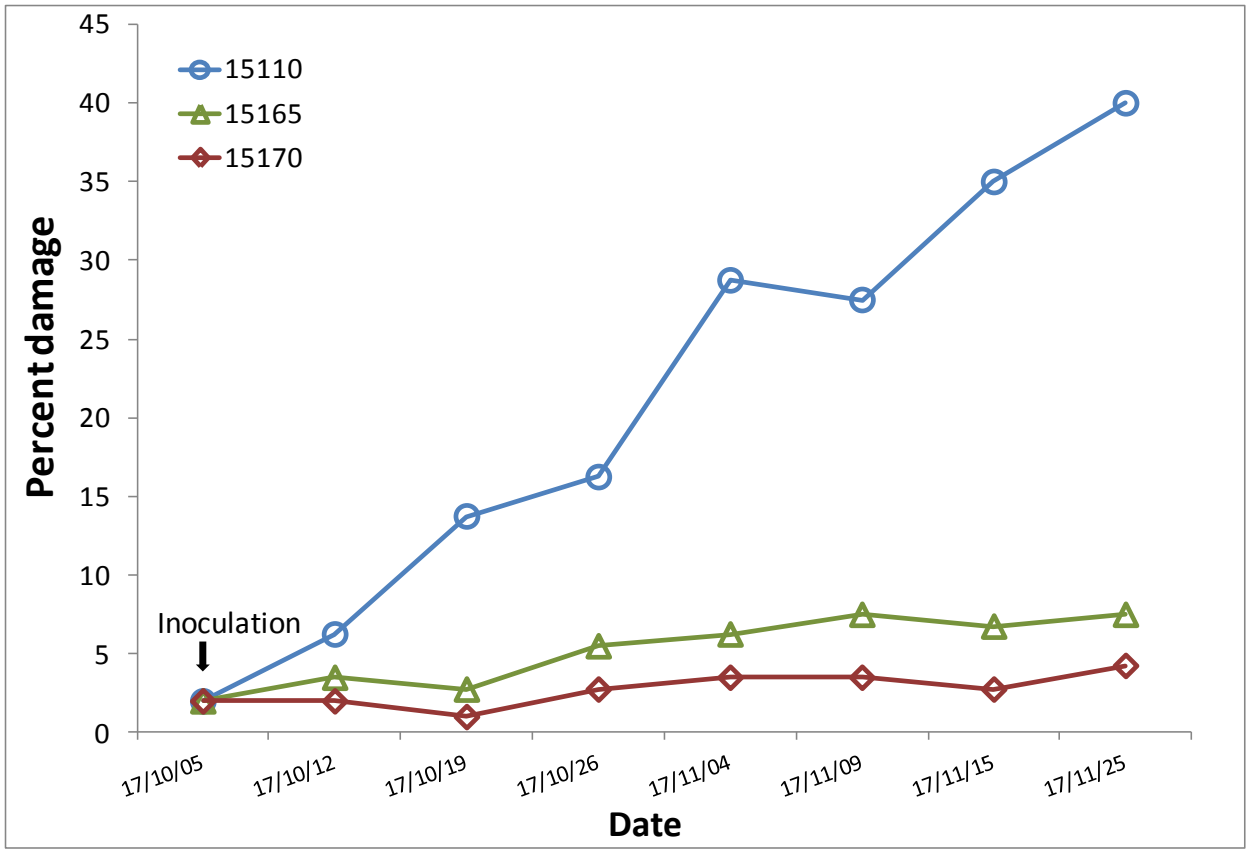
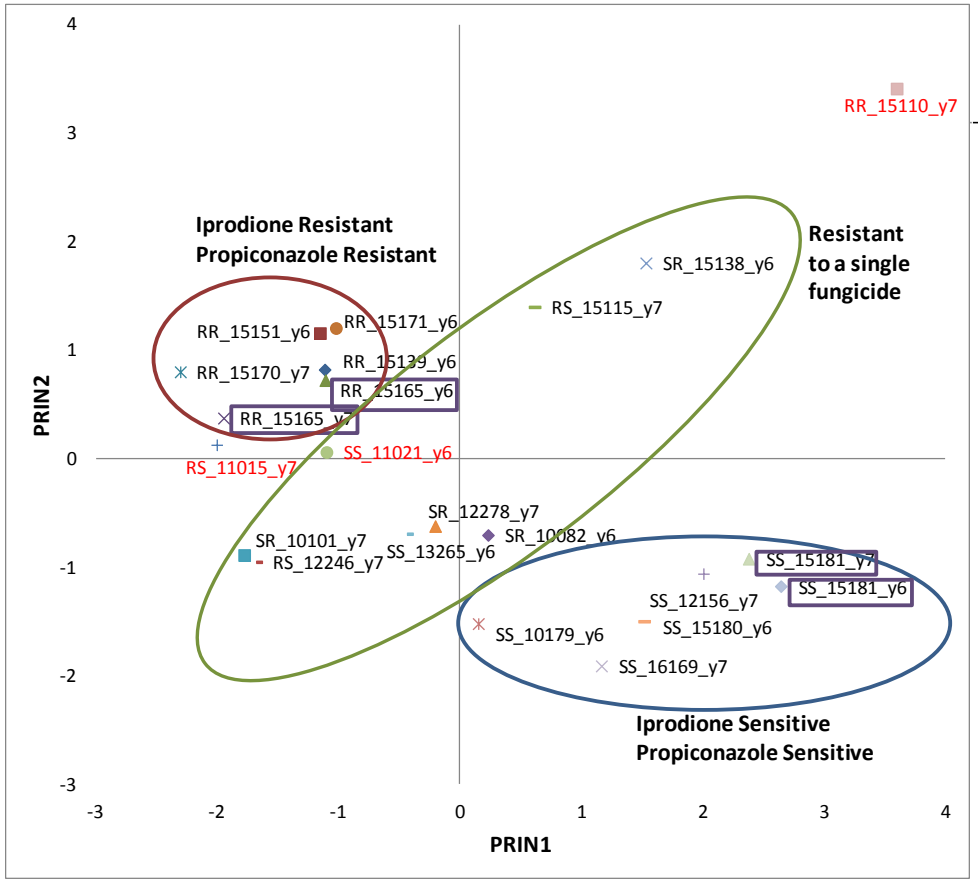


Figure 4.6 The average percent damage to *A. stolonifera* without any fungicide treatments when inoculated by three *M. nivale* isolates resistant to iprodione and propiconazole during fall 2017 field trial.

Figure 4.7 Principal component analysis (PCA) based on growth on non-amended PDA, % growth on discriminatory concentrations of iprodione and propiconazole, as well as AUDPC values from 2016 and 2017 field trials (all four treatments: untreated, iprodione, propiconazole, iprodione + propiconazole). Isolate numbers include sensitivity to iprodione and propiconazole based on % growth on, discriminatory concentrations in lab tests where isolates with >50% growth were deemed resistant. (y6 = field data from 2016; y7 = field data from 2017; purple box indicates isolate was tested in both field trials; red isolates are not clustered with others of similar sensitivity)



Principal Component	Contribution to variance (%)
1	41.9
2	24.7
3	14.5

Prin1 Component	Contribution to variance %
AUDPC – Iprodione + Propiconazole	25.4
AUDPC – Iprodione	25.1
AUDPC – Propiconazole	20.5
AUDPC – Untreated	18.0
Discriminatory growth - iprodine	5.6
Discrimnatory growth - propiconazole	5.2
Growth on PDA	0.2

Appendices

Appendix 4.1 Literature regarding fitness costs associated with resistance to the dicarboximide and DMI fungicides in fungal plant pathogens (low = significantly lower than sensitive isolates; same = no difference to sensitive isolates; -- = not tested)

Species	Fungicide	Tested fitness parameter			Reference
		Growth	Sporulation	Virulence	
Botrytis cinerea	vinclozolin, iprodione, procymidone	low[1]	--	low	(Katan, 1982)
Botrytis cinerea	vinclozolin	low	low	low	(Pollastro et al., 1996)
Botrytis cinerea	iprodione	same	low	--	(Raposo et al., 2000)
Botrytis cinerea	iprodione	same	--	--	(Lamondia and Douglas, 1997)
Botrytis cinerea	iprodione, vinclozolin	low[2]	same	--	(Cui et al., 2004)
Botrytis cinerea	iprodione, vinclozolin	low[2]	same	same	(Oshima et al., 2006)
Botrytis cinerea	iprodione	same	same	same	(Grabke et al., 2014)
Botrytis elliptica	iprodione	low	low	low	(Hsiang and Chastagner, 1991)
Microdochium nivale	iprodione	low	--	--	(Chastagner and Vassey, 1982)
Monilinia fructicola	iprodione	same	low	low	(Elmer and Gaunt, 1994)
Monilinia laxa	iprodione, vinclozolin	--	low	low	(Katan and Shabi, 1982)
Neurospora crassa	vinclozolin	low[2]	low	--	(Grindle and Temple, 1985)
Penicillium expansum	iprodione	low[5]	--	low	(Karaoglanidis et al., 2011)
Rhizoctonia cerealis	iprodione	low[2]	--	low	(Hamada et al., 2011)
Sclerotinia minor	iprodione	same	--	low	(Hubbard et al., 1997)

Sclerotinia minor	iprodione, vinclozolin	--	--	same	(Brenneman et al., 1987)
Alternaria alternata	vinclozolin, iprodione, procymidone	--	--	same	(Hutton, 1988)
Erysiphe graminis f.sp. *tritici*	fenpropimorph	--	same	same[4]	(Engels and DeWaard, 1996)
S. homoeocarpa	propiconazole	-	--	low	(Hsiang et al., 1998)
Penicillium expansum	tebuconazole	same[5]	--	low	(Karaoglanidis et al., 2011)
Penicillium digitatum	thiabendazole	--	same	low	(Kinay et al., 2007)
Cercospora beticola	flutriafol	-	low	low[4]	(Karaoglanidis et al., 2001)
Pyrenophora tritici-repentis	epoxyconazole	low	--	--	(Reimann and Deising, 2005)
Pyrenophora teres	propiconazole	--	same	same[3]	(Peever and Milgroom, 1994)
Moniliana fructicola	propiconazole	same	same	low[3]	(Cox et al., 2007)
Moniliana fructicola	propiconazole	same	same	same	(Chen et al., 2013)
Fusarium gramniearum	tebuconazole	--	--	same[4]	(Spolti et al., 2014)
Rhynchosporium secalis	propiconazole, tebuconazole	--	--	same	(Kendall et al., 1993)

[1]a small number of resistant isolates grew as fast as the sensitive isolates

[2]growth compromised by osmotically variable environment

[3]latent infection period for resistant isolates

[4]decreased frequency of resistant isolates in mixed inoculum experiments

[5]growth was reduced for isolates with resistance to both iprodione and propiconazole, but not isolates resistant to a single fungicide

Appendix 4.2 Example of SAS statement used for growth rate of different *M. nivale* fungicide sensitivity groups on non-amended PDA.

```
data temperature;
options pagesize=180 linesize=80;
infile cards expandtabs;
 input pop$ local$ sens$ isolate rep1 rep2 rep3 rep4 rep5 rep6;
growth = rep1; output;
growth = rep2; output;
growth = rep3; output;
growth = rep4; output;
growth = rep5; output;
growth = rep6; output;
cards;
ON GTI SS 10173 18 17 17 17 17 16
ON GTI SS 10179 16 17 16 15 17 16
ON GTI SS 11042 11 11 10 11 12 12
;
run;
data temp;
set;
;
run;
proc glm;
class sens;
model growth = sens;
means sens / LSD lines;
run;
```

Appendix 4.3 Example of SAS statement used for field trials to check significance between *M. nivale* fungicide sensitivity groups and the treatment applications

```
data temp;
options pagesize=80 linesize=100;
infile cards;
input date TRT$ isolate sens$ rep1 rep2 rep3 rep4;
effect=cats (sens||TRT);
 injury=rep1; output;
 injury=rep2; output;
 injury=rep3; output;
 injury=rep4; output;
cards;
170112 IP 15180 ISPS 95 85 90 95
170112 IP 15181 ISPS 75 90 95 90
170112 C 13265 ISPS 20 20 10 10
170112 C 15171 IRPR 20 30 15 20
;
run;
data temp;
set;
run;
proc sort; by date;
proc glm; by date;
class effect;
model injury = effect;
means effect / LSD lines;
output out=glmout;
run;
```

Appendix 4.4 Example of SAS statement used for principal component analysis to assess if isolates could be grouped. Analysis included AUDPC for four treatments during field trails 2016 and 2017, percent growth on discriminatory concentrations of iprodione and propiconazole, and growth on PDA in the absence of fungicides.

```
data PCAin;
title 'PCA';
 options pagesize=80 linesize=80;
length isolate $ 9;
input isolate$ PDA iproLab propLab conFld iproFld propFld ipprFld;
datalines;
RR15139y6 8 87.85 64.58 217.125 166.125 206.875 250
RR15151y6 7.692.74 72.33 234.5 196.125 204.625 227.125
;

proc sort data=PCAin; by isolate;
data temp; set PCAin;
proc print; * Print the new data ;
run;
ods pdf file="D:/MSc/M.nivale/PCA/output/all/test.pdf";
proc princomp outstat=PCAstats out=PCAout;
run;
ods pdf close;
proc export data=PCAout dbms=tab replace outfile="D:/MSc/M.nivale/PCA/output/all/test.tsv";
run;
```

Chapter 5 Identifying mutations associated with decreased sensitivity to iprodione and propiconazole in isolates of *Microdochium nivale*

5.1 Introduction

5.1.1 Mutations previously associated with iprodione resistance

Early experiments investigating the mode of action of the dicarboximide fungicides suggested a link between insensitivity to the fungicides and increased sensitivity to osmotic pressure (Pappas and Fisher, 1979). Shortly after this discovery, Perkins et al. (1982) categorized six chromosomal loci associated with osmotic sensitivity in *Neurospora crassa*. Further experiments showed that mutations in four of the six loci (*os-1*, *os-2*, *os-4*, and *os-5*) were associated with resistance to dicarboximide group of fungicides, while 2 of 6 (*cut* and *gla*) were not associated with resistance (Grindle and Temple, 1982).

The *os-1* locus was later shown to encode an osmosensing-histidine kinase similar to osmosensors found in bacteria and yeast (Schumacher et al., 1997). Histidine protein kinases form two-component signaling systems and play key roles in signal transduction and signal perception (Wolanin et al., 2002). Mutations in homologs of the *N. crassa os-1* gene have since been identified in at least seven other species with documented field resistance to the dicarboximide fungicides: *Alternaria brassicicola* (Avenot et al., 2005), *A. alternata* (Dry et al., 2004), *Botrytis cinerea* (Grabke et al., 2014), *Cochliobolus heterostrophus* (Yoshimi et al., 2004), *Sclerotinia homoeocarpa* (Sang et al., 2015), *S. sclerotiorum* (Duan et al., 2014), and *Stemphylium vesicarum* (Alberoni et al., 2010).

The *os-2*, *os-4*, and *os-5* gene all encode mitogen-activated protein kinases (Zhang et al., 2002; Fujimura et al., 2003; Yang et al., 2012) and are directly linked to *os-1* in an

osmo-regulation pathway (Figure 5.1). Further experiments with *N. crassa* and *Saccharomyces cerevisiae* confirmed that mutations in the genes *os-2*, *os-4*, and *os-5* were associated with resistance to the dicarboximide group of fungicides in those species (Zhang et al., 2002; Fujimura et al., 2003). Ochiai et al., (2007) also reported mutations in *os-2*, *os-4*, and *os-5* could cause decreased sensitivity to dicarboximides in *Fusarium graminearum*. Interestingly, Yang et al., (2012) found *os-4* mutants of *B. cinerea* had increased sensitivity to iprodione. Additionally, full deletion of the *os-2*, *os-4*, or *os-5* genes was not associated with resistance to dicarboximides in *B. cinerea* (Liu et al., 2008; Yan et al., 2010; Yang et al., 2012).

In *Cochliobolus heterostrophus*, two other loci associated with resistance to iprodione have been identified. The first locus, *dic2*, was found to contain a *Skn7*-type response regulator (*ChSkn7*), and mutant strains exhibited decreased sensitivity to iprodione (Izumitsu et al., 2009). The authors also associated a mutation in the locus *dic3* with decreased sensitivity; however the specific gene has not been identified.

5.1.2 Mutations previously associated with DMI resistance

Resistance to the demethylation inhibitor class of fungicides is well documented in a variety of fungal plant pathogens. A review of DMI resistance revealed several resistance mechanisms, and that increased levels of resistance to DMIs are often under polygenic control (Becher and Wirsel, 2012; Cools et al., 2013; Angelini et al., 2015). The gene *cyp51* is a target of DMI fungicides (Siegel, 1981; Köller, 1988). *Cyp51* encodes a cytochrome P450 enzyme, lanosterol-14α-demethylase, which is involved in ergosterol synthesis (Délye et al., 1998; Hamamoto et al., 2000; Wyand and Brown, 2005). Ergosterol is an essential component of ascomycetous and basidiomycetous fungal cell membranes, and many fungi cannot survive

without it (Hollomon et al. 1990; Weete et al. 2010). A number of DMI resistance cases were associated with mutations in, or increased expression of, the target-site *cyp51* (Délye et al., 1998; Hamamoto et al., 2000; Wyand and Brown, 2005; Ma et al., 2006; Becher and Wirsel, 2012; Cools and Fraaije, 2013; Angelini et al., 2015). Additionally, species such as *F. graminearum* with multiple copies of *cyp51* are reported to be intrinsically less sensitive to DMI fungicides (Liu et al., 2011; Cools et al., 2013).

The over-expression of efflux transporters have also been investigated as a source of dicarboximide resistance. Kretchmer et al. (2009) reported the over-expression of the major facilitator transporter, *mfsM2*, induced low levels of resistance to iprodione and tebuconazole in *B. cinerea*. However, at this time, no other reports were found which implicated *mfsM2* as a cause of fungicide resistance. Increased expression of the efflux transporters *mfs1*, *atrD* and *atrB* (through the transcription factor *mmr1),* have also been reported as a cause of resistance to dicarboximide and DMI fungicides (Nakaune et al., 1998; Kretschmer et al., 2009; Hulvey et al., 2012; Sang et al., 2015).

5.1.3 Genomic analyses

Mutations in the genes noted above may be a source of insensitivity to propiconazole or iprodione. One approach to identifying mutations is whole genome sequencing. The decreasing cost of high throughput sequencing over the last 30 years has allowed smaller research labs access to large amounts of genomic data (Haridas et al., 2011; Lee et al., 2016). Although the traditional Sanger sequencing method is still used for small sequences such as PCR products, a variety of ‘next-gen’ high-throughput sequencing (NGS) platforms allow sequencing of entire genomes (Buermans and Den Dunnen, 2014). Regardless of which NGS platform is used, the

data usually obtained as millions of separate reads (sequence fragments). These reads must then be joined (i.e. assembled) into contiguous sequences (contigs) for further analysis. Contigs can be further joined into scaffolds stitching together contigs linked by N's, with the largest scaffolds as chromosome. In one assembly strategy, overlapping reads allow consensus sequences to be developed and a probable nucleotide order to be resolved using de Brujin graphs (Zerbino and Birney, 2008). A generalized hidden Markov chain, and use of known coding regions from related species, can then be used to guide gene prediction and establish putative gene sequences (Stanke et al., 2004)

As the amount of genomic data increased, large databases such as GenBank were created. Originally, these databases were searched using full alignment procedures such as the Smith-Waterman algorithm (Schuler, 2001), however full alignment methods were very time consuming when dealing with increasingly larger data sets. The heuristic method BLAST (Basic Local Alignment Search Tool) was developed to expedite the search process (Altschul et al., 1990). BLAST initially breaks a query sequence into short partial sequences of length k (e.g. ATTCG would be broken into ATT, TTC, and TCG when $k = 3$). The partial sequences are used to build a list of potential matches by assigning alignment scores using an alignment scoring matrix (e.g. BLOSUM) and having a cut-off value for inclusion (Altschul et al., 1990). Full alignments between the query and the potential matches are then performed and output generated.

BLAST is an algorithm which can be used to query assembled genomes for genes of interest (Altschul et al., 1990), such as those associated with fungicide resistance. Once BLAST queries a database it will generate a list of high-scoring segment pairs, each of which consists of two aligned sequence fragments (one query, one match) ranked according to their e-value

(Altschul et al., 1990). The e-value for each sequence match is the number of times that match would be expected by chance from a database of the same size. The cut-off e-value for inclusion in the list of matches can be adjusted to be inclusive. The default e-value is generally set at 1E-5, which is strict enough to suggest that homology exists between the query and any hits (Altschul et al., 1990). The output also includes p-values for each e-value, an alignment score (derived from BLOSUM matrix), and the number of identical (nucleotide or protein) or similar residue (protein) matches (Bergeron, 2003). Top hits can then be extracted from the assembly (or predicted gene set) and aligned to identify conserved sequences and mutations (Darling et al., 2004).

The genes which are associated with resistance to the dicarboximide or DMI fungicides may be extracted from the genomes of sensitive and resistant isolates and subsequently compared to identify polymorphisms. Previous works have utilized whole genome sequencing to facilitate the identification of genes and mutations associated with fungicide resistance. Catlett et al. (2003) performed whole-genome analysis on the two-component signaling genes in fungi, this set of genes included those involved in the HOG pathway which had been linked with dicarboximide-resistance. Sequencing of azole-resistant *Aspergillus fumigatus* isolates revealed a novel tandem repeat in the promoter of cyp51A which was common to all the resistant isolates (Abdolrasouli et al., 2015). Similarly, a genome wide association study in azole-resistant *Rhynchosporium commune* found mutations in three genes associated with resistance, these genes were not previously known to confer resistance (Mohd-Assaad et al., 2016). Other groups, such as Zheng et al. (2015) have used genome sequencing to identify genes and mutations associated with resistance to other types fungicides such as the cyanoacrylate phenamacril. Additionally, the identification of ABC efflux transporters associated with multi-drug resistance

has also been facilitated by the use of whole-genome sequencing (De Waard et al., 2006). The amplification of genes previously known to be associated with resistance via PCR is a more commonly used technique for identifying mutations associated with resistance. However this type of work is generally supported by pre-existing genome assemblies (i.e. NCBI databases). The use of genome sequencing to identify mutations associated with fungicide resistance has not previously been done with *M. nivale*.

Major objectives of this chapter were:

(1) to sequence genomes of isolates with varying sensitivity to iprodione and/or propiconazole;

(2) to identify differences in gene sequences correlated with decreased sensitivity to iprodione and propiconazole; and

(3) to identify differences promoter regions which could be associated with increased expression of genes linked to resistance to iprodione or propiconazole.

5.2 Methods and Materials

5.2.1 Selection of isolates for genome sequencing

The selection criterion for isolates was to ensure representative isolates from each sensitivity group established in Section 2.3.3. Isolates of *M. nivale* which were identified as having high or low sensitivity to iprodione or propiconazole based on discriminatory concentration tests (Section 2.3.3) were selected for sequencing. Another criterion was to have isolates from different sampling locations, and have multiple isolates from each location. Out of 296 isolates assessed for sensitivity to both iprodione and propiconazole, six were selected for full genome sequencing.

5.2.2 DNA extraction

DNA extraction followed Jewell and Hsiang (2013). Briefly, genomic DNA was extracted by first placing a 5-mm plug from the actively growing edge of a culture in a 9-cm-diameter petri plate containing fresh antibiotic amended PDA overlaid with a 5 cm x 5 cm sheet of cellophane (Flexel Inc., Atlanta, Ga., U.S.A.). Cellophane sheets had been pre-cut and autoclaved twice (20 minutes, 121°C) in de-ionized water prior to use. Mycelium was left to grow over the cellophane sheet (5 d at 25°C). Mycelium was collected by scraping from the top of the cellophane into autoclaved 1.5 mL tubes without also collecting any agar which could interfere with DNA extraction. Approximately 50 mg of acid-washed and autoclaved sea sand (Fisher, Fair Lawn NJ, USA) was added to each tube along with 200 µL Edwards buffer (Tris HCl (pH 7.5) 200 mM, NaCl 250 mM, EDTA 25 mM, SDS 0.5% (w/v)) (Edwards et al., 1991). Mycelia was ground in the tubes using a plastic pestle (Froggabio, Toronto, Canada) powered with a Mastercraft Lithium Ion screwdriver (3.6V, Canada) for 60 seconds. An additional 200 µL of Edwards buffer was then added and mixtures were left at room temperature for 2 to 3 hours. Tubes were centrifuged at 12,000 x g using an Eppendorf 5415 D centrifuge (Eppendorf, Mississauga, Canada) for 10 min. Supernatant was transferred to a new 1.5 mL tube, an equal volume of cold isopropanol (-20°C) was added, then the tube was placed for at least 1 h at -20°C. The tubes were again centrifuged at 12,000 x g for 10 min to obtain a DNA pellet. Supernatant was discarded and pellet was washed with ~400 µL of cold 70% ethanol. Ethanol was discarded and tubes were inverted and left open to dry. DNA was resuspended in 100 µL PCR water and tubes were placed at 4°C for 12 h to allow dissolution. DNA was then stored at -20°C.

Quality and quantity of DNA was assessed using gel electrophoresis. Samples were run through 1% agarose gel (UltraPureTM Agarose, Invitrogen, Carlsbad, CA, USA) prepared with

0.5 M TBE buffer. The TBE buffer was created using 90 mM Tris base, 90 mM boric acid, and 2 mM EDTA. Agarose gels were placed in 0.5 M TBE buffer and wells loaded with 6 µL mixtures (2:1) of DNA to loading buffer (0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol, 30% v/v/ glycerol, and 60 mM EDTA). Gels were subject to electrophoresis at 100 V for 20 to 30 minutes before being stained in an ethidium bromide solution and DNA visualized with UV light. The concentration was assessed by comparison to a molecular weight and mass ladder loaded in every gel (GeneRuler 1000bp DNA ladder, Fermentas, Canada).

5.2.3 Genome sequencing and assembly

Genomic DNA, ~1 µg of each isolate, was sent to Genome Quebec, Montreal, Canada, for fragmentation, library preparation, and sequencing using an Illumina HiSeq 2000 machine. Library inserts were approximately 400 bp and sequencing was 100 bp paired end. After sequencing, raw sequence reads were downloaded from ftp servers. Sequence reads were assembled using many kmer values and repeated with three assembly programs, SOAPdenovo ver. 1.1.2 (with GAPcloser ver 1.12) (Li et al., 2008), ABySS ver. 1.2.3 (Simpson et al., 2009), and Velvet ver. 1.0.12 (Zerbino and Birney, 2008), generating a large number of assemblies for each genome (Haridas et al., 2011). The assemblies with the highest N50 value (50% of all bases in the assembly are contained in contigs larger than this value) were selected for gene prediction with AUGUSTUS ver 3.2.2 (Stanke et al., 2004) based on the *Fusarium graminearum*, *Magnaporthe grisea*, and *Neurospora crassa* gene models. The model which generated the largest number of genes was selected for further genomic comparisons. Genome completeness was assessed by querying predicted gene sets with the fungal BUSCO set of highly conserved single-copy orthologs (1E-5) (Simao et al., 2015).

5.2.4 Comparison of sequenced isolates from the same locations

The pairs of isolates 15109/15110 and 15165/15170 were collected from the locations BC-1 and BC-3 respectively. To assess whether these isolate pairs were the same genotype, a full genomic comparison was conducted. The genomes of select species (*Cladorrhinum flexuosum*, *Nectria nigrescens*, and *Ophiosphaerella korrae*) which were previously sequenced twice were used as a check comparison. Additionally, two pairs of *M. nivale* isolates which were known to be different (collected at different times from two different locations) were included (Table 5.2). Standalone BLAST databases were created for each genome using makeblastdb.exe (BLAST 2.6.0+). For each pair of genomes (e.g. isolates 15109 and 15110), the predicted gene sets (including introns) were used as a set of queries and the assemblies (Table 5.1) were used as databases in two reciprocal BLASTN comparisons (i.e. one isolate acted as the query in the first search, and acted as the database in the second search). The percent identity between isolate pairs for each individual search was calculated using the equation: (percent identity between isolates) = [(average percent identity of all hits) * (number of unique BLAST hits)] / (total number of query genes). Missing genes are accounted for in this equation by dividing with the total number of query genes, any genes which did not produce a hit will become part of the calculated percentage. The percent identity calculated from each search was subject to SAS PROC GLM and the calculated means of the reciprocal pairs were separated by Fishers Least Significant Difference ($p = 0.05$). An example of the SAS statement can be found in Appendix 5.1.

5.2.5 Identification of mutations associated with dicarboximide resistance

Standalone BLAST databases were created for each of the 13 sequenced *M. nivale* genomes (Table 5.1) using makeblastdb.exe (BLAST 2.6.0+). Amino acid sequences of *os-1*

(Schumacher et al., 1997), *os-2* (Zhang et al. 2002), *os-4* (Fujimura et al. 2003), *os-5* (Fujimura et al. 2003), *skn7* (*dic2*) (Izumitsu et al., 2009), *mfsM2* (Kretschmer et al., 2009), and *mrr1* (Kretschmer et al., 2009) were downloaded from GenBank in FASTA format (accession numbers in Appendix 5.2). Amino acid sequences were then queried against each *M. nivale* genome using BLASTp with an e-value of 1E-50. The top hits for each *M. nivale* isolate were then copied from each genome to create putative homolog sequence FASTA files. Each putative gene was queried remotely against GenBank using BLASTP (1E-50) to confirm identity. Predicted protein sequences of *M. nivale* were then used to query the draft genome assemblies using TBLASTN (1E-50), and nucleotide sequences (+1000 bp upstream and downstream) were extracted as FASTA files using fastcmd.exe (v2.2.10). The *M. nivale* putative homologs were aligned using MUSCLE (v3.6). Alignments of amino acid sequences and nucleotide sequences were viewed using CLUSTAL (v1.83) or BioEdit (v7.0.5). Dendograms were created using the Maximum Parsimony method with MEGA based on distance algorithms, and viewed using TREEVIEW (v3.2) or MEGA. Mutations previously associated with dicarboximide resistance in the homologs of *os-1*, *os-2*, *os-4*, *os-5*, *skn7*, *mfsM2*, and *mrr1* from other species were compiled (Appendix 5.3 for full list of *os-1* mutations) and each genome was checked for these, and any novel, mutations in these target genes.

5.2.6 Identification of mutations associated with demethylation inhibitor resistance

Previously created BLAST databases were used (Section 5.2.4). *Fusarium graminearum*, another Sordariomycetes, is a well studied and closely related to *M. nivale*. Select gene sequences from *F. graminearum*: *cyp51A*, *cyp51B*, and *cyp51C* (Liu et al., 2011) were downloaded from GenBank. Other genes previously associated with DMI or azole resistance in

other fungal species were also downloaded from GenBank in both nucleotide and amino acid: *atrD* (Sang et al., 2015), *mfsM2* (Kretschmer et al., 2009), *mfs1* (Hayashi and Waard, 2002), and *mrr1* (Kretschmer et al., 2009) (accession numbers in Appendix 5.2). These genes were used to query each *M. nivale* genome using StandAlone BLASTP (1E-50) and the top hits were extracted to create putative *M. nivale* homolog amino acid sequences. The *M. nivale* amino acid sequences were used to query the local nucleotide databases, and sequences, including 1000 bp upstream and 1000 bp downstream, were extracted from assemblies using fastcmd.exe (v2.2.10). The *M. nivale* putative homologs were aligned using MUSCLE (v3.6). Alignments of amino acid sequences and nucleotide sequences were viewed using CLUSTAL (v1.83) or BioEdit (v7.0.5). Cladograms were created using CLUSTAL or MEGA (v7.0.26) and viewed using either TREEVIEW (v32) or MEGA based on nucleotide sequences. Mutations previously associated with DMI resistance in homologs of *cyp51A*, *cyp51B, atrD*, *mfsM2*, *mfs1*, and *mrr1* from other species were compiled (Appendix 5.4 for *cyp51* coding region mutations and Appendix 5.5 for *cyp51* promoter region mutations) and each genome was checked for specific mutations in these genes, and scrutinized for any novel mutations.

5.3 Results

5.3.1 Isolate selection and genome assembly

Based on the selection criteria outlined in Section 5.2.1 the isolates 13172, 15109, 15110, 15141, 15165, and 15170 were selected for whole genome sequencing. Genome completeness based on BLAST results of the BUSCO (fungi v1, containing 1438 genes, www.busco.ezlab.org/v1) fungal gene set showed, with one exception, all genomes contained 99.9% of BUSCO genes (isolate 11037 contained >99.5%). Isolates 15109 and 15110 were

selected from the first group of isolates tested, BC-1, having exhibited resistance to both iprodione and propiconazole. Isolates 15165 and 15170, from BC-3, were selected as both were highly resistant to both iprodione and propiconazole, with EC_{50} values much higher than those of 15109 and 15110 (Appendix 2.1). Isolate 15141 was selected as a fully sensitive BC isolate for comparison with resistant BC isolates. Isolate 13172, collected from the GTI (ON), exhibited low levels of resistance to both iprodione and propiconazole. Other isolates which were previously sequenced and had their sensitivity to iprodione and propiconazole assessed were included in the analysis, these isolates included fully sensitive isolates from ON. Genome and assembly details can be found in Table 5.1.

5.3.2 Comparison of sequenced isolates from the same location

The average identity (calculated from two reciprocal BLAST queries, Section 5.2.4) between the genomes of *M. nivale* isolates 15109 and 15110 was 98.2% across 12250 genes, and the identity between isolates 15165 and 15170 was 98.5% across 12271 genes (Table 5.2). The average identity for isolates which were sequenced twice (*C. flexuosum*, *N. nigrescens*, and *O. korrae*) ranged from 96.7% to 99.4%. The average identity of the isolate pair 12099 and 12262 which were known to be different was 93.6%, and 94.8% for isolates 10106 and 10082 which were also known to be different. There was no significant difference between the average identity of the isolate pairs 15109/15110 and 15165/15170 to the twice sequenced isolates (*C. flexuosum*, *N. nigrescens*, and *O. korrae*). Additionally, there was a significant difference between the average identity of isolate pairs known to be different (12099/12262 and 10106/10082) and all other isolate pairs based on an ANOVA test ($p = 0.05$).

5.3.3 Mutations associated with dicarboximide resistance

Alignment of *mnos-1* amino acid sequences revealed two different mutations associated with isolates with insensitivity to iprodione in the lab. The first mutation was a 63 bp deletion from position 430 to 462 (Figure 5.2), corresponding to a 21 amino acid deletion from positions 144 to 164 (Figure 5.3). The deletion was found in two isolates from BC-3 (isolates 15165 and 15170). The second mutation detected was a point mutation of adenine (A) to guanine (G) at position 3503 (Figure 5.2), leading to an amino acid change of glutamic acid (E) to glycine (G) at position 1168 (Figure 5.3). The E1168G point mutation was found in two isolates from BC-1 (isolates 15109 and 15110). A fifth isolate, 13172 (GTI, Ontario), which showed insensitivity to iprodione, did not have either of these mutations, and showed no other mutations in *mnos-1* that were not also shared by at least one iprodione-sensitive isolate. In addition, none of the previously associated *os-1* mutations from the literature were present in our sequenced *M. nivale* genomes (Appendix 5.3).

The alignments of *mnos-2* and *mnos-5* did not reveal any mutations present in iprodione-resistant isolates which were not also shared by at least one sensitive isolate. Alignments of *mnos-4* revealed two point mutations present in two dicarboximide-resistant isolates. The first point mutation was a guanine (G) to adenine (A) exchange at position 49, leading to an amino acid exchange of alanine (A) to threonine (T) at position 17 (Figure 5.4). The second was cytosine (C) to thymine (T) at position 395 leading to an amino acid exchange isoleucine (I) to threonine (T) at position 132 (Figure 5.5).

Aligned *mn-skn7* showed the amino acid and nucleotide sequences are similar between all but one isolate. Isolate 13172 had 214 point mutations, 6 deletions in the 5’ end, and 2 insertions in the 3’ end (Appendix 5.6), with 53 of the point mutations leading to amino

exchange. Isolate 13172 tested as moderately insensitive to iprodione based on EC_{50} tests but did not have mutations in *mnos-1* or *mnos-4* like the other insensitive isolates.

Amino acid and nucleotide alignments of the transcription factor *mn-mrr1*, which regulates the efflux transporter *atrb1*, revealed two mutations associated only with iprodione-resistant isolates 15109, 15110, 15165, and 15170. The mutations were G2237A and G2392A which lead to the amino acid exchanges R747Q and E799K (Figure 5.6 and Figure 5.7). Similar to mutations in *mnos-1* and *mnos-4*, these mutations were not present in the iprodione insensitive isolates 13172. Table 5.3 summarizes the results for mutations associated with dicarboximide resistance.

5.3.4 Mutations associated with demethylation inhibitor resistance

No amino acid or nucleotide mutations were identified in the coding sequences of *cyp51A*, *cyp51B*, *atrD*, *mfs1* or *mfsM2* in isolates with decreased sensitivity to propiconazole (Table 5.4). The transcription factor *mn-mrr1*, which regulates the efflux transporter *atrb1*, is also associated with the resistant isolates 15109, 15110, 15165, and 15170. The mutations were G2237A and G2392A which lead to the amino acid exchanges R747Q and E799K (Figure 5.6 and Figure 5.7). Of the 132 amino acid exchanges which were previously associated with azole resistance in *cyp51A*, five (V37A, F105L, A313G, F449L/S/V/Y, and Y491H) were present in all *M. nivale* genomes, and two (L98H, Q474K) were present in only isolate 13172 (Appendix 5.4).

5.3.5 Promoter region mutations associated with fungicide resistance

Over-expression of *cyp51* paralogs (A and B) and the major efflux transporters *mfs1*, *mfsM2*, *atrD* (*PDR1*), and *atrB* (controlled by transcription factor *mrr1*) have been reported as

causes of DMI insensitivity (Kretschmer et al., 2009; Ishii and Hollomon, 2015; Sang et al., 2015). The promoter regions for the genes of interest were compared for mutations which could indicate increased gene expression. However, none of the 10 previously characterized *cyp51* promoter insertions previously associated with azole resistance were present in any *M. nivale* isolate (Appendix 5.5). Additionally, we did not identify any novel differences in the promoter sequences for *cyp51A, cyp51B, mfsM2, atrD,* or *mrr1*.

5.4 Discussion

The full genomic comparisons between isolate pairs 15109/15110 and 15165/15170 suggest that the isolates 15109 and 15110 may be the same genotype, and that isolate 15165 may be the same genotype as 15170. These comparisons suggest that a number of isolates collected from within a population (e.g. BC-3) could be the same or extremely similar, implying that some or many of the resistant isolates from a location may be clonal. Genotyping using microsatellite markers or other such markers could be used to test this hypothesis, but these tests were beyond the scope of this work. There were minor differences in the comparisons between reciprocal pairs (i.e. the results from using 15109 as the query and 15110 as the database produced slightly different results than when 15110 was the query and 15109 the database) (Table 5.1). The discrepancies (e.g. the relatively large difference in the number of reads) could be explained by sequencing or assembly errors such as minor miss-assembly, higher DNA fragmentation during sequencing (may disrupt gene prediction), differences in the assembly programs (SOAP vs. ABySS) or other errors in the sequencing or assembly pipeline. As a result of the genotype similarity of some isolates there are potential biases in the results presented in this thesis, namely the number of isolates in the fungicide-resistant group may be artificially inflated. For example,

the proportion of isolates which exhibited resistance in the BC populations may in actuality be smaller than what is presented in Chapter 2. Similarly, the averages calculated for growth rates in the absence of fungicide may be skewed by the inclusion of clonal isolates which are not true biological replicates as is presented in Chapter 4. As a result, the lack growth rate fitness costs associated with the double-resistant isolates could be explained by multiple copies of a single isolate which was able to grow quickly. Future work should attempt to include more robust genotyping using microsatellite markers to remove such biases.

Past research evaluating dicarboximide resistance have shown that single point mutations which confer single amino acid exchanges in the *os-1* gene are able to decrease sensitivity to the dicarboximides including iprodione (Appendix 5.3). Although the observed *os-1* mutations in *M. nivale* have not been reported in the *os-1* homologs of other species, it is a good causal candidate for the observed iprodione insensitivity found in the BC isolates. Deletions and duplications causing frameshifts in *os-1* have also been linked to dicarboximide insensitivity (Appendix 5.3). The 63 base pair deletion (Δ430-462) found in two of the iprodione-insensitive isolates does not appear to cause a frameshift; however, such a large deletion may cause significant alterations in protein structure. The dicarboximide insensitivity seen in British Columbia populations of *M. nivale* appears to be paraphyletic, having arisen independently in different populations (Figure 5.8). Iprodione-insensitive isolate 13172 did not share the above mutations in *os-1*. The lack of mutations in the *mnos-1* gene of 13172 could indicate differences in regards to the cause of resistance. Firstly, mutations in *mnos-1* are not a cause of dicarboximide insensitivity in *M. nivale*, implying that mutations are present elsewhere in the genome which have not been identified. Secondly, it is possible that *mnos-1* is over or under-expressed in isolate 13172. Future work could use RT-PCR to investigate this possibility. Additionally, it is possible that

mutations in *mnos-1* are the cause of insensitivity in the four BC isolates, but a mutation in another gene is the cause in this Ontario isolate. Finally, the sensitivity of 13172 to iprodione was at the outer range of what was considered resistant in this study, and may actually fall into a zone of normal sensitivity to iprodione.

Previous research, namely Dry et al. (2004), Avenot et al. (2005), Ma et al. (2007), and Tanaka and Izumitsu (2010), indicated that moderate levels of resistance can be conferred via a single point mutation in *os-1*, but the source of high levels of resistance to dicarboximide fungicides still requires further investigation. (Grabke et al., 2014) also found mutations in *os-1* could cause low and moderate levels of resistance in *B. cinerea* based on growth on two discriminatory concentrations of iprodione, but could not find mutations in *os-1* associated with isolates exhibiting high levels of resistance. Using the criteria of Grabke et al. (2014) as a guide, isolate 13172 demonstrated a low level of resistance, growing ~50% on 10 μg mL^{-1} but 0% on 100 μg mL^{-1}. The isolates 15109 and 15110 exhibited moderate resistance, growing ~80% on 10 μg mL^{-1} and ~20% on 100 μg mL^{-1}. Isolates 15165 and 15170 exhibited high levels of resistance, growing >80% on 10 μg mL^{-1} and >50% on 100 μg mL^{-1}.

There has been relatively little work done investigating the association of *os-2, os-4,* and *os-5* mutations with dicarboximide resistance. This is may be due to the identification of *os-1* mutations in dicarboximide-resistant isolates, which appear to be far more common. Fujimara et al. (2003) did not make it clear which mutations in *os-4* were responsible for resistance in their isolates, as their protocol involved transforming resistant isolates with wild-type *os* genes and then testing for sensitivity. No previous literature was found describing which mutations in *os-4* could cause resistance; it is possible that the three mutations in *os-4* observed in isolates 15165 and 15170 are responsible for or contributing to their iprodione insensitivity. Additionally,

isolates 15165 and 15170 had much higher iprodione EC_{50} values relative to isolates 15109 and 15110, which are also insensitive to iprodione (Table 5.1). All four of these isolates had mutation in *os-1*, and it is possible that the mutations in found in *os-4* are contributing to the increased insensitivity of 15165 and 15170. Mutations in *os-4* were not explored in the work of Grabke et al. (2014), who were unable to find mutations to account for their highly resistant group of isolates. However, as there were only two sequenced isolates with these mutation, there is not enough evidence to state with certainty that *os-4* mutations are a contributing factor in *M. nivale* iprodione resistance. *Botrytis cinerea* with mutations in *os-4* showed increased sensitivity to dicarboximide fungicides (Yang et al., 2012). Although work with *N. crassa* and *B. cinerea* (Zhang et al., 2002; Fujimura et al., 2003) identified mutations in *os-2* and *os-5*, which cause dicarboximide resistance, no mutations in *mnos-2* or *mnos-5* were found which could explain the observed insensitivity to iprodione in the sequenced isolates.

As no mutations were found in the *os* genes of the iprodione-insensitive isolate 13712, it is possible that the large number of mutations in *mn-skn7* is the cause of insensitivity seen in this isolate. However, use of only a single representative is not strong support for this hypothesis. Additionally, as isolate 13172 was at the outer range of what was considered resistant to iprodione in this study, isolate 13172 may fall in the range of normal sensitivity. This would suggest that the mutations found in *mn-skn7* may be meaningless in the context of dicarboximide resistance.

Mutations in *cyp51A* previously associated with DMI resistance were identified but were present in all isolates, and so are unlikely to be relevant to propiconazole insensitivity. Mutations present only in isolate 13172 may be a cause of resistance in this isolate, but similar to iprodione, the resistance to propiconazole in this isolate is on the border of what was considered resistant.

As no previously identified or novel gene sequence variations in *cyp51A* could be correlated with propiconazole insensitivity, we have ruled out target gene mutations as the cause of resistance in this case. The paralog *cyp51B* was ruled out as well, as no mutations were found in propiconazole-resistant isolates. Although no promoter mutations were found for either *cyp51* gene, over-expression of either *cyp51A* or *cyp51B* may still be responsible for the observed DMI insensitivity. Future work should include gene expression analysis to test this hypothesis. Furthermore, in *F. graminearum*, multiple paralogs of *cyp51* (A, B, and C) are present and confer increased natural insensitivity in this species (Liu et al., 2011). In *M. nivale*, the two paralogous copies of *cyp51* do not appear to decrease the species' natural sensitivity to propiconazole, as EC_{50} tests show similar levels of sensitivity as species with a single *cyp51* such as *M. graminicola* (Leroux and Walker, 2011).

Increased expression of major efflux transporters can cause resistance to multiple fungicides simultaneously (Sang et al., 2015). Although no coding or promoter region mutations were found for transporters *mfsM2* or *atrD*, they may still be over-expressed in the fungicide-resistant isolates. For this reason, future work should include expression analysis of these efflux transporters to test this hypothesis. Mutations in the transcription factor *mrr1* were identified in isolates resistant to both iprodione and propiconazole. As *mrr1* regulates the transcription of the efflux transporter *atrB*, it is possible *atrB* is being over-expressed in resistant isolates. Once again, future work should include expression analysis to confirm this hypothesis. The single point mutation in the *os-1* gene of 15109/15110 may not cause fungicide resistance; rather the cause of resistance for these isolates may only be the mutations in *mrr1*. The 63 bp deletion in the *os-1* gene of isolates 15165/15170, in addition to the *mrr1* and *os-4* mutations could be the cause for the markedly lower fungicide sensitivity in these isolates. In the future, use of *os-1*,

os-4, or *mrr1* specific primers could be of use for the rapid identification of iprodione or propiconazole-resistant isolates of *Microdochium nivale*. Future work should also include gene replacement experiments for *os-1*, *os-4*, and *skn7* to determine if resistance may be conferred to sensitive isolates and whether sensitivity can be restored to resistant isolates.

Tables

Table 5.1 Sequencing and assembly details of *Microdochium nivale* isolates.

Isolate	Location	EC_{50} (µg mL^{-1})		Reads (millions)	Program	N50 (kb)	Contigs
		Iprodione	Propiconazole				
10082*	United Kingdom	1.4	0.31	22.7	ABySS	356	8732
10106*	Medicina, ITL	1.3	0.56	22.2	Velvet	477	3741
11037*	GTI, Guelph, ON	1.6	0.28	70.0	SOAP	155	2642
12099*	GTI, Guelph, ON	2.3	0.03	19.1	SOAP	467	3943
12262*	Ottawa, ON	1.9	0.16	28.1	ABySS	383	7527
13172	GTI, Guelph, ON	10.4	0.14	9.6	SOAP	241	2035
13407*	Upland Res. Center, JPN	4.3	0.06	20.2	SOAP	526	4482
13408*	Kitasato Univ. Farm, JPN	2.6	0.06	20.6	SOAP	381	10170
15109	BC-1, BC	26.7	0.11	16.7	SOAP	235	806
15110	BC-1, BC	40.8	0.81	15.7	ABySS	281	791
15141	BC-3, BC	5.6	1.83	9.1	SOAP	108	23726
15165	BC-3, BC	115	0.36	8.2	SOAP	121	3669
15170	BC-3, BC	191	0.44	11.5	SOAP	207	3974

*assembled previously by Hsiang Lab

Table 5.2 Genome comparisons of isolate pairs 15109-15110 and 15165-15170. Isolates 12262 and 12099 were known to be different, and other genomes were isolates sequenced twice. BLAST searches used predicted gene sets as queries (nucleotide sequences produced by AUGUSTUS, Section 5.2.3) and assemblies as databases (assemblies from Table 5.1).

Query (predicted gene set)	Number of genes in query	Database (assembly)	Number of unique hits	Identity (%)	Average identity between reciprocal pairs (%)
15109.nt	12250	15110.assembly	12230	99.00	98.20
15110.nt	12238	15109.assembly	12022	97.39	
15170.nt	12171	15165.assembly	12110	98.72	98.52
15165.nt	12271	15170.assembly	12173	98.32	
12262.nt	11634	12099.assembly	11244	94.93	93.59*
12099.nt	12158	12262.assembly	11540	92.25	
10106.nt	11716	10082.assembly	11532	95.84	94.84*
10082.nt	12228	10106.assembly	11832	93.85	
cf53.nt	10628	cf51.assembly	10578	98.82	98.83
cf51.nt	10596	cf53.assembly	10545	98.85	
hp79.nt	13541	he65.assembly	13530	99.52	99.39
he65.nt	13566	hp79.assembly	13523	99.27	
ok91.nt	11425	ok83.assembly	11352	96.66	96.69
ok83.nt	11330	ok91.assembly	11245	96.72	

-The identity (%) column was calculated as follows: identity (%) = [(average % identity of all hits) * (# unique hits)] / (# total query genes)
-Asterisks indicate significant difference from the other isolate pairs based on ANOVA ($p = 0.05$).

Table 5.3 Sequenced isolates of *Microdochium nivale* ordered by their sensitivity to iprodione with presence of mutations in genes previously associated with dicarboximide resistance.

Isolate	Location	EC_{50} ($\mu g\ mL^{-1}$)	*os1*	*os2*	*os4*	*os5*	*skn7*	*mfsM2*	*mrr1*
10106	Medicina, ITL	1.3	-	-	-	-	-	-	-
10082	United Kingdom	1.4	-	-	-	-	-	-	-
11037	GTI, Guelph, ON	1.6	-	-	-	-	-	-	-
12262	Ottawa, ON	1.9	-	-	-	-	-	-	-
12099	GTI, Guelph, ON	2.3	-	-	-	-	-	-	-
13408	Kitasato Univ. Farm, JPN	2.6	-	-	-	-	-	-	-
13407	Upland Ag Res. Center, JPN	4.3	-	-	-	-	-	-	-
15141	Cordova Bay, BC	5.6	-	-	-	-	-	-	-
13172	GTI, Guelph, ON	10.4	-	-	-	-	+[6]	-	-
15109[1]	Victoria, BC	26.7	+[3]	-	-	-	-	-	+[7]
15110[1]	Victoria, BC	40.8	+[3]	-	-	-	-	-	+[7]
15170[2]	Cordova Bay, BC	115	+[4]	-	+[5]	-	-	-	+[7]
15165[2]	Cordova Bay, BC	191	+[4]	-	+[5]	-	-	-	+[7]

[1] likely the same genotype based on Table 5.2, average 98.2% identity
[2] likely the same genotype based on Table 5.2, average 98.5% identity
[3] point mutation A3503G
[4] deletion Δ430-462
[5] point mutations T182C, T395C
[6] six deletion, three insertions, and 214 point mutations, see Appendix 5.6 for full details
[7] point mutations G2237A, G2392A

Table 5.4 Sequenced isolates of *Microdochium nivale* ordered by their sensitivity to propiconazole with presence of mutations in genes previously associated with resistance.

Isolate	Location	EC_{50} (µg mL^{-1})	*cyp51A*	*cyp51B*	*atrD*	*mfsM2*	*mrr1*	*mfs1*
12099	GTI, Guelph, ON	0.03	-	-	-	-	-	-
13407	Upland Ag Res. Center, JPN	0.06	-	-	-	-	-	-
13408	Kitasato Univ. Farm, JPN	0.06	-	-	-	-	-	-
15109	Victoria, BC	0.11	-	-	-	-	+[1]	-
13172	GTI, Guelph, ON	0.14	-	-	-	-	-	-
12262	Ottawa, ON	0.16	-	-	-	-	-	-
11037	GTI, Guelph, ON	0.28	-	-	-	-	-	-
10082	United Kingdom	0.31	-	-	-	-	-	-
15165	Cordova Bay, BC	0.36	-	-	-	-	+[1]	-
15170	Cordova Bay, BC	0.44	-	-	-	-	+[1]	-
10106	Medicina, ITL	0.56	-	-	-	-	-	-
15110	Victoria, BC	0.81	-	-	-	-	+[1]	-
15141	Cordova Bay, BC	1.83	-	-	-	-	-	-

[1] point mutations G2237A, G2392A

Figures

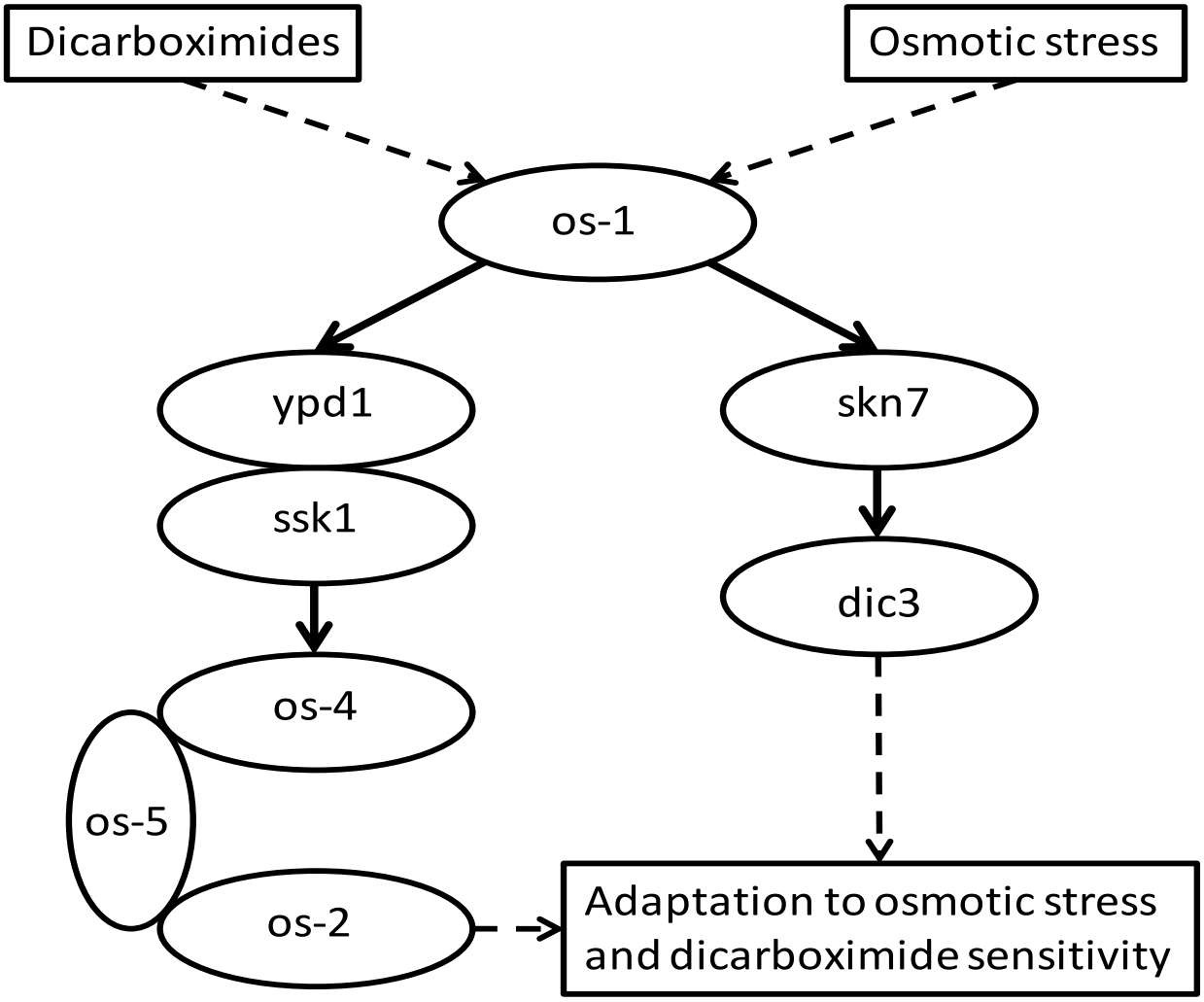


Figure 5.1 Diagram of the osmo-regulation pathway in filamentous fungi and its association with dicarboximide and DMI sensitivity, adapted from Izumitsu et al. (2009) and Kim et al. (2011).

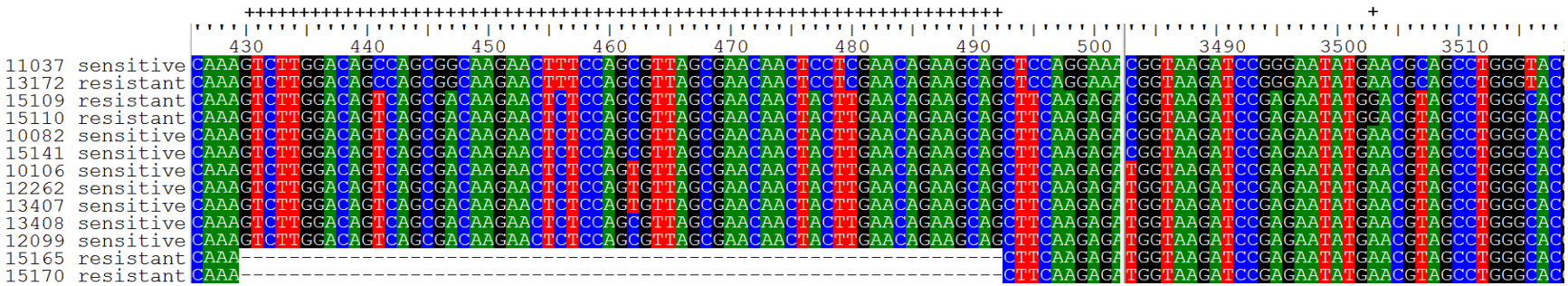


Figure 5.2 Nucleotide sequence alignment of *mnos-1* genes from *Microdochium nivale* isolates show two mutations, a 63 base pair deletion from position 430 to 492 and a point mutation of adenine (A) to guanine (G) at position 3503, associated with only iprodione-resistant isolates. Asterisk indicates the location of a mutation present only in iprodione-resistant isolates.

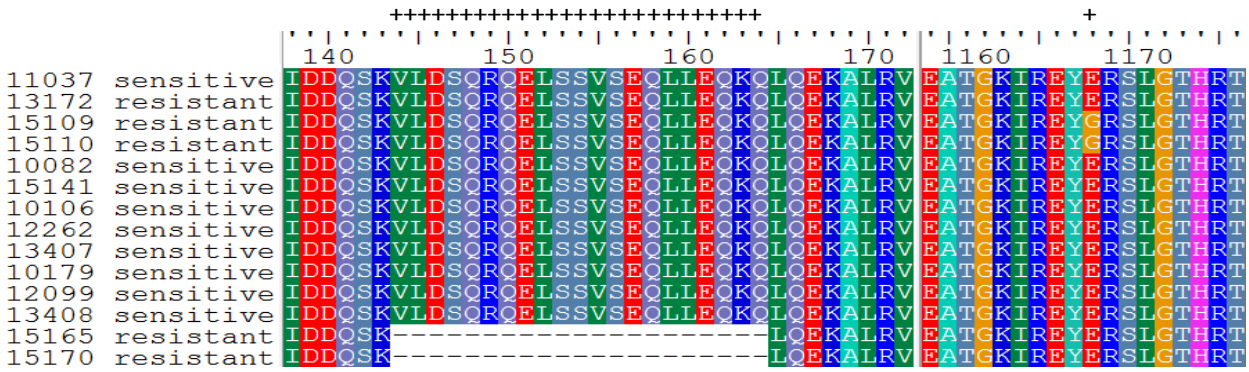


Figure 5.3 Amino acid sequence alignment of *mnos-1* genes from *Microdochium nivale* isolates show two mutations: a 21 amino acid deletion from position 144 to164 and a single amino acid exchange of glutamate (E) to glycine (G) at position 1168, associated with only iprodione-resistant isolates. Asterisk indicates the location of a mutation present only in iprodione-resistant isolates.

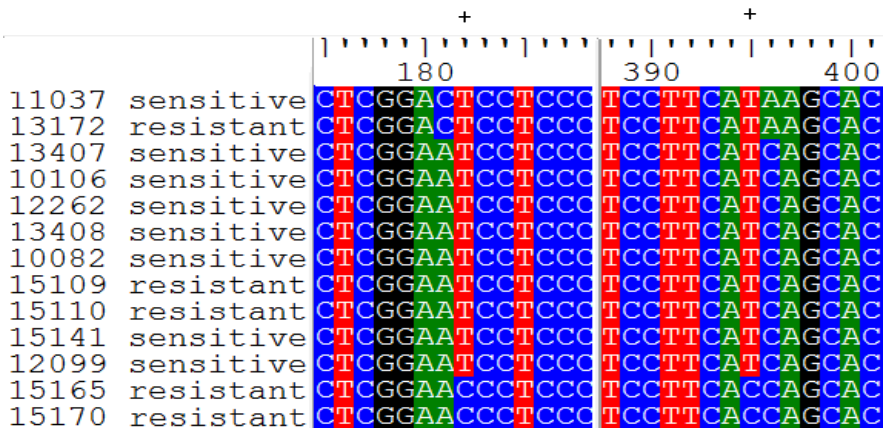


Figure 5.4 Nucleotide sequence alignment of *mnos-4* genes from *Microdochium nivale* isolates show two mutations, T182C and T395C, associated with only iprodione-resistant isolates. Asterisk indicates the location of a mutation present only in iprodione-resistant isolates.

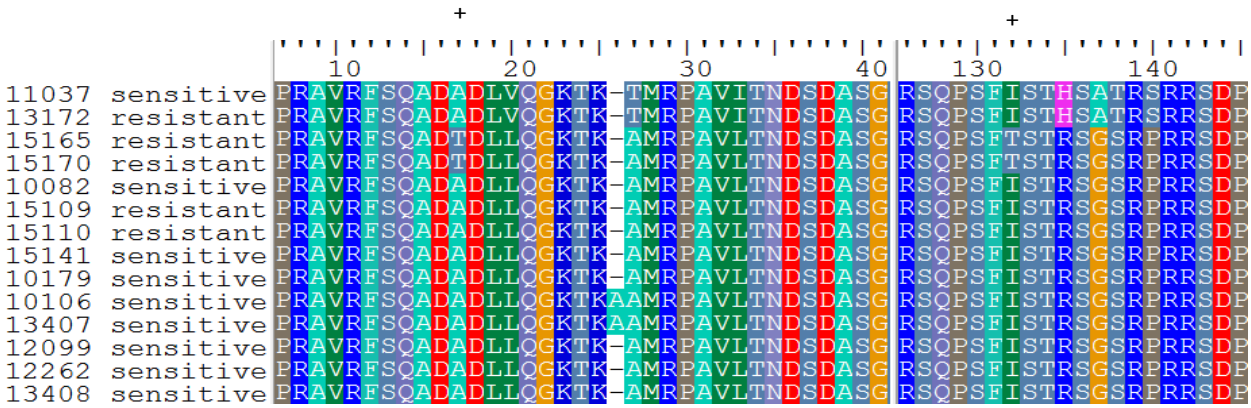


Figure 5.5 Amino acid sequence alignment of *mnos-4* genes from *Microdochium nivale* isolates show two mutations, A17T and I132T, associated with only iprodione-resistant isolates. Asterisk indicates the location of a mutation present only in iprodione-resistant isolates.

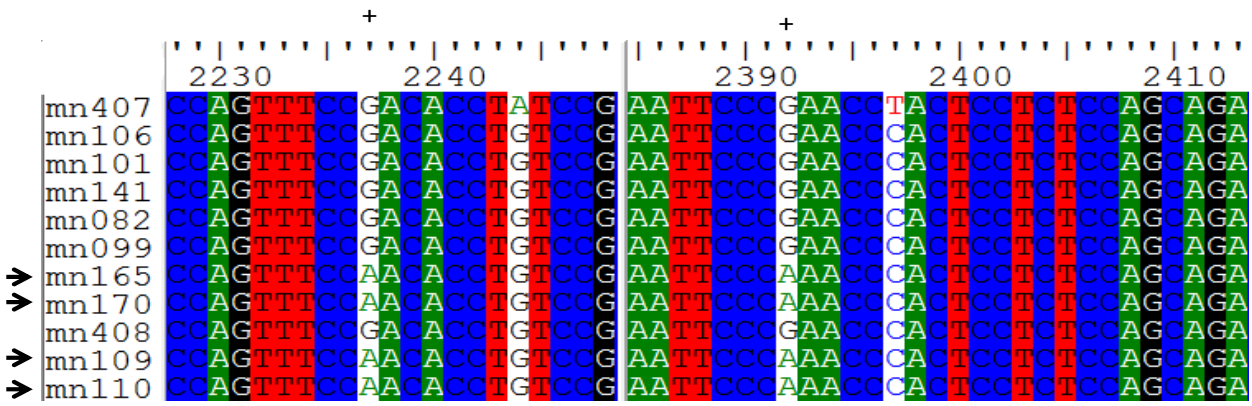


Figure 5.6 Nucleotide sequence alignment of *mn-mrr1* genes from *Microdochium nivale* isolates show two mutations, G2237A and G2392A, associated with isolates resistant to both iprodione and propiconazole (arrows). Asterisk indicates the location of a mutation present only in isolates resistant to both iprodione and propiconazole.

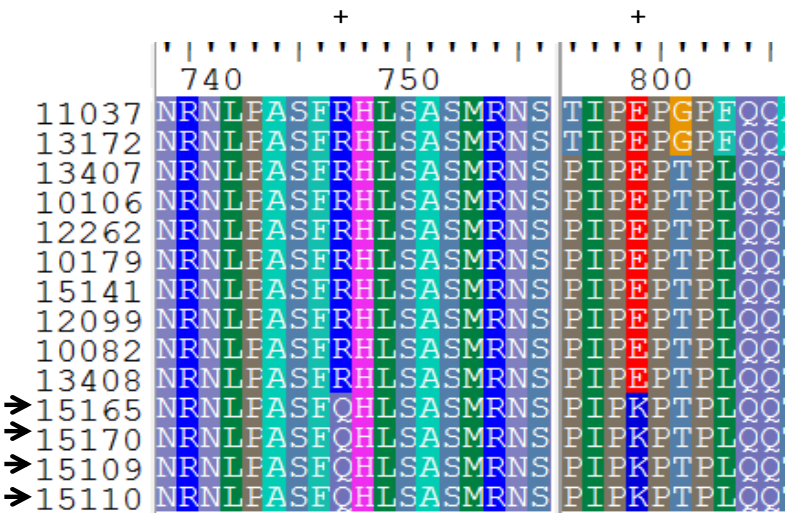


Figure 5.7 Amino acid sequence alignment of *mn-mrr1* genes from *Microdochium nivale* isolates show two mutations, R747Q and E799K, associated with isolates resistant to both iprodione and propiconazole (arrows). Asterisk indicates the location of a mutation found only in sequenced isolates resistant to both iprodione and propiconazole.

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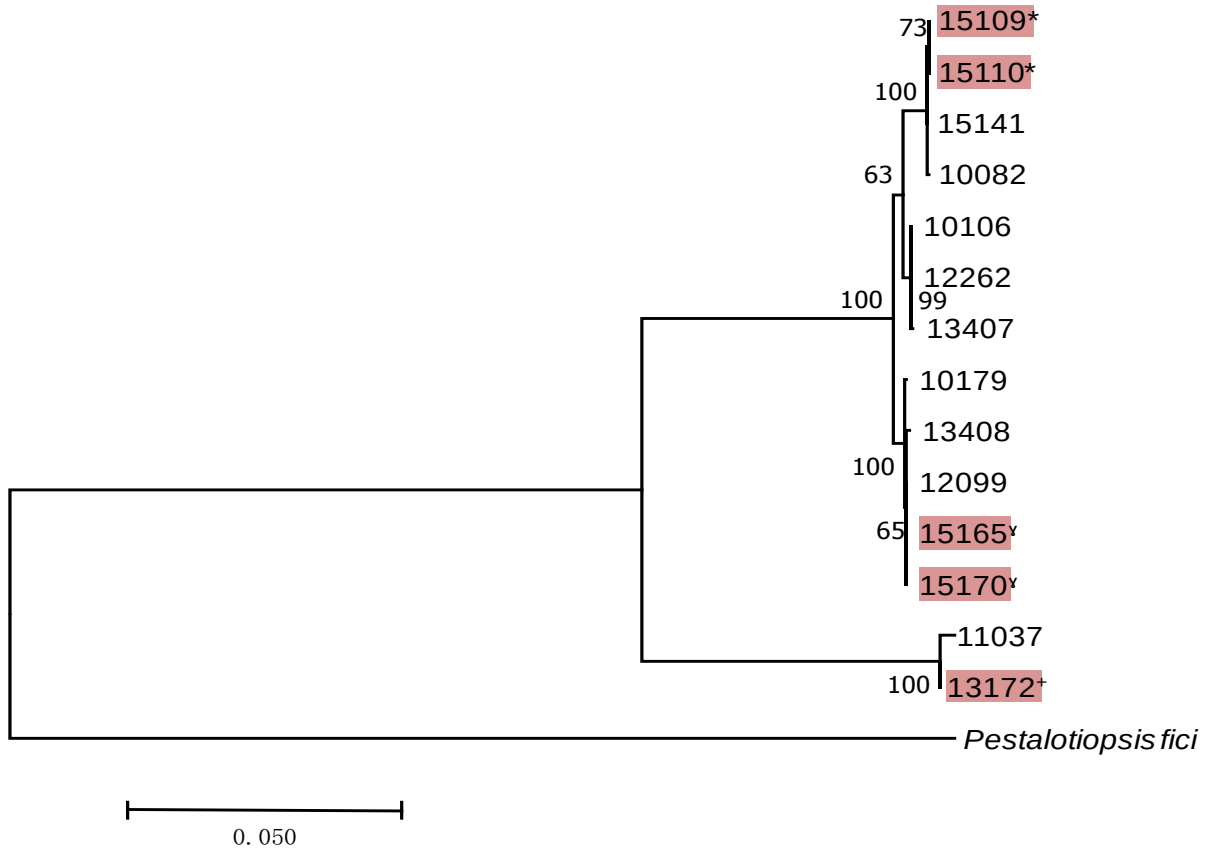

Figure 5.8 Cladogram of the *mnos-1* gene (nucleotide sequences) in *Microdochium nivale* isolates (Table 5.1) inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest likelihood is shown. The bootstrap consensus tree was inferred from 1000 replicates, and branches reproduced in less than 50% bootstrap replicates are collapsed with percentage shown at the nodes (Felsenstein, 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Isolates shaded red were resistant to iprodione based on EC_{50} tests where an EC_{50} greater than 10 µg mL^{-1} was considered resistant.

*deletion from position 430 to 462

ɣ single point mutation A3503G leading to an amino acid exchange E1168G

+ no mutation in *mnos-1*

Appendices

Appendix 5.1 SAS statement for genome comparisons of isolate pairs 15109/15110 and 15165/1570. Pair = reciprocal pair (e.g. 15109-15110), pid1 = average percent identity from first BLAST search, pid2 = average percent identity for all genes from second BLAST search. Average percent identity was calculated by: identity (%) = [(average % identity of all hits) * (# unique hits)] / (# total query genes).

```
data temp;
options pagesize=80 linesize=100;
infile cards;
input pair$ pid1 pid2;
pid = pid1; output;
pid = pid2; output;
cards;
15109-15110 99.00 97.39
15165-15170 98.72 98.32
12262-12099 94.93 92.25
cf51-53 98.82 98.85
hp65-79 99.52 99.27
ok83-91 96.66 96.72
108-082 95.84 93.85
;
run;
data temp;
set;
run;
proc glm;
class pair;
model pid = pair;
means pair / LSD lines;
output out=glmout;
run;
```

Appendix 5.2 Accession numbers of gene sequences, previously associated with resistance to iprodione or propiconazole, used to query *Microdochium nivale* genomes.

Gene (syn.)	Association	GenBank Accession	Reference
os-1(*tco1*, *dic1*)	Iprodione	AAB01979	(Schumacher et al., 1997)
os-2 (*hog1*)	Iprodione	AAK83125	(Zhang et al., 2002)
os-4(*ssk2*)	Iprodione	BAC56234	(Fujimura et al., 2003)
os-5(*pbs2*)	Iprodione	BAC56235	(Fujimura et al., 2003)
skn7 (*dic2*)	Iprodione	BAI48020	(Izumitsu et al., 2009)
mfsM2	Iprodione + Propiconazole	CCD50602	(Kretschmer et al., 2009)
mrr1	Iprodione + Propiconazole	CCD43682	(Kretschmer et al., 2009)
cyp51A (*erg11*)	Propiconazole	XP_011321548	(Liu et al., 2011)
cyp51B	Propiconazole	XP_011316750	(Liu et al., 2011)
atrD (*PDR1*)	Iprodione + Propiconazole	JQ612525	(Hulvey et al., 2012)

Appendix 5.3 Catalog of mutations in *os-1* gene previously associated with resistance to iprodione in several fungal species, as well as their presence in our sequenced *Microdochium nivale* isolates. (+) indicates mutation is present, (-) indicates mutation is not present, (Δ) indicates a deletion, letters indicate amino acid difference not present in literature,.

Species	Homolog	Mutation associated with dicarboximide resistance	Presence of mutation in isolate														Reference
			10082	10179	11037	10106	12099	12262	13407	13408	15109	15110	13172	15141	15165	15170	
Microdochium nivale	mnos1	E1168G	-	-	-	-	-	-	-	-	+	+	-	-	-	-	This study
Microdochium nivale	mnos1	Δ149-169	-	-	-	-	-	-	-	-	-	-	-	-	+	+	This study
Botrytis cinerea	bos1	I365S/N/R	V	V	V	V	V	V	V	V	V	V	V	V	V	V	Many
Botrytis cinerea	bos1	V368F, Q369H, plus T447S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Banno et al., 2008)
Botrytis cinerea	bos1	N373S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Ma et al., 2007)
Botrytis cinerea	bos1	L386F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cui et al., 2002)
Botrytis cinerea	bos1	S531N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cui et al., 2002)
Botrytis cinerea	bos1	L570W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cui et al., 2002)
Botrytis cinerea	bos1	V346A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cui et al., 2002)
Botrytis cinerea	bos1	G538R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cui et al., 2002)
Botrytis cinerea	bos1	A585H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cui et al., 2002)
Botrytis cinerea	bos1	E345G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cui et al., 2002)
Botrytis cinerea	bos1	Δ482-483	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cui et al., 2002)
Botrytis cinerea	bos1	S1040STOP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Ma et al., 2007)
Botrytis cinerea	bos1	F127S	G	G	G	G	G	G	G	G	G	G	G	G	G	G	(Grabke et al., 2014)
Botrytis cinerea	bos1	V1136I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Grabke et al., 2014)
Botrytis cinerea	bos1	G311R/E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al., 2012)
Botrytis cinerea	bos1	E692K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	G278D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	G323C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	G415D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	A493T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	T581P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	G81STOP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	A157T	S	S	S	S	S	S	S	S	S	S	S	S	**del***	**del***	(Fillinger et al. 2012)
Botrytis cinerea	bos1	M427T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	V239F	T	T	T	T	T	T	T	T	T	T	T	T	T	T	(Fillinger et al. 2012)
Botrytis cinerea	bos1	E529A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Fillinger et al. 2012)
Botrytis cinerea	bos1	V368F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Oshima et al., 2006)
Botrytis cinerea	bos1	T447S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Oshima et al., 2006)
Botrytis cinerea	bos1	Q369H/P	K	K	K	K	K	K	K	K	K	K	K	K	K	K	(Oshima et al., 2006)
Botrytis cinerea	bos1	N373S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Oshima et al., 2006)
Sclerotinia homoeocarpa	Shos1	I366N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Sang et al., 2017)
Sclerotinia sclerotiorum	shk1	T489I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Duan et al., 2014)
Sclerotinia sclerotiorum	shk1	E599K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Duan et al., 2014)
Sclerotinia sclerotiorum	shk1	G736Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Duan et al., 2014)
Neurospora crassa	os1	Q308STOP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Ochiai et al., 2001)
Neurospora crassa	os1	Δ294	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Ochiai et al., 2001)
Neurospora crassa	os1	L625P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Ochiai et al., 2001)

Neurospora crassa	os1	A578V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Ochiai et al., 2001)
Neurospora crassa	os1	G580R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Ochiai et al., 2001)
Alternaria brassicicola	AbNik1	Q998STOP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Avenot et al., 2005)
Alternaria brassicicola	AbNik1	Δ495-496	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Avenot et al., 2005)
Alternaria brassicicola	AbNik1	634STOP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Avenot et al., 2005)
Alternaria brassicicola	AbNik1	Q343STOP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Avenot et al., 2005)
Alternaria brassicicola	AbNik1	E753K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Avenot et al., 2005)
Cochilobolus heterostrophus	bmhk1	W573STOP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Yoshimi et al., 2004)
Cochilobolus heterostrophus	bmhk1	Δ226–234	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Yoshimi et al., 2004)
Cochilobolus heterostrophus	bmhk1	Δ834	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Yoshimi et al., 2004)
Cochilobolus heterostrophus	bmhk1	G282D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Yoshimi et al., 2004)
Cochilobolus heterostrophus	bmhk1	G616S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Yoshimi et al., 2004)
Cochilobolus heterostrophus	bmhk1	G647D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Yoshimi et al., 2004)
Cochilobolus heterostrophus	bmhk1	M781I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Yoshimi et al., 2004)
Cochilobolus heterostrophus	bmhk1	R1210C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Yoshimi et al., 2004)
Alternaria alternata	AaHK1	95STOP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Dry et al., 2004)
Alternaria alternata	AaHK1	4 bp deletion, 1349-1352	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Dry et al., 2004)

*part of novel 21 amino acid deletion noted at the top of this table

Appendix 5.4 Catalog of mutations in *cyp51A* gene previously associated with resistance to iprodione in several fungal species, as well as their presence in our sequenced *Microdochium nivale* isolates. (+) indicates mutation is present, (-) indicates mutation is not present, letters indicate amino acid difference not present in literature.

Species	Mutations associated with azole resistance	Presence of mutation in isolate														Reference
		10082	10179	11037	10106	12099	12262	13407	13408	15109	15110	13172	15141	15165	15170	
Aspergillus fumigatus	V101F	L	L	L	L	L	L	L	L	L	L	L	L	L	L	(Becher and Wirsel, 2012)
Aspergillus fumigatus	N125I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	G138C/R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	Q141H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	H147Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	V172M	I	I	I	I	I	I	I	I	I	I	-	I	I	I	(Abdolrasouli et al., 2015)
Aspergillus fumigatus	P216L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	M220K/I/T/V	L	L	L	L	L	L	L	L	L	L	L	L	L	L	(Becher and Wirsel, 2012)
Aspergillus fumigatus	N22D	S	S	S	S	S	S	S	S	S	S	S	S	S	S	(Becher and Wirsel, 2012)
Aspergillus fumigatus	M236K/T/V	H	H	H	H	H	H	H	H	H	H	H	H	H	H	(Becher and Wirsel, 2012)
Aspergillus fumigatus	E255D	V	V	V	V	V	V	V	I	V	V	A	V	V	V	(Abdolrasouli et al., 2015)
Aspergillus fumigatus	S297T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Abdolrasouli et al., 2015)
Aspergillus fumigatus	P394L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	K427E	T	T	T	T	T	T	T	T	T	T	T	T	T	T	(Abdolrasouli et al., 2015)
Aspergillus fumigatus	Y431C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	G434C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	T440A	S	S	S	S	S	S	S	S	S	S	S	S	S	S	(Becher and Wirsel, 2012)
Aspergillus fumigatus	G448S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	Y46F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Abdolrasouli et al., 2015)
Aspergillus fumigatus	Y491H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(Becher and Wirsel, 2012)
Aspergillus fumigatus	F495I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Abdolrasouli et al., 2015)
Aspergillus fumigatus	S52T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	(Becher and Wirsel, 2012)
Aspergillus fumigatus	G54E/K/V/R/W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Aspergillus fumigatus	Q88H	D	D	D	D	D	D	D	D	D	D	D	D	D	D	(Becher and Wirsel, 2012)
Aspergillus fumigatus	L98H	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	+	Q	Q	Q	(Abdolrasouli et al., 2015)
Blumeria graminis f.sp. hordei	Y136F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Blumeria graminis f.sp. hordei	K147Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Blumeria graminis f.sp. tritici	Y136F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Candida albicans	V509	F	F	F	F	F	F	F	F	F	F	F	F	F	F	(Morio et al., 2010)
Candida albicans	**F**105L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(Morio et al. 2010)
Candida albicans	A107T	G	G	G	G	G	G	G	G	G	G	G	G	G	G	(Morio et al. 2010)
Candida albicans	K108E	-	-	-	-	-	-	-	-	-	-	R	-	-	-	(Morio et al. 2010)
Candida albicans	A114S	V	V	V	V	V	V	V	V	V	V	**I**	V	V	V	(Morio et al. 2010)

Candida albicans	K119L	G	G	G	G	G	G	G	G	G	G	G	G	G	G	(Morio et al. 2010)
Candida albicans	F126L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	V130I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	Y132F/H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	N136Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	K143E/R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	A149V	G	G	G	G	G	G	G	G	G	G	G	G	G	G	(Morio et al. 2010)
Candida albicans	T199I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	T229A	S	S	S	S	S	S	S	S	S	S	S	S	S	S	(Morio et al. 2010)
Candida albicans	P230L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	I253V	M	M	M	M	M	M	M	M	M	M	M	M	M	M	(Morio et al. 2010)
Candida albicans	Y257H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	R267H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	D278E/N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	(Morio et al. 2010)
Candida albicans	S279F	N	N	N	N	N	N	N	N	N	N	N	N	N	N	(Morio et al. 2010)
Candida albicans	H283D/R	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	(Morio et al. 2010)
Candida albicans	K287R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	G307S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	Y33C	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	DEL	(Morio et al. 2010)
Candida albicans	F380S	M	M	M	M	M	M	M	M	M	M	M	M	M	M	(Morio et al. 2010)
Candida albicans	Y39C	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	(Morio et al. 2010)
Candida albicans	V404I/L	A	A	A	A	A	A	A	A	A	A	A	A	A	A	(Morio et al. 2010)
Candida albicans	S405G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	D446N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	Y447G/H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	G448E/R/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	F449L/S/V/ Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(Morio et al. 2010)
Candida albicans	G450E/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	V452A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	V456I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	(Morio et al. 2010)
Candida albicans	G464S	-	-	-	-	-	-	-	-	-	-	+	+	+	+	(Morio et al. 2010)
Candida albicans	G465S	A	A	A	A	A	A	A	A	A	A	A	A	A	A	(Morio et al. 2010)
Candida albicans	R467I/K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	I471T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	Q474K	+	+	+	+	R	+	+	+	R	R	+	R	R	R	(Morio et al. 2010)
Candida albicans	T486P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	L491V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	T494A	S	S	S	S	S	S	S	S	S	S	-	S	S	S	(Morio et al. 2010)
Candida albicans	P49R/T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	P503L	T	T	T	T	T	T	T	T	T	T	T	T	T	T	(Morio et al. 2010)
Candida albicans	W54Stop	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	A61V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	Y79C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Morio et al. 2010)
Candida albicans	K99T	D	D	D	D	D	D	D	D	D	D	D	D	D	D	(Morio et al. 2010)
Cercospora beticola	I330T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Cercospora beticola	P384S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Cercospora beticola	E297K	P	P	P	P	P	P	P	P	P	P	P	P	P	P	(Becher and Wirsel, 2012)
Cryptococcus neoformans	G484S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Cryptococcus neoformans	*Y*145F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Erysiphe necator	Y136F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Délye et al., 1998)

Histoplasmosis capsulatum	Y136F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Becher and Wirsel, 2012)
Mycosphaerella fijensis	G462A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Canas-Guiterrez et al., 2009)
Mycosphaerella fijensis	Y136F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Canas-Guiterrez et al., 2009)
Mycosphaerella fijensis	Y461D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Canas-Guiterrez et al., 2009)
Mycosphaerella fijensis	Y463D/H/N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Canas-Guiterrez et al., 2009)
Mycosphaerella fijensis	A313G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(Canas-Guiterrez et al., 2009)
Mycosphaerella fijensis	A381G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(Canas-Guiterrez et al., 2009)
Mycosphaerella graminicola	D107V	E	E	E	E	E	E	E	E	E	E	-	E	E	E	(Leroux and Walker, 2011)
Mycosphaerella graminicola	D134G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Leroux and Walker, 2011)
Mycosphaerella graminicola	V136A	I	I	I	I	I	I	I	I	I	I	I	I	I	I	(Leroux and Walker, 2011)
Mycosphaerella graminicola	V136C/G	I	I	I	I	I	I	I	I	I	I	I	I	I	I	(Cools et al., 2013)
Mycosphaerella graminicola	Y137F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Leroux and Walker, 2011)
Mycosphaerella graminicola	M145L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Leroux and Walker, 2011)
Mycosphaerella graminicola	N178S	K	K	K	K	K	K	K	K	K	K	K	K	K	K	(Cools et al., 2013)
Mycosphaerella graminicola	S188N	G	G	G	G	G	G	G	G	G	G	G	G	G	G	(Cools et al., 2013)
Mycosphaerella graminicola	S208T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Mycosphaerella graminicola	N284H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Mycosphaerella graminicola	H303Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	(Cools et al., 2013)
Mycosphaerella graminicola	A311G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(Cools et al., 2013)
Mycosphaerella graminicola	G312A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Mycosphaerella graminicola	A379G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(Leroux and Walker, 2011)
Mycosphaerella graminicola	I381V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Leroux and Walker, 2011)
Mycosphaerella graminicola	A410T	S	S	S	S	S	S	S	S	S	S	S	S	S	S	(Cools et al., 2013)
Mycosphaerella graminicola	G412A	M	M	M	M	M	M	M	M	M	M	M	M	M	M	(Cools et al., 2013)
Mycosphaerella graminicola	Y459C/D/N/P/S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Mycosphaerella graminicola	G460D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Leroux and Walker, 2011)
Mycosphaerella graminicola	Y461D/S/H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Mycosphaerella graminicola	V490L	M	M	M	M	M	M	M	M	M	M	M	M	M	M	(Cools et al., 2013)
Mycosphaerella graminicola	L50S	K	K	K	K	K	K	K	K	K	K	K	K	K	K	(Cools et al., 2013)
Mycosphaerella graminicola	G510C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Mycosphaerella graminicola	N513K	G	G	G	G	G	G	G	G	G	G	G	G	G	G	(Cools et al., 2013)
Mycosphaerella graminicola	S524T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	(Leroux and Walker, 2011)
Mycosphaerella graminicola	ΔG460	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Leroux and Walker, 2011)
Mycosphaerella graminicola	ΔY459	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Leroux and Walker, 2011)
Oculimacula acuformis	Q167H	E	E	E	E	E	E	E	E	E	E	E	E	E	E	(Albertini et al., 2003)
Oculimacula acuformis	A29P	-	-	-	-	-	-	-	-	-	-	V	-	-	-	(Albertini et al. 2003)
Oculimacula acuformis	V37A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(Albertini et al. 2003)
Oculimacula acuformis	Y486H	E	E	E	E	E	E	E	E	E	E	E	E	E	E	(Albertini et al. 2003)
Oculimacula acuformis	S505Q	R	R	R	R	R	R	R	R	R	R	S	R	R	R	(Albertini et al. 2003)
Oculimacula yallundae	E106K	E	E	E	E	E	E	E	E	E	E	D	E	E	E	(Albertini et al. 2003)
Oculimacula yallundae	N244S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Albertini et al. 2003)
Oculimacula yallundae	S35T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Albertini et al. 2003)
Oculimacula yallundae	Q43H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Albertini et al. 2003)
Oculimacula yallundae	S505Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Albertini et al. 2003)
Oculimacula yallundae	D78Y	K	K	K	K	K	K	K	K	K	K	K	K	K	K	(Albertini et al. 2003)
Puccinia triticina	Y134F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Stammler et al. 2009
Venturia nasicola	Y133	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2013)
Pyrenopeziza brassicae	G460S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Carter et al., 2014)
Pyrenopeziza brassicae	S508T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Carter et al., 2014)

Appendix 5.5 Promoter mutations know to confer resistance to the DMI fungicides via over-expression of the *cyp51* gene and their presence in *Microdochium nivale* isolates. (+) indicates mutation is present, (-) indicates mutation is not present, (/) mutation not characterized by authors.

Species	Homolog	Mutations associated with azole resistance	Presence of mutation in isolate														Reference
			10082	10179	11037	10106	12099	12262	13407	13408	15109	15110	13172	15141	15165	15170	
Aspergillus fumigatus	cyp51A	1.8 Kb transposon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Albarrag et al., 2011)
Blumeria jaapii	cyp51A	2 Kb and 5 Kb insertions	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Ma et al., 2006)
Cercospora beticola	cyp51A	not characterized	/	/	/	/	/	/	/	/	/	/	/	/	/	/	(Nikou et al., 2009)
Moniliana fructicola	cyp51A	65 bp repeat element	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Luo & Schnabel, 2008a)
Mycosphaerella graminicola	cyp51A	120 bp insertion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Cools et al., 2012)
Penicillium digitalum	cyp51A	126 bp 5x tandem repeat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Hamamoto et al., 2000)
Puccinia tritici	cyp51A	not characterized	/	/	/	/	/	/	/	/	/	/	/	/	/	/	(Stammler et al., 2009)
Pyrenopeziza brassicae	cyp51A	46 bp upstream insertion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Carter et al., 2014)
Pyrenopeziza brassicae	cyp51A	151 bp upstream insertion*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Carter et al., 2014)
Pyrenopeziza brassicae	cyp51A	232 bp upstream insertion*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Carter et al., 2014)
Rhynchosporium commune	cyp51A	not characterized	/	/	/	/	/	/	/	/	/	/	/	/	/	/	(Hawkins et al., 2011)
Venturia inequalis	cyp51A	553 bp insertion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Schnabel & Jones, 2001)
Sclerotinia homoeocarpa	cyp51B	not characterized	/	/	/	/	/	/	/	/	/	/	/	/	/	/	(Hulvey et al., 2012)
Penicillium digitalum	cyp51B	199 bp element	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(Sun et al., 2011)

*the 46bp insertion is present within this insertion

Appendix 5.6 Cataloged mutations present in the coding region of the *skn7* gene of isolate 13172.

Δ8-130	A1134G	A1455G	C1677A	A1944G	G2367A	G2625A
G34T	A1143T	T1464C	T1686C	C1968T	T2371G	A2636C
Δ141-286	C1167T	A1482G	T1692A	T1974C	G2373A	A2637G
C288T	A1176G	C1490G	C1698A	T1980C	2388_*ins*CATGGC	G2638C
Δ292-294	C1179T	C1491A/T	C1716T	A1989G	T2392A	A2640G
A296G	G1191C	T1503A	C1725T	C2013T	A2403G	C2641A
C302A	T1194A	C1506T	C1746T	C2019T	G2404C	G2648C
C303G	C1197T	A1508T	T1749C	T2049C	C2406T	A2655G
C305G	A1203G	T1509C	G1758A	A2070G	C2409T	G2659A
C309A	T1212C	T1510C	G1761A	C2085T	T2415C	A2673G
C310A	A1221G	T1515C	T1773C	A2109G	G2419A	T2676C
G312A	G1226C	G1519A	A1776G	G2115T	A2424G	G2688A
T314C	G1230C	A1521T	C1782G	T2118A	C2427T	C2706G
Δ316-339	T1242C	T1522G	A1794C	T2154C	A2436G	G2708A
Δ351-655	T1257C	C1527T	T1812C	T2157C	G2502A	C2709T
Δ662-804	C1260T	G1530T	G1821C	C2178T	T2508C	T2717A
T894C	G1263A	A1534G	G1831A	G2181A	A2519C	
T930C	T1266C	T1536G	A1833C	C2188A	A2523T	
T936A	C1269T	C1545T	T1842C	T2199C	C2526T	
A948G	A1296G	T1563C	C1845G	A2229C	C2532T	
C591G	G1311A	A1569T	G1848A	G2232A	T2538C	
C993G	T1315C	C1572T	A1858G	G2235T	G2553A	
C999T	C1353T	C1578T	1863_*ins*GAACAA	C2247T	G2568A	
G1002C	G1359A	G1596A	A1867T	G2250T	C2571A	
C1017T	T1365G	T1614C	A1879T	T2277C	G2574C	
C1132T	T1389C	T1617C	A1881G	T2280C	T2589G	
T1045C	G1395A	T1623C	T1885C	T2283C	G2592A	
C1059T	T1404C	G1626A	G1891A	C2286T	T2601C	
T1062C	C1410G	A1633G	C1893T	A2293G	A2607C	
A1074G	T1419G	T1641G	T1896C	A2294T	G2608A	
T1080C	G1423A	A1644G	G1908A	A2311C	C2609T	
A1095T	C1438A	G1647A	T1915C	C2316A	T2616A	
A1110C	A1439T	A1656G	G1917T	G2331C	T2619G	
C1116T	C1451A	A1657G	A1929C	A2346G	C2624G	

Chapter 6 General discussion and conclusions

6.1 Major Conclusions

The major conclusions drawn from this body of research and presented in this thesis are as follows:

1. Repeated annual use of iprodione (>75 g 100 m^{-2} $year^{-}$) increases the proportion of *Microdochium nivale* isolates which exhibit decreased sensitivity to iprodione in vitro (measured by EC_{50} and growth on a discriminatory concentration) as evidenced by the high proportion of resistant isolates in the BC populations (82%, average RF = 38) relative to the ON populations (12%, average RF = 2.1) (Chapter 2 – Figures 2.7 and 2.11).

2. Repeated annual use of propiconazole (>35 g 100 m^{-2} $year^{-}$) increases the proportion of *M. nivale* isolates which exhibit decreased sensitivity to propiconazole in vitro (measured by EC_{50} and percentage growth on a discriminatory concentration) as evidenced by the high proportion of resistant isolates in the BC populations (77%, average RF = 20.4) relative to the ON populations (24%, average RF = 7.9) (Chapter 2 – Figures 2.8 and 2.12).

3. Isolates of *M. nivale* with decreased sensitivity to iprodione or propiconazole grow 17% slower on PDA relative to sensitive isolates (Chapter 4 – Table 4.2).

4. Isolates of *M. nivale* with decreased sensitivity to both iprodione and propiconazole grow at the same rate as sensitive isolates on PDA indicating that compensatory mutations may be present in these isolates (Chapter 4 – Table 4.2).

5. There is limited evidence (fall 2016 field trial only) to suggest that applications of iprodione and propiconazole in the field will decrease the damage caused by isolates which exhibited decreased sensitivity to propiconazole and iprodione in the lab (Chapter 3 - Table 3.2)

6. Isolates of *M. nivale* with decreased sensitivity to iprodione and/or propiconazole cause between 53% and 64% less damage in the field relative to sensitive isolates as measured by percent yellowing (Chapter 4 – Table 4.2).

7. Novel mutations in the coding sequence of the *mnos-1* gene may be associated with decreased sensitivity to iprodione in *M. nivale* as evidenced by their presence in four iprodione-resistant isolates and absence in eight sensitive isolates (Chapter 5 – Table 5.3).

8. Novel mutations in the coding sequence of the *mnos-4* gene may be associated with further decreasing sensitivity to iprodione as evidenced by their presence in two iprodione-resistant isolates and absence in eight sensitive isolates (Chapter 5 – Table 5.3).

9. Novel mutations in the transcription factor *mrr1* may decrease sensitivity to both iprodione and propiconazole as evidenced by their presence in four isolates with decreased sensitivity to both fungicides and absence in three isolates sensitive to both fungicides (Chapter 5 – Tables 5.3 and 5.4).

10. There is insufficient direct evidence to show that mutations in the coding sequences of the *mnos-2* and *mnos-5* genes are associated with decreased sensitivity to iprodione in *M. nivale* since no mutations were found in five iprodione-resistant isolates compared to eight sensitive isolates (Chapter 5 – Table 5.3)

11. There is insufficient evidence to suggest that mutations in the coding sequences of *cyp51A, cyp51B, atrD, mfsM2,* and *mfs1* are associated with decreased sensitivity to propiconazole in *M. nivale* since no mutations were found in ten propiconazole-resistant isolates or three sensitive isolates (Chapter 5 – Table 5.4).

12. Comparison of de novo genome assemblies is a feasible method for genotyping isolates as evidenced by the successful separation of *M. nivale* isolates which were known to be different from isolates which had been sequenced two separate times (Chapter 5 – Table 5.2)

6.2 General Discussion and Conclusions

Continuous use of chemical controls products creates a strong selection pressure on a pathogen population. As a result, in many high fungicide use sectors the development of field resistance is a constant threat, as evidenced in the parallel circumstances of antibiotic use for human and livestock diseases (Costelloe et al., 2010). The results of the sensitivity testing found in Chapter 2 addressed the first objective of this work by providing quantitative measures of how sensitivity has decreased to iprodione and propiconazole in Ontario and British Columbia, as well as what proportion of isolates from the sample locations exhibited resistance. The fungicide iprodione has been widely used in Canada since 1987 and propiconazole since 1994 (Health Canada, 2017). Populations of *Microdochium nivale* with continuous high exposure to these chemicals, such as those sampled from the locations in British Columbia, exhibited a decline in sensitivity (iprodione RF = 38, propiconazole RF = 20.4) (Section 2.3). Although there are previous reports of iprodione-resistance in *M. nivale* (Chastagner and Vassey, 1982; Pennucci et al., 1990), this is the first report of resistance in Canada. This is the first report of resistance to the DMI class of fungicides (propiconazole) in *M. nivale* world-wide. Previously, it has been suggested that a population with a resistance factor less than 50 would not be sufficiently intolerant to observe control failure in the field (Golembiewski et al., 1995; Hsiang et al., 2007). The resistance factors for iprodione and propiconazole-resistant isolates were found to be less 50 which suggests that field resistance may not be an issue for golf turf managers. Anecdotally

however, the *M. nivale* isolates from some locations in British Columbia were not controlled adequately in the field by label rates of iprodione or propiconazole.

The results of the field testing in Chapter 3 only partially addressed the second objective of this work in providing limited and inconsistent data. The in vitro fitness tests and the field tests in Chapter 4 addressed the third objective of determining whether fitness costs were associated with decreased sensitivity to iprodione or propiconazole. In a single field experiment (2016) iprodione was able to control those isolates which had tested as insensitive in the lab (Section 3.3). Applications of propiconazole, however, did not reduce the Microdochium patch symptoms caused by some of the lab-insensitive isolates. The inconsistency between field trial results, and the limited data from the two successful trials, highlights the need for further field work with these isolates to reach meaningful conclusions regarding the efficacy of control products for managing lab-insensitive isolates. The field experiments did show consistent results when fungicides were absent, revealing that both iprodione and propiconazole insensitivity may be associated with a reduced ability to cause disease (Section 4.3). Again, the limited number of successful field trials means that these results are only suggestive and are not considered a conclusive result. Isolates with lab insensitivity to a single fungicide, on average, grew at a slower rate relative to sensitive isolates on fungicide-free PDA. However, isolates with resistance to both iprodione and propiconazole did not exhibit a lower growth rate compared to sensitive isolates, additionally, one double-resistant isolate showed a higher level of field damage than its sensitive counterparts.

The results presented in Chapter 5 addressed the fourth and final objective of this work to identify mutations which may underlie the observed fungicide insensitivity. The genomic comparisons between iprodione sensitive and resistant isolates revealed a number of mutations

which may be associated with resistance to iprodione. Of the mutations identified, the mutations in *mnos-1* are the most likely to be a cause of insensitivity to iprodione as previous research with other fungi suggests (Oshima et al., 2002; Grabke et al., 2014). If the osmo-regulation pathway of *M. nivale* is similar to other filamentous fungi, then the genes *mnos-4* lies downstream from *mnos-1* (Kim et al., 2011). This suggests that the mutations identified in *mnos-4* may contribute to a higher degree of insensitivity to iprodione in *M. nivale,* as *os-4* mutations are associated with dicarboximide resistance in other species (Fujimura et al., 2003). Other genes in the osmo-regulation pathway such as *mnos-2* and *mnos-5* were not found to have mutations in our resistant *M. nivale* isolates, suggesting that iprodione resistance in *M. nivale* may not be associated with these genes. Finally, many point mutations, deletions, and insertions were found in the *skn7* gene of a single iprodione insensitive isolate. The *skn7* gene may also be involved with osmo-regulation in filamentous fungi and mutations in this gene were previously identified in field-resistant isolates of other species (Izumitsu et al., 2009). The results derived from de novo sequencing are considered draft genomes, as there are many sources of error in the sequencing pipeline (e.g. DNA fragmentation/quality, mistakes in assembly, mistakes in gene prediction, etc.). As a result, future experiments are needed to confirm that mutations in *os-1, os-4*, and *skn7* are contributing to iprodione resistance in *M. nivale*. This work should include direct gene sequencing by PCR, and possibly gene replacement experiments to determine if sensitivity can be restored to insensitive isolates and if resistance can be conferred to sensitive isolates.

Similar comparisons were made between propiconazole sensitive and resistant isolates. No mutations were identified in the coding regions of either *cyp51* paralogs, which are known targets of the DMI fungicides (Cañas-Gutiérrez et al., 2009). Additionally, no mutations were found in the promoter region of *cyp51* which, if present, could have suggested expression

alterations, another known cause of resistance to DMI fungicides. Gene expression analysis should be done in the future to determine if increased expression of *cyp51* is the cause of propiconazole insensitivity in *M. nivale*.

Increased expression of efflux transporters is another mechanism by which resistance to dicarboximide or DMI fungicides may be conferred (Ma and Tredway, 2013). Previously, the increased expression of four efflux transporters: *atrD*, *mfsM2*, *atrB*, and *mfs1*, have been associated with resistance to DMI fungicides (Kretschmer et al., 2009; Sang et al., 2015). Of these four transporters, *mfsM2* and *atrB*, were also associated with resistance to dicarboximides. No coding region mutations or promoter regions mutations were found for *atrD*, *mfsM2*, or *mfs1* in resistant isolates. The efflux transporter atrB is thought to be regulated through the transcription factor *mrr1*, as there is evidence that mutations in *mrr1* lead to increased expression of *atrB* and resistance to multiple fungicide groups (Kretschmer et al., 2009). *Mrr1* mutations were identified in isolates with resistance to both iprodione and propiconazole. These mutations may play a role in the observed resistance but expression analysis of *atrB* is needed to test this hypothesis.

The results of the fungicide sensitivity testing, fitness experiments, and genomic work may contain biases, and should be interpreted with caution. Firstly, the sampling of the British Columbia isolates occurred in only two locations and those locations may not be representative of the entire area. Additionally, the sample locations in B.C were selected because there was anecdotal evidence that resistance may be developing. Although there was a larger range of samples locations in Ontario, a relatively large proportion of the Ontario isolates were collected from the Guelph Turfgrass Institute. Finally, based on the full genome comparison of several isolate pairs from B.C, it is likely that there are clonal isolates within the collection (i.e. isolates

which were treated as individuals, but are identical genotypes) which have artificially inflated averages and skewed correlations.

This is the first work which has attempted to identify mutations conferring fungicide resistance in *M. nivale* and, overall, these results suggest that field resistance to iprodione may be conferred by different mutations, and that resistance to propiconazole is likely not caused by mutations in the coding region of the identified target gene *cyp51*. Depending on which mutations arise, resistance to iprodione and propiconazole may not be a major concern for turfgrass managers due to the associated fitness costs (i.e. lack of field damage for some resistant isolates) although further field testing is required.

Although the efficacy of resistance management strategies may be dependent on the presence of fitness costs, these strategies should be implemented before insensitivity is observed in the field. Additionally, resistance management should be considered an essential component of 'integrated pest management'. The most reliable method for preventing resistance is to eliminate the use of fungicides. Realistically however, most disease management programs will continue to rely on fungicide inputs at least for the near future. Alternating or mixing the mode of actions used and limiting use to high disease pressure areas or times are accepted strategies for reducing the risk of resistance developing (Angelini et al., 2015). There is evidence which supports that implementing resistance management programs can reduce problems associated with decreasing sensitivity and prolong to useful life of these chemicals.

Turfgrass managers dealing with fungicide resistance have a number of control options available to them. The apparent presence of fitness costs associated with iprodione and propiconazole resistance suggest that reducing or discontinuing the use of those chemicals may be an effective management tool. By eliminating the fungicide selection pressure the sensitivity

of the population may revert to predominantly sensitive. The length of time needed for a population to revert is not clear, and may involve a time span greater than a typical career length. There is evidence to suggest tank-mixing iprodione or propiconazole with a secondary mode of action with multi-site action (e.g. chlorothalonil) may boost the efficacy and reduce to risk of further resistance development (Ishii and Hollomon, 2015). Additionally, the use of cultural control measures such as shade reduction, timing of irrigation, dew removal and possibly maintaining lower soil pH may provide some benefit in disease reduction (Smiley et al., 2005).

Monitoring of fungicide resistance in *M. nivale* should be continued and those isolates already collected should be examined for cross-resistance to other fungicides on the Canadian market (e.g. SDHIs, QoIs, other DMIs). Further work using gene replacement is needed to confirm that the mutations identified in *os-1* and *os-4* contributes to dicarboximide resistance. These tests should attempt to engineer resistance and test whether sensitivity can be restored. Primers could also be designed for the rapid identification of iprodione-resistant isolates. Gene expression work (e.g. rtPCR, RNA-seq) should be performed to identify other potential gene differences, to assess if *cyp51* is over-expressed in propiconazole-resistant isolates, and if *atrB* is over-expressed is those isolates with multiple resistances.

Fungicide resistance is an increasing problem, particularly in high use sectors like golf turf management. Monitoring for resistant isolates is an essential first step in preventing the development of field resistance. In addition, determining if fitness costs are associated with resistance provides information which is fundamental for the management of resistant isolates by suggesting which approaches or methods may be effective. Finally, identifying the genetic determinants of resistance is an integral component for the long-term management of resistant strains, for example, by facilitating the use of rapid PCR-based identification of resistant isolates.

This work provides an assessment on the sensitivity of *Microdochium nivale* to iprodione and propiconazole in Canada, and a framework for further investigation of the genetic basis for the resistance.

Literature Cited

Abdolrasouli, A., J. Rhodes, M. Beale, F. Hagen, T.R. Rogers, A. Chowdhary, J.F. Meis, and M.C. Fisher. 2015. Genomic context of azole-resistance mutations in *Aspergillus fumigatus* using whole-genome sequencing. MBio 6: 1–11.

Agrawal, M., and S. Sunder. 2014. Variations in sensitivity of *Rhizoctonia solani* AG-1 IA isolates from rice to carbendazim and hexaconazole. J. Mycol. Plant Pathol. 43: 394–398.

Agrios, G.N. (Ed). 2005. Plant Pathology. 5th ed. Elsevier Academic Press, Burlington, MA.

Albarrag, A.M., M.J. Anderson, S.J. Howard, G.D. Robson, P.A. Warn, D. Sanglard, and D.W. Denning. 2011. Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in cyp51A, upregulation of cyp51A, and integration and activation of transposon Atf1 in the cyp51A promoter. Antimicrob. Agents Chemother. 55: 5113–5121.

Alberoni, G., M. Collina, C. Lanen, P. Leroux, and A. Brunelli. 2010. Field strains of *Stemphylium vesicarium* with a resistance to dicarboximide fungicides correlated with changes in a two-component histidine kinase. Eur. J. Plant Pathol. 128: 171–184.

Alberoni, G., M. Collina, D. Pancaldi, and A. Brunelli. 2005. Resistance to dicarboximide fungicides in *Stemphylium vesicarium* of Italian pear orchards. Eur. J. Plant Pathol. 113: 211–219.

Albertini, C., M. Gredt, and P. Leroux. 2003. Polymorphism of 14α-demethylase gene (CYP51) in the cereal eyespot fungi *Tapesia acuformis* and *Tapesia yallundae*. Eur. J. Plant Pathol. 109: 117–128.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–10.

Anderson, J.B. 2005. Evolution of antifungal drug resistance: mechanisms and pathogen fitness. Nat. Rev. Microbiol. 3: 547–556.

Andersson, D.I., and D. Hughes. 2010. Antibiotic resistance and its cost: Is it possible to reverse resistance? Nat. Rev. Microbiol. 8: 260–271.

Angelini, R.M.D.M., S. Pollastro, and F. Faretra. 2015. Genetics of fungicide resistance. p. 13–34. *In* Ishii, H., Holloman, D.W. (eds.), Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management. Springer, Japan.

Annonymous. 1998. Golf course pesticide use and monitoring. Alberta Environmental Protection, Chemicals Assessment and Management Division, Pesticide Management Branch, Edmonton.

Annonymous. 2017. Golf course fungicide records.

Avenot, H., P. Simoneau, B. Iacomi-Vasilescu, and N.B. Simoneau. 2005. Characterization of mutations in the two-component histidine kinase gene AbNIK1 from *Alternaria brassicicola* that confer high dicarboximide and phenylpyrrole resistance. Curr. Genet. 47: 324–243.

Banno, S., F. Fukumori, A. Ichiishi, K. Okada, H. Uekusa, M. Kimura, and M. Fujimura. 2008. Genotyping of benzimidazole-resistant and dicarboximide-resistant mutations in *Botrytis cinerea* using real-time polymerase chain reaction assays. Phytopathology 98: 397–404.

Bardas, G.A., C.K. Myresiotis, and G.S. Karaoglanidis. 2008. Stability and fitness of anilinopyrimidine-resistant strains of *Botrytis cinerea*. Phytopathology 98: 443–450Available at http://apsjournals.apsnet.org/doi/10.1094/PHYTO-98-4-0443.

Becher, R., and S. Wirsel. 2012. Fungal cytochrome P450 sterol 14 α -demethylase (CYP51) and

azole resistance in plant and human pathogens. Appl. Microbiole. Biotechnol 95: 825–840.
Beever, R.E. 1983. Osmotic sensitivity of fungal variants resistant to dicarboximide fungicides. Trans. Br. Mycol. Soc. 80: 327–331.
Bergeron, B. 2003. Bioinformatics computing. Prentice Hall PTR, New Jersey.
Bishop, P., J. Sorochan, B.H. Ownley, T.J. Samples, A.S. Windham, M.T. Windham, and R.N. Trigiano. 2008. Resistance of *Sclerotinia homoeocarpa* to iprodione, propiconazole, and thiophanate-methyl in Tennessee and northern Mississippi. Crop Sci. 48: 1615–1620.
Björkholm, B., M. Sjölund, P.G. Falk, O.G. Berg, L. Engstrand, and D.I. Andersson. 2001. Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. Proc. Natl. Acad. Sci. U. S. A. 98: 14607–14612.
Bjorkman, J., I. Nagaev, O.G. Berg, D. Hughes, and D. Andersson. 2000. Effect of environment on compensatory mutants to ameliorate costs of antibiotic resistance. Science (80-.). 287: 1479–1482.
Brenneman, T.B., P.M. Phipps, and R.J. Stipes. 1987. Sclerotinia blight of peanut: Relationship between in vitro resistance and field efficacy of dicarboximide fungicides. Phytopathology 77: 1028–1032.
Brownback, L.. 2002. Survey of isolates of *Sclerotinia homoeocarpa* in Indiana for sensitivity to three fungicides. Phytopathology 92: S10.
Buermans, H.P.J., and J.T. Den Dunnen. 2014. Next generation sequencing technology: advances and applications. Biochim. Biophys. Acta 1842: 1932–1941.
Cañas-Gutiérrez, G.P., M.J. Angarita-Velásquez, J.M. Restrepo-Flórez, P. Rodríguez, C.X. Moreno, and R. Arango. 2009. Analysis of the CYP51 gene and encoded protein in propiconazole-resistant isolates of *Mycosphaerella fijiensis*. Pest Manag. Sci. 65: 892–899.
Carter, H.E., B.A. Fraaije, J.S. West, S.L. Kelly, A. Mehl, M.W. Shaw, and H.J. Cools. 2014. Alterations in the predicted regulatory and the predicted coding regions of the sterol 14-demethylase gene (CYP51) confer decreased azole sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae*. Mol. Plant Pathol. 15: 513–522.
Catlett, N.L., O.C. Yoder, and B.G. Turgeon. 2003. Whole-genome analysis of two-component signal transduction genes in fungal pathogens. Eukaryot. Cell 2: 1151–1161.
Cevat, H. 1992. Japanse roest terug van nooit weggeweest. nu maatregelen nemen om aantasting in najaar te voorkomen. Vakbl. voor Bloemist. 29: 32–33.
Chastagner, G.A., and K. Riley. 1987. Occurrence and control of benzimidazole and dicarboximide resistant *Botrytis* spp on bulb crops in western Washington and Oregon. Phytopathology 77: 1237.
Chastagner, G., and W. Vassey. 1982. Occurence of iprodione-tolerant *Fusarium nivale* under field conditions. Plant Dis. 66: 112–114.
Chen, F., X. Liu, S. Chen, E. Schnabel, and G. Schnabel. 2013. Characterization of *Monilinia fructicola* strains resistant to both propiconazole and boscalid. Plant Dis. 97: 645–651.
Cools, H.J., C. Bayon, S. Atkins, J.A. Lucas, and B.A. Fraaije. 2012. Overexpression of the sterol 14α-demethylase gene (MgCYP51) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. Pest Manag. Sci. 68: 1034–1040.
Cools, H.J., and B.A. Fraaije. 2013. Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. Pest Manag. Sci. 69: 150–155.
Cools, H.J., N.J. Hawkins, and B.A. Fraaije. 2013. Constraints on the evolution of azole resistance in plant pathogenic fungi. Plant Pathol. 62: 36–42.

Cortes-Barco, A.M., T. Hsiang, and P.H. Goodwin. 2010. Induced systemic resistance against three foliar diseases of *Agrostis stolonifera* by (2R,3R)-butanediol or an isoparaffin mixture. Ann. Appl. Biol. 157: 179–189.

Costelloe, C., C. Metcalfe, A. Lovering, D. Mant, and A.D. Hay. 2010. Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis. Bmj 340: 1–11.

Cowen, L.E., L.M. Kohn, and J.B. Anderson. 2001. Divergence in fitness and evolution of drug resistance in experimental populations of *Candida albicans*. J. Bacteriol. 183: 2971–2978.

Cox, K.D., P.K. Bryson, and G. Schnabel. 2007. Instability of propiconazole resistance and fitness in *Monilinia fructicola*. Phytopathology 97: 448–453.

Cristani, C., and P. Gambogi. 1993. Isolamento in laboratorio di ceppi di *Microdochium (Fusarium) nivale* con sensibilita' ridotta a prodotti inibitori della diosntesi degli steroli (IBS). Riv. di Patalogia Veg. 3: 49–57.

Cui, W., R.E. Beever, S.L. Parkes, and M.D. Templeton. 2004. Evolution of an osmosensing histidine kinase in field strains of *Botryotinia fuckeliana* (*Botrytis cinerea*) in response to dicarboximide fungicide usage. Phytopathology 94: 1129–1135.

Cui, W., R.E. Beever, S.L. Parkes, P.L. Weeds, and M.D. Templeton. 2002. An osmosensing histidine kinase mediates dicarboximide fungicide resistance in *Botryotinia fuckeliana* (*Botrytis cinerea*). Fungal Genet. Biol. 36: 187–198.

Darling, A.C.E., B. Mau, F.R. Blattner, and N.T. Perna. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. 14(7): 1394–403.

Délye, C., L. Bousset, and M.-F. Corio-Costet. 1998. PCR cloning and detection of point mutations in the eburicol 14 alpha -demethylase (CYP51) gene from *Erysiphe graminis* f. sp. *hordei* , a recalcitrant fungus. Curr. Genet. 34: 399–403.

Dennis, C., and R.P. Davis. 1979. Tolerance of *Botrytis cinerea* to iprodione and vinclozolin. Plant Pathol. 28: 131–133.

Detweiler, A.., J. Vargas, and T. Danneberger. 1983. Resistance of *Sclerotinia homoeocarpa* to iprodione and benomyl. Plant Dis. 67: 627–630.

Devries, R.E., R.N. Trigiano, M.T. Windham, A.S. Windham, and J.M. Vargas. 2008. Genetic analysis of fungicide-resistant *Sclerotinia homoeocarpa* isolates from Tennessee and northern Mississippi. Plant Dis. 92: 83–90.

Dry, I.B., K.H. Yuan, and D.G. Hutton. 2004. Dicarboximide resistance in field isolates of *Alternaria alternata* is mediated by a mutation in a two-component histidine kinase gene. Fungal Genet. Biol. 41: 102–108.

Duan, Y., G. Chang-Yan, and M. Zhou. 2014. Molecular and biochemical characterization of *Sclerotinia sclerotiorum* laboratory mutants resistant to dicarboximide and phenylpyrrole fungicides. J Pest Sci 87: 221–230.

Edwards, K., C. Johnstone, and C. Thompson. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19: 1349.

Elad, Y. 1992. Reduced sensitivity of *Botrytis cinerea* to two sterol, biosynthesis inhibiting inhibiting fungicides: fenetrazole and fenethanil. Plant Pathol. 41: 47–54.

Elmer, P., M. Braithwaite, and D. Saville. 1992. Changes in triforine sensitivity in populations of *Monilinia fructicola* from Hawkes Bay orchards. Proc. 45th. New Zeal. Plant Prot. Conf. 45: 138–140.

Elmer, P.A.G., and R.E. Gaunt. 1994. The biological characteristics of dicarboximide-resistant isolates of *Monilinia fructicola* from New Zealand stone-fruit orchards. Plant Pathol. 43:

130–137.
Engels, A.J.G., and M.A. de Waard. 1996. Fitness of isolates of *Erysiphe graminis* f.sp. *tritici* with reduced sensitivity to fenpropimorph. Crop Prot. 15: 771–777.
Fillinger, S., S. Ajouz, P.C. Nicot, P. Leroux, and M. Bardin. 2012. Functional and structural comparison of pyrrolnitrin- and iprodione-induced modifications in the class III histidine-kinase Bos1 of *Botrytis cinerea* (Z Zhang, Ed.). PLoS One 7: 1–14.
Fletcher, J., and M. Wolfe. 1981. Insensitivity of *Erysiphe graminis* f. sp. *hordei* to triadimefon, triadimenol and other fungicides. *In* 1981 British Crop Protection Conference: pests and diseases (11th British Insecticide and Fungicide Conference): proceedings, held at Hotel Metropole, Brighton, England, November 16-19, 1981. BCPC Publications, Croydon [England].
FRAC. 2015. FRAC Code List 2015: Fungicides sorted by mode of action.
FRAC. 2016. Expert fora dicarboxamides SOA and mechanism(s) of resistance. Available at http://www.frac.info/expert-fora/dicarboxamides/soa-and-mechanism(s)-of-resistance (verified 28 June 2016).
FRAC. 2017. List of plant pathogenic organisms resistant to disease control agents.
Fujimura, M., N. Ochiai, A. Ichiishi, R. Usami, K. Horikoshi, and I. Yamaguchi. 2000. Fungicide resistance and osmotic stress sensitivity in os mutants of *Neurospora crassa*. Pestic. Biochem. Physiol. 67: 125–133.
Fujimura, M., N. Ochiai, M. Oshima, T. Motoyama, A. Ichiishi, R. Usami, K. Horikoshi, and I. Yamaguchi. 2003. Putative homologs of SSK22 MAPKK kinase and PBS2 MAPK kinase of *Saccharomyces cerevisiae* encoded by os-4 and os-5 genes for osmotic sensitivity and fungicide resistance in *Neurospora crassa*. Biosci. Biotechnol. Biochem 67: 186–191.
Golembiewski, R.., J.. Vargas, A.. Jones, and A.. Detweiler. 1995. Detection of demethylation inhibitor (DMI) resistance in *Sclerotinia homoeocarpa* populations. Plant Dis. 79: 491–493.
Goszczynski, W., J. Cimanowski, and R. Bachnacki. 1988. First note on the occurrence of a strain of *Sphaerotheca morsuvae* Schw. –Berk. with decreased ensitivity to triadimefon. Fruit Sci. Reports 15: 181–184.
Grabke, A., D. Fernández-Ortuño, A. Amiri, X. Li, N.A. Peres, P. Smith, and G. Schnabel. 2014. Characterization of iprodione resistance in *Botrytis cinerea* from strawberry and blackberry. Phytopathology 104: 396–402.
Grindle, M., and W. Temple. 1982. Fungicide-resistant of os mutants of *Neurospora crassa*. Neurospora News 29: 16.
Grindle, M., and W. Temple. 1985. Sporulation and osmotic sensitivity of dicarboximide-resistant mutants of *Neurospora crassa*. Trans. Br. Mycol. Soc. 84: 369–372.
Guarnaccia, V., D. Aiello, G. Polizzi, G. Perrone, G. Stea, and A. Vitale. 2014. Emergence of prochloraz-resistant populations of *Calonectria pauciramosa* and *Calonectria polizzii* in ornamental nurseries of Southern Italy. Plant Dis. 98: 344–350.
Hamada, M.S., Y. Yin, and Z. Ma. 2011. Sensitivity to iprodione, difenoconazole and fludioxonil of *Rhizoctonia cerealis* isolates collected from wheat in China. Crop Prot. 30: 1028–1033.
Hamamoto, H., K. Hasegawa, R. Nakaune, Y.J. Lee, Y. Makizumi, K. Akutsu, and T. Hibi. 2000. Tandem repeat of a transcriptional enhancer upstream of the sterol 14alpha - demethylase gene (CYP51) in *Penicillium digitatum*. Appl. Environ. Microbiol. 66: 3421–3426.
Haridas, S., C. Breuill, J. Bohlmann, and T. Hsiang. 2011. A biologist's guide to de novo

genome assembly using next-generation sequence data: A test with fungal genomes. J. Microbiol. Methods 86: 368–375.
Hawkins, N.J., H.J. Cools, M.W. Shaw, and H. Sierotzki. 2011. Molecular mechanisms of altered triazole sensitivity in *Rhynchosporium secalis*. p. 129–134. *In* Modern Fungicides and Antifungal Compounds. Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany.
Hayashi, K., and M.A. De Waard. 2002. Bcmfs1, a novel major facilitator superfamily transporter from *Botrytis cinerea*, provides tolerance towards the natural toxic compounds camptothecin and cercosporin and towards fungicides. Appl. Environ. Microbiol. 68: 4996–5004.
Health Canada. 2016a. Consum. Prod. Saf. Label search results.Available at http://pr-rp.hc-sc.gc.ca/ls-re/lbl_detail-eng.php?p_disp_regn='20110'&p_regnum=20110 (verified 16 June 2016a).
Health Canada. 2016b. Consum. Prod. Saf. Label search results.Available at http://pr-rp.hc-sc.gc.ca/ls-re/lbl_detail-eng.php?p_disp_regn=%2723706%27&p_regnum=23706.
Hollomon, D.W. (University of Bristol, Bristol, U., J.A. Butters, and J.A. Hargreaves. 1990. Resistance to sterol biosynthesis-inhibiting fungicides: current status and biochemical basis. ACS Symp. Ser. - Am. Chem. Soc.
Hollomon, D.W., J.A. Butters, and S.J. Kendall. 1997. Mechanisms of Resistance to Fungicides. Springer Berlin Heidelberg.
Hsiang, T. 2005. Turfgrass fungicides in Canada.
Hsiang, T. 2009. All you ever wanted to know about Fusarium patch, Microdochium patch, pink snow mold or whatever that disease is called. Greenmaster 44: 18–19.
Hsiang, T., and G.A. Chastagner. 1991. Growth and virulence of fungicide-resistant isolates of three species of *Botrytis*. Can. J. Plant Pathol. 13: 226–231.
Hsiang, T., A. Liao, and D. Benedetto. 2007. Sensitivity of *Sclerotinia homoeocarpa* to demethylation-inhibiting fungicides in Ontario, Canada, after a decade of use. Plant Pathol. 56: 500–507.
Hsiang, T., N. Matsumoto, and S. Millet. 1999. Biology and management of *Typhula* snow molds of turfgrass. Plant Dis. 83: 788–798.
Hsiang, T., L. Yang, and W. Barton. 1997. Baseline sensitivity and cross-resistance to demethylation-inhibiting fungicides in Ontario isolates of *Sclerotinia homoeocarpa*. Eur. J. Plant Pathol. 103: 409–416.
Hsiang, T., L. Yang, and W. Barton. 1998. Relative virulence of isolates of *Sclerotinia homoeocarpa* with varying sensitivity to propiconazole. Eur. J. Plant Pathol. 104: 163–169.
Hubbard, J.C., K. V Subbarao, and S.T. Koike. 1997. Development and significance of dicarboximide resistance in *Sclerotinia minor* isolates from commercial lettuce fields in California. Plant Dis. 81: 148–153.
Hulvey, J., J.T. Popko, H. Sang, A. Berg, and G. Jung. 2012. Overexpression of ShCYP51B and ShatrD in *Sclerotinia homoeocarpa* isolates exhibiting practical field resistance to a demethylation inhibitor fungicide. Appl. Environ. Microbiol. 78: 6674–6682.
Hunter, T., V. Jordan, and S.J. Kendall. 1986. Fungicide sensitivity changes in Rhynchosporium secalis in glasshouse experiments. Proc. Br. Crop Prot. Conf. Pests Dis.: 523–536.
Hutton, D.. 1988. The appearance of dicarboximide resistance in *Alternaria alternats* [sic] in passionfruit in south-east Queensland. Australas. Plant Pathol. 17: 34–36.
IPM Council of Canada. 2017. Registered Golf Course Search. Available at

http://public.ipmcouncilcanada.org/ (verified 21 January 2018).
Ishii, H., and D.W. Hollomon. 2015. Fungicide resistance in plant pathogens (H Ishii and DW Hollomon, Eds.). Springer.
Izumitsu, K., A. Yoshimi, S. Hamada, A. Morita, Y. Saitoh, and C. Tanaka. 2009. Dic2 and Dic3 loci confer osmotic adaptation and fungicidal sensitivity independent of the HOG pathway in *Cochliobolus heterostrophus*. Mycol. Res. 113: 1208–1215.
Jewell, L.E., and T. Hsiang. 2013. Multigene differences between *Microdochium nivale* and *Microdochium majus*. Botany 91: 99–106.
Jo, Y., A.L. Niver, J.W. Rimelspach, and M.J. Boehm. 2006. Fungicide sensitivity of *Sclerotinia homoeocarpa* from golf courses in Ohio. Plant Dis. 90: 807–813.
Karaoglanidis, G.S., P. Ionnidis, and C.C. Thanossoulopoulos. 2000. Reduced sensitivity of *Cercospora beticola* isolates to sterol demethylation inhibiting fungicides. Plant Pathol. 49: 267–272.
Karaoglanidis, G.S., A.N. Markoglou, G.A. Bardas, E.G. Doukas, S. Konstantinou, and J.F. Kalampokis. 2011. Sensitivity of *Penicillium expansum* field isolates to tebuconazole, iprodione, fludioxonil and cyprodinil and characterization of fitness parameters and patulin production. Int. J. Food Microbiol. 145: 195–204.
Karaoglanidis, G.S., C.C. Thanassoulopoulos, and P.M. Ioannidis. 2001. Fitness of *Cercospora beticola* field isolates – resistant and – sensitive to demethylation inhibitor fungicides. Eur. J. Plant Pathol. 107: 337–347.
Katan, T. 1982. Resistance to 3,5 dichlorophenyl N cyclic imide ('dicarboximide') fungicides in the grey mould pathogen *Botrytis cinerea* on protected crops. Plant Pathol. 31: 133–141.
Katan, T., and E. Shabi. 1982. Characterization of a dicarboximide-fungicides-resistant laboratory isolate of *Monilinia laxa*. Phytoparasitica 10: 241–245.
Kendall, S.O., D.W. Hollomon, L.R. Cooke, and D.R. Jones. 1993. Changes in sensitivity to DMI fungicides in *Rhynchosporium secalis*. Crop Prot. 12: 357–362.
Kim, S.-Y., Y.-J. Ko, K.-W. Jung, A. Strain, K. Nielsen, and Y.-S. Bahn. 2011. Hrk1 plays both Hog1-dependent and -independent roles in controlling stress response and antifungal drug resistance in *Cryptococcus neoformans*. PLoS One 6: e18769.
Kinay, P., M.F. Mansour, F.M. Gabler, D.A. Margosan, and J.L. Smilanick. 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop Prot. 26: 647–656.
Köller, W. 1988. Sterol demethylation inhibitors: mechanism of action and resistance. p. 79–88. *In* Delp, C.. (ed.), Fungicide Resistance in North America. APS Press, St. Paul,Minnesota.
Köller, W., and H. Scheinpflug. 1987. Fungal resistance to sterol biosynthesis inhibitors: a new challenge. Plant Dis. 71: 1066–1074.
Köller, W., and J. Wubben. 1988. Variable resistance factors of fungicides acting as sterol demethylation inhibitors. Pestic. Sci. 26: 133–145.
Kretschmer, M., M. Leroch, A. Mosbach, A.-S. Walker, S. Fillinger, D. Mernke, H.-J. Schoonbeek, J.-M. Pradier, P. Leroux, M.A. De Waard, M. Hahn, and A. Andrianopoulos. 2009. Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. PLoS Pathog 5: 1–13.
Kuck, K.. 1994. Evaluation of anti-resistance strategies. Monogr. Crop Prot. Counc. 43: 43.
Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: molecular evolutionary genetics analysis bersion 7.0 for bigger datasets. Mol. Biol. Evol. 33: 1870–1874.
Lalève, A., S. Fillinger, and A. Walker. 2014. Fitness measurement reveals contrasting costs in

homologous recombinant mutants of *Botrytis cinerea* resistant to succinate dehydrogenase inhibitors. Fungal Genet. Biol. 67: 24–36.
Lamondia, J.A., and S.M. Douglas. 1997. Sensitivity of *Botrytis cinerea* from Connecticut greenhouses to benzimidazole and dicarboximide fungicides. Plant Dis. 81: 729–732.
Lee, H., J. Gurtowski, S. Yoo, M. Nattestad, S. Marcus, S. Goodwin, W.R. Mccombie, and M.C. Schatz. 2016. Third-generation sequencing and the future of genomics. bioRxivAvailable at http://biorxiv.org/content/biorxiv/early/2016/04/13/048603.full.pdf (verified 2 April 2017).
Leroux, P. 2007. Chemical control of *Botrytis* and its resistance to chemical fungicides. p. 195–217. *In* Elad, Y., Williamson, B., Tudzynski, P., Delen, N. (eds.), *Botrytis*: Biology, Pathology and Control. Springer, Dordrecht, The Netherlands.
Leroux, P., C. Lanen, and R. Fritz. 1992. Similarities in the antifungal activities of fenpiclonil, iprodione and tolclofos-methyl against *Botrytis cinerea* and *Fusarium nivale*. Pestic. Sci. 36: 255–261.
Leroux, P., and P. Marchegay. 1991. Caractérisation des souches de *Pseudocercosporella herpotrichoides* agent du Piétin-verse des céréales, resistantes au prochloraze, isolées en France sur blé tendre d'hiver. Agronomie 11: 767–776.
Leroux, P., and A.S. Walker. 2011. Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. Pest Manag. Sci. 67: 44–59.
Li, R., Y. Li, K. Kristiansen, and J. Wang. 2008. SOAP: Short oligonucleotide alignment program. Bioinformatics 24(5): 713–714.
Liu, W., P. Leroux, and S. Fillinger. 2008. The HOG1-like MAP kinase Sak1 of *Botrytis cinerea* is negatively regulated by the upstream histidine kinase Bos1 and is not involved in dicarboximide- and phenylpyrrole-resistance. Fungal Genet. Biol. 45: 1062–1074.
Liu, X., F. Yu, G. Schnabel, J. Wu, Z. Wang, and Z. Ma. 2011. Paralogous cyp51 genes in *Fusarium graminearum* mediate differential sensitivity to sterol demethylation inhibitors. Fungal Genet. Biol. 48: 113–123.
Lucas, J.A., N.J. Hawkins, and B.A. Fraaije. 2015. The Evolution of Fungicide Resistance. Adv. Appl. Microbiol. 90: 29–92Available at https://ac.els-cdn.com/S0065216414000021/1-s2.0-S0065216414000021-main.pdf?_tid=265726c8-ac64-4932-858e-02f3bef9a851&acdnat=1521747940_fb3e0d0d4c2c4c3e9090112524245567 (verified 22 March 2018).
Luo, C.X., and G. Schnabel. 2008. Adaptation to fungicides in *Monilinia fructicola* isolates with different fungicide resistance phenotypes. Phytopathology 98: 230–238.
Ma, Z., and T.J. Michailides. 2004. Characterization of iprodione-resistant *Alternaria* isolates from pistachio in California. Pestic. Biochem. Physiol. 80: 75–84.
Ma, Z., T.J. Proffer, J.L. Jacobs, and G.W. Sundin. 2006. Overexpression of the 14 -demethylase target gene (CYP51) mediates fungicide resistance in *Blumeriella jaapii*. Appl. Environ. Microbiol. 72: 2581–2585.
Ma, B., and L.P. Tredway. 2013. Induced overexpression of cytochrome P450 sterol 14-demethylase gene (CYP51) correlates with sensitivity to demethylation inhibitors (DMIs) in *Sclerotinia homoeocarpa*. Pest Manag. Sci. 69: 1369–1378.
Ma, Z., L. Yan, Y. Luo, and T.J. Michailides. 2007. Sequence variation in the two-component histidine kinase gene of *Botrytis cinerea* associated with resistance to dicarboximide fungicides. Pestic. Biochem. Physiol. 88: 300–306.
McKnight, T.. 2000. Climate zones and types. *In* Physical geography: a landscape appreciation.

Prentice Hill, Upper Saddle River, NJ.
McPhee, W. 1980. Some characteristics of *Alternaria alternata* strains resistant to iprodione. Plant Dis. 64: 847–849.
Metcalfe, R.J., M.W. Shaw, and P.E. Russell. 2000. The effect of dose and mobility on the strength of selection for DMI fungicide resistance in inoculated field experiments. Plant Pathol. 49: 546–557.
Mikaberidze, A., B.A. Mcdonald, and S. Bonhoeffer. 2014. Can high-risk fungicides be used in mixtures without selecting for fungicide resistance? Phytopathology 104: 324–331.
Mikaberidze, A., B.A. Mcdonald, A. Mikaberidze, and B.A. Mcdonald. 2015. Fitness cost of resistance: impact on management. p. 77–89. *In* Ishii, H., Hollomon, D.W. (eds.), Fungicide Resistance in Plant Pathogens. Springer.
Miller, G.L., K.L. Stevenson, and L.L. Burpee. 2002. Sensitivity of *Sclerotinia homoeocarpa* isolates to propiconazole and impact on control of dollar spot. Plant Dis. 86: 1240–1246.
Mohammad, P. 2007. Handbook of turfgrass management and physiology. CRC Press.
Mohanta, T.K., and H. Bae. 2015. The diversity of fungal genome. Biol. Proced. Online 17: 1–9.
Mohd-Assaad, N., B.A. McDonald, and D. Croll. 2016. Multilocus resistance evolution to azole fungicides in fungal plant pathogen populations. Mol. Ecol. 25(24): 6124–6142.
Monteith, J.., and A.. Dahl. 1932. Turf diseases and their control. US Golf Assoc. Green Sect.
Morio, F., C. Loge, B. Besse, C. Hennequin, and P. Le Pape. 2010. Screening for amino acid substitutions in the *Candida albicans* Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. Diagn. Microbiol. Infect. Dis. 66: 373–384.
Morton, V., and T. Staub. 2008. A short history of fungicides. Available at http://www.apsnet.org/publications/apsnetfeatures/Pages/Fungicides.aspx.
Myresiotis, C.K., and F. Agriculture. 2007. Resistance of *Botrytis cinerea* isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide, benzimidazole, and dicarboximide fungicides. Plant Dis. 91: 407–413.
Myresiotis, C.K., G.S. Karaoglanidis, and K. Tzavella-Klonari. 2007. Resistance of *Botrytis cinerea* isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide, benzimidazole, and dicarboximide fungicides. Plant Dis. 91: 407–413.
Nakaune, R., K. Adachi, O. Nawata, M. Tomiyama, K. Akutsu, and T. Hibi. 1998. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Appl. Environ. Microbiol. 64: 3983–8.
Van Den Nieuwelaar, A.-M., and T. Hsiang. 2014. Changes in sensitivity of the dollar spot fungus, *Scelrotinia homoeocarpa*, to the demethylation inhibitor fungicide propiconazole 20 years after first use. Eur. J. Turfgrass Sci. 45: 43–44.
Nikou, D., A. Malandrakis, M. Konstantakaki, J. Vontas, A. Markoglou, and B. Ziogas. 2009. Molecular characterization and detection of overexpressed C-14 alpha-demethylase-based DMI resistance in *Cercospora beticola* field isolates. Pestic. Biochem. Physiol. 95: 18–27.
Nuninger-Ney, C., F.-J. Schwinn, and T. Staub. 1989. In vitro selection of sterol-biosynthesis-inhibitor (SBI)- resistant mutants in *Monilinia fructicola* (Wint.) Honey*. Netherl. J. Plant Pathol. 95: 137–150.
O ’leary, N.A., M.W. Wright, J.R. Brister, S. Ciufo, D. Haddad, R. Mcveigh, B. Rajput, B. Robbertse, B. Smith-White, D. Ako-Adjei, A. Astashyn, A. Badretdin, Y. Bao, O. Blinkova, V. Brover, V. Chetvernin, J. Choi, E. Cox, O. Ermolaeva, C.M. Farrell, T. Goldfarb, T. Gupta, D. Haft, E. Hatcher, W. Hlavina, V.S. Joardar, V.K. Kodali, W. Li, D. Maglott, P.

Masterson, K.M. Mcgarvey, M.R. Murphy, K.O.' Neill, S. Pujar, S.H. Rangwala, D. Rausch, L.D. Riddick, C. Schoch, A. Shkeda, S.S. Storz, H. Sun, F. Thibaud-Nissen, I. Tolstoy, R.E. Tully, A.R. Vatsan, C. Wallin, D. Webb, W. Wu, M.J. Landrum, A. Kimchi, T. Tatusova, M. Dicuccio, P. Kitts, T.D. Murphy, and K.D. Pruitt. 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 44: D733–D745.

Ochiai, N., M. Fujimura, T. Motoyama, A. Ichiishi, R. Usami, K. Horikoshi, and I. Yamaguchi. 2001. Characterization of mutations in the two-component histidine kinase gene that confer fludioxonil resistance and osmotic sensitivity in the os-1 mutants of *Neurospora crassa*. Pest Manag. Sci. 57: 437–442.

Ochiai, N., T. Tokai, T. Nishiuchi, N. Takahashi-Ando, M. Fujimura, and M. Kimura. 2007. Involvement of the osmosensor histidine kinase and osmotic stress-activated protein kinases in the regulation of secondary metabolism in *Fusarium graminearum*. Biochem. Biophys. Res. Commun. 363: 639–644.

OMAFRA. 2017. Protection Guide for Turfgrass.

Oshima, M., S. Banno, K. Okada, T. Takeuchi, M. Kimura, A. Ichiishi, I. Yamaguchi, and M. Fujimura. 2006. Survey of mutations of a histidine kinase gene BcOS1 in dicarboximide-resistant field isolates of *Botrytis cinerea*. J. Gen. Plant Pathol. 72: 65–73.

Oshima, M., M. Fujimura, S. Banno, C. Hashimoto, T. Motoyama, A. Ichiishi, and I. Yamaguchi. 2002. A point mutation in the two-component histidine kinase BcOS-1 gene confers dicarboximide resistance in field isolates of *Botrytis cinerea*. Phytopathology 92: 75–80.

Pappas, A.C., and D.J. Fisher. 1979. A Comparison of the Mechanisms of Action of Vinclozolin, Procymidone, Iprodione and Prochloraz against *Botrytis cinerea*. Pestic. Sci. 10: 239–246.

Peever, T.L., and M.G. Milgroom. 1994. Lack of correlation between fitness and resistance to sterol biosynthesis-inhibiting fungicides in *Pyrenophora teres*. Phytopathology 84: 515–519.

Pennucci, A., R.. Beever, and E.. Laracy. 1990. Dicarboximide-resistant strains of *Microdochium nivale* in New Zealand. Australas. Plant Pathol. 19: 38–41.

Penrose, L.J., W. Koffmann, and M.R. Nicholls. 1985. Field occurrence of vinclozolin resistance in *Monilinia fructicola*. Plant Pathol. 34: 228–234.

Perkins, D.D., A. Radford, D. Newmeyer, and M. Bjorkman. 1982. Chromosomal loci of *Neurospora crassa*. Microbiol. Rev. 46: 426–570.

Pollastro, S., F. Faretra, A. Santomauro, M. Miazzi, and P. Natale. 1996. Studies on pleiotropic effects of mating type, benzimidazole-resistance and dicarboximide-resistance genes in near-isogenic strains of *Botryotinia fuckeliana* (*Botrytis cinerea*). Phytopathol. Mediterr. 35: 48–57.

Popko, J.T., C.-H. Ok, K. Campbell-Nelson, and G. Jung. 2012. The association between in vitro propiconazole sensitivity and field efficacy of five New England *Sclerotinia homoeocarpa* populations. Plant Dis. 96: 552–561.

Pringle, A., and J.W. Taylor. 2002. The fitness of filamentous fungi. Trends Microbiol. 10: 474–481.

Proffer, T.J., R. Berardi, Z. Ma, J.E. Nugent, G.R. Ehret, P. McManus, A.L. Jones, and G.W. Sundin. 2006. Occurrence, distribution, and polymerase chain reaction-based detection of resistance to sterol demethylation inhibitor fungicides in populations of *Blumeriella jaapii* in Michigan. Phytopathology 96: 709–717.

Raposo, R., V. Gomez, T. Urrutia, and P. Melgarejo. 2000. Fitness of *Botrytis cinerea* associated with dicarboxamide resistance. Phytopathology 91: 1246–1249.

Reimann, S., and H.B. Deising. 2005. Inhibition of efflux mediated fungicide resistance in *Pyrenophora tritici-repentis* by a derivative of 4 ‘-hydroxyflavone and enhancement of fungicide activity. Appl. Environ. Microbiol. 71: 3269–3275.

Resseler, H., and H. Buchenauer. 1988a. Physiologische untersuchu ngen an dicarboximid und benzimidazol resistenten *Gerlachia nivalis*-Stammen. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 95(2): 156–168.

Resseler, H., and H. Buchenauer. 1988b. Resistenzentwicklung von *Gerlachia nivalis* gegenuber dicarboximid- und benzimidazol-fungiziden sowie untersuchungen zur vitalitat der doppeltresistenten Stamme. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 95(2): 143–155.

Reuveni, M. 2001. Improved control of powdery mildew (*Sphaerotheca pannosa*) of nectarines in Israel using strobilurin and polyoxin B fungicides; mixtures with sulfur; and early bloom applications. Crop Prot. 20: 663–668.

Russell, P.E. 2005. A century of fungicide evolution. J. Agric. Sci. 143: 11–25.

Salgado-Salazar, C., L.A. Beirn, A. Ismaiel, M.J. Boehm, I. Carbone, A.I. Putman, L.P. Tredway, B.B. Clarke, and J.A. Crouch. 2018. *Clarireedia*: A new fungal genus comprising four pathogenic species responsible for dollar spot disease of turfgrass. Fungal Biol. 122: 761–773.

Samuels, G.J., and I.C. Hallett. 1983. *Microdochium stoveri* and *Monographella stoveri*, new combinations for *Fusarium stoveri* and *Micronectriella stoveri*. Trans. Br. Mycol. Soc. 81(3): 473–483.

Sanchez, P. 2002. Fitness of in vitro selected *Pseudomonas aeruginosa* nalB and nfxB multidrug resistant mutants. J. Antimicrob. Chemother. 50: 657–664.

Sander, P., B. Springer, T. Prammananan, A. Sturmfels, M. Kappler, M. Pletschette, C. Böttger, and E.C. Böttger. 2002. Fitness cost of chromosomal drug resistance conferring mutations. Antimicrob. Agents Chemother. 46: 1204–1211.

Sang, H., J. Hulvey, J.T. Popko, J. Lopes, A. Swaminathan, T. Chang, and G. Jung. 2015. A pleiotropic drug resistance transporter is involved in reduced sensitivity to multiple fungicide classes in *Sclerotinia homoeocarpa* (F.T. Bennett). Mol. Plant Pathol. 16: 251–261.

Sang, H., J.T. Popko, T. Chang, and G. Jung. 2017. Molecular mechanisms involved in qualitative and quantitative resistance to the dicarboximide fungicide iprodione in *Sclerotinia homoeocarpa* field isolates. Phytopathology 107: 198–207.

Schepers, H.T.A.M. 1983. Decreased sensitivity of *Sphaerotheca fuliginea* to fungicides which inhibit ergosterol biosynthesis. Netherlands J. Plant Pathol. 89: 185–187.

Schmitz, H.K., C.-A. Medeiros, I.R. Craig, and G. Stammler. 2014. Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. Pest Manag. Sci. 70: 378–388.

Schnabel, G., P.K. Bryson, and P.M. Brannen. 2004. Reduced sensitivity in *Monilinia fructicola* to propiconazole in Georgia and implications for disease management. Plant Dis. 88: 1000–1004.

Schnabel, G., and A.L. Jones. 2001. Genetics and resistance the 14α−demethylase (CYP51A1) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. Phytopathology 91: 102–110.

Schuler, G.D. 2001. Sequence alignment and database searching (Baxevanis and Ouellette, Eds.). 2nd ed. Wiley-Interscience.

Schulthess, H. 1761. Vorschlag einiger durch die Erfahrung bewa¨ hrter Hilfsmittel gegen den Brand im Korn. Abhandlung Zurich Naturfor schenden Gesellschaft 1: 498–506.

Schumacher, M.M., C.S. Enderlin, and C.P. Selitrennikoff. 1997. The osmotic-1 locus of *Neurospora crassa* encodes a putative histidine kinase similar to osmosensors of bacteria and yeast. Curr. Microbiol. 34: 340–347.

Semar, M., D. Strobel, A. Koch, K. Klappach, and G. Stammler. 2007. Field efficacy of pyraclostrobin against populations of *Pyrenophora teres* containing the F129L mutation in the cytochrome b gene. J. Plant Dis. Prot. 114: 117–119.

Sheridan, J., N. Grbavac, and N. Sheridan. 1985. Triadimenol insensitivity in *Pyrenophora teres*. Trans. Br. Mycol. Soc. 85: 338–341.

Siegel, M.R. 1981. Sterol-inhibiting fungicides: effect on sterol biosynthesis and sites of action. Plant Dis. 65: 986–989.

Simao, F.A., R.M. Waterhouse, P. Ioannidis, E. V. Kriventseva, and E.M. Zdobnov. 2015. BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31: 3210–3212.

Simpson, D.R., H.N. Rezanoor, D.W. Parry, and P. Nicholson. 2000. Evidence for differential host preference in *Microdochium nivale* var. *majus* and *Microdochium nivale* var. *nivale*. Plant Pathol. 49: 261–268.

Simpson, J.T., K. Wong, S.D. Jackman, J.E. Schein, and S.J.M. Jones. 2009. ABySS : A parallel assembler for short read sequence data ABySS : A parallel assembler for short read sequence data. : 1117–1123.

Sleator, R.D. 2010. An overview of the current status of eukaryote gene prediction strategies. Gene 461: 1–4.

Smiley, R.W., P.H. Dernoeden, and B.B. Clarke (Eds). 2005. Compendium of Turgrass Diseases. Third. APS Press.

Snider, C.S., T. Hsiang, G. Zhao, and M. Griffith. 2000. Role of ice nucleation and antifreeze activities in pathogenesis and growth of snow molds. Phytopathology 90: 354–361.

Sokal, R., and F. Rohlf. 1981. Biometry, the principles and practice of statistics in biological research. W.H. Freeman and Co., New York.

Solel, Z., L.W. Tmmer, and M. Kimchi. 1996. Iprodione resistance of *Alternaria alternata* pv. *citri* from *Minneola Tangelo* in Israel and Florida. Plant Dis. 80: 291–293.

Sparks, A., P. Esker, M. Bates, W. Dall'Acqua, Z. Guo, V. Segovia, S. Silwal, S. Tolos, and K. Garrett. 2008. Ecology and epidemiology in R: disease progress over time. Plant Heal. Instr.Available at http://www.apsnet.org/edcenter/advanced/topics/EcologyAndEpidemiologyInR/DiseasePro gress/Pages/default.aspx (verified 25 May 2018).

Spolti, P., E.M. Del Ponte, J.A. Cummings, and G.C. Bergstrom. 2014a. Triazole sensitivity in a contemporary population of *Fusarium graminearum* from New York wheat and competitiveness of a tebuconazole-resistant isolate. Plant Dis. 98: 607–613.

Spolti, P., E.M. del Ponte, Y.H. Dong, J.A. Cummings, G.C. Bergstrom, and E.M. del Ponte. 2014b. Triazole sensitivity in a contemporary population of *Fusarium graminearum* from New York wheat and competitiveness of a tebuconazole-resistant isolate. Plant Dis. 98: 607–613.

Stammler, G., M. Carstensen, A. Koch, M. Semar, D. Strobel, and S. Schlehuber. 2008.

Frequency of different CYP51-haplotypes of *Mycosphaerella graminicola* and their impact on epoxiconazole-sensitivity and -field efficacy. Crop Prot. 27: 1448–1456.
Stammler, G., J. Cordero, A. Koch, M. Semar, and S. Schlehuber. 2009. Role of the Y134F mutation in cyp51 and overexpression of cyp51 in the sensitivity response of *Puccinia triticina* to epoxiconazole. 2Crop Prot. 28: 891–897.
Stamp, M. 2012. A revealing introduction to hidden markov models.
Stanis, V., and A. Jones. 1985. Reduced sensitivity to sterol inhibiting fungicides in field isolates of *Venturia inaequalis*. Phytopathology 75: 1098–1101.
Stanke, M., R. Steinkamp, S. Waack, and B. Morgenstern. 2004. AUGUSTUS: a web server for gene finding in eukaryotes. Nucleic Acids Res. 32(Web Server): W309–W312.
Van Steekelenburg, N.A.M. 1987. Resistance to benzimidazole and dicarboximide fungicides in *Botrytis cinerea* and *Didymella bryoniae* in cucumbers in the Netherlands. Medelingen van Fac. Landbouwwet. Univ. Gent 53: 875–880.
Stehmann, C., and M.A. De Waard. 1996. Sensitivity of populations of *Botrytis cinerea* to triazoles, benomyl and vinclozolin. Eur. J. Plant Pathol. 102(171): 171–180.
Steva, H. 1994. Evaluating anti-resistance strategies for control of *Uncinula necator*. Monogr. Crop Prot. Counc. 59: 59.
Steva, H., P. Cartolaro, and M. Gomes da Silva. 1990. Tolerance of powdery mildew of SBI fungicides: situation for 1989. Phytoma 419: 41–44.
Sun, X., J. Wang, D. Feng, Z. Ma, and H. Li. 2011. PdCYP51B, a new putative sterol 14α-demethylase gene of *Penicillium digitatum* involved in resistance to imazalil and other fungicides inhibiting ergosterol synthesis. Appl. Genet. Mol. Biotechnol. 91: 1107–1119.
Sztejnberg, A., and A.. Jones. 1978. Tolerance of the brown rot fungus *Monilinia fructicola* to iprodione, vinclozolin, and procymidone fungicides. Phytopathol. News 12: 187.
Talas, F., and B.A. Mcdonald. 2015. Significant variation in sensitivity to a DMI fungicide in field populations of *Fusarium graminearum*. Plant Pathol. 64: 664–670.
Tanaka, C., and K. Izumitsu. 2010. Two-component signaling system in filamentous fungi and the mode of action of dicarboximide and phenylpyrrole fungicides. p. 523–538. *In* Carisse, O. (ed.), Fungicides. InTech.
Tanaka, F., I. Saito, K. Miyajima, S. Tsuchiya, and K. Tsuboki. 1983. Occurrence of thiophanate methyl tolerant isolates of *Fusarium nivale* synonymy *Gerlachia nivalis* a causal fungus of snow mold of winter wheat in Japan. Ann. Phytopathol. Soc. Japan 49: 565 – 566.
Taylor, E.L., A.G. Holley, and M. Kirk. 2007. Pesticide development: a brief look at the history. South. Reg. Ext. For. SREF-FM-01.
Tremblay, D.M., and B.G. Talbot. 2003. Sensitivity of *Botrytis squamosa* to different classes of fungicides. Plant Dis. 87: 573–578.
Tronsmo, A.M., T. Hsiang, H. Okuyama, and T. Nakajima. 2001. Low temperature diseases caused by *Microdochium nivale*. Low Temp. Plant Microbe Interact. Under Snow (January): 75–86.
Tsiplova, K., G. Fox, K. Jordan, and E. Lyons. 2008. Economic profile of the Ontario turfgrass industry. Ontario Turfgrass Res. Found.
Vargas, J.M., R. Golembiewski, and A.R. Detweiler. 1992. Dollar spot resistance to DMI fungicides. Golf Course Manag. 60: 50–54.
Veloukas, T., P. Kalogeropoulou, A.N. Markoglou, and G.S. Karaoglanidis. 2014. Fitness and competitive ability of *Botrytis cinerea* field isolates with dual resistance to SDHI and QoI fungicides, associated with several sdhB and the cytb G143A mutations. Phytopathology

104: 347–356.
Vincelli, P., and G. Munshaw. 2015. Chemical control of turfgrass diseases 2015. Lexington, KY.
De Waard, M.A., A.C. Andrade, K. Hayashi, H.J. Schoonbeek, I. Stergiopoulos, and L.H. Zwiers. 2006. Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence. Pest Manag. Sci. 62(3): 195–207.
De Waard, M.A., E. Kipp, N. Horn, and J. Van Nistelrooy. 1986. Variation in sensitivity to fungicides which inhibit ergosterol biosynthesis in wheat powdery mildew. Netherlands J. Plant Pathol. 92: 21–32.
Walker, A.S., C. Auclair, M. Gredt, and P. Leroux. 2009. First occurrence of resistance to strobilurin fungicides in *Microdochium nivale* and *Microdochium majus* from French naturally infected wheat grains. Pest Manag. Sci. 65: 906–915.
Washington, W.S., N. Shanmuganathan, and C. Forbes. 1992. Fungicide control of strawberry fruit rots, and the field occurrence of resistance of *Botrytis cinerea* to iprodione, benomyl and dichlofluanid. Crop Prot. 11: 355–260.
Weber, R.W.S. 2011. Resistance of *Botrytis cinerea* to multiple fungicides in northern German small-fruit production. Plant Dis. 95: 1263–1269.
Weete, J.D., M. Abril, and M. Blackwell. 2010. Phylogenetic distribution of fungal sterols. PLoS One 5(5): e10899.
Wiese, M.. 1987. Compendium of wheat diseases. 2nd ed. APS Press, Minnesota.
Williamson, B., B. Tudzynski, P. Tudzynkski, and J.A.L. Van Kan. 2007. *Botrytis cinerea*: the cause of grey mould disease. Mol. Plant Pathol. 8: 561–580.
Wolanin, P.M., P.A. Thomason, and J.B. Stock. 2002. Histidine protein kinases: key signal transducers outside the animal kingdom. Genome Biol. 3: 3013.1-1303.8.
Wong, F.P., and S.L. Midland. 2007. Sensitivity distributions of California populations of *Colletotrichum cereale* to the DMI fungicides propiconazole, myclobutanil, tebuconazole, and triadimefon. Plant Dis. 91: 1547–1555.
Wyand, R.A., and J.K.M. Brown. 2005. Sequence variation in the CYP51 gene of *Blumeria graminis* associated with resistance to sterol demethylase inhibiting fungicides. Fungal Genet. Biol. 42: 726–735.
Yamaguchi, I., and M. Fujimura. 2005. Recent topics on action mechanisms of fungicides. J. Pestic. Sci. 30: 67–74.
Yamaguchi, J., M. Inada, and M. Matsuzaki. 2000. Occurrence of DMI fungicide resistant isolates of *Mycovellosiella nattrassii* Deighton, causal fungus of leaf mold of eggplant. Japanese J. Phytopathol. 66: 78–84.
Yan, L., Q. Yang, G.W. Sundin, H. Li, and Z. Ma. 2010. The mitogen-activated protein kinase kinase BOS5 is involved in regulating vegetative differentiation and virulence in *Botrytis cinerea*. Fungal Genet. Biol. 47: 753–760.
Yang, H.F., M.X. Ji, K.B. Yao, and Z.L. Shu. 2013. Study on resistance of *Fusarium monilifore* to Prochloraz and its management. Acta Agric. Jiangxi 25: 94–96.
Yang, Q., L. Yan, Q. Gu, and Z. Ma. 2012. The mitogen-activated protein kinase kinase kinase BcOs4 is required for vegetative differentiation and pathogenicity in *Botrytis cinerea*. Appl. Microb. Cell Physiol. 96: 481–492.
Yin, Y., X. Liu, B. Li, and Z. Ma. 2009. Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. Phytopathology 99: 487–497.

Yoshimi, A., A.M. Tsuda, and A.C. Tanaka. 2004. Cloning and characterization of the histidine kinase gene Dic1 from *Cochliobolus heterostrophus* that confers dicarboximide resistance and osmotic adaptation. Mol. Genet. Genomics 271: 228–236.

Zehr, E.I., L.A. Luszcz, W.C. Olien, and W.C. Newall. 1999. Reduced sensitivity in *Monilinia fructicola* to propiconazole following prolonged exposure in peach orchards. Plant Dis. 83: 913–916.

Zerbino, D.R., and E. Birney. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18: 821–9.

Zhan, J., and B. a McDonald. 2013. Experimental measures of pathogen competition and relative fitness. Annu. Rev. Phytopathol. 51: 131–53.

Zhang, Y., R. Lamm, C. Pillonel, S. Lam, and J.R. Xu. 2002. Osmoregulation and fungicide resistance: the *Neurospora crassa* os-2 gene encodes a HOG1 mitogen-activated protein kinase homologue. Appl. Environ. Microbiol. 68: 532–538.

Zheng, Z., Y. Hou, Y. Cai, Y. Zhang, Y. Li, and M. Zhou. 2015. Whole-genome sequencing reveals that mutations in myosin-5 confer resistance to the fungicide phenamacril in Fusarium graminearum. Sci. Rep. 5(2021): 8248Available at http://www.nature.com/articles/srep08248.

Zhou, F., X.L. Zhang, J.L. Li, and F.X. Zhu. 2014. Dimethachlon resistance in *Sclerotinia sclerotiorum* in China. Plant Dis. 98: 1221–1226.

Ziogas, B., and A. Malandrakis. 2015. Sterol biosynthesis inhibitors: C14 demethylation (DMIs). p. 199–216. *In* Ishii, H., Hollomon, D.W. (eds.), Fungicide Resistance in Plant Pathogens. Springer, Tokyo.