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RESEARCH

Resistance to Dicarboximide Fungicides in a Canadian Population of *Microdochium nivale*

Ryan Gourlie and Tom Hsiang*

ABSTRACT

Isolates of *Microdochium nivale* suspected of resistance to iprodione [3-(3,5-Dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] were collected from a golf course in Victoria, British Columbia (BC), Canada, in Fall 2015. The 9 BC isolates and 12 other reference isolates from around the world were tested with four concentrations of iprodione: 0, 1, 10, and 100 μg mL^{-1}. The BC isolates showed a half maximal effective concentration (EC_{50}) range of 14.2 to 40.8 μg mL^{-1} with a mean of 24.2 μg mL^{-1}, while the non-BC isolates showed an EC_{50} range of 1.2 to 4.3 μg mL^{-1} with a mean of 1.9 μg mL^{-1}. The yielded resistance factor (EC_{50} resistant/EC_{50} sensitive) of 12.7 implies that these BC isolates have been selected for reduced sensitivity to iprodione. This level of resistance may be responsible for the reduced efficacy, particularly the observed reduced interval of control, but further research is needed to assess this. A test of >100 isolates of *M. nivale* from a worldwide collection using a discriminatory concentration of 10 μg mL^{-1} of iprodione showed four isolates with similar levels of reduced sensitivity, but these were the only isolates from their respective populations.

R. Gourlie and T. Hsiang, School of Environmental Sciences, Univ. of Guelph, Guelph, Ontario N1G 2W1, Canada. Received 3 June 2016. Accepted 21 Jan. 2017. *Corresponding author (thsiang@uoguelph.ca). Assigned to Associate Editor John Inguagiatio.

Abbreviations: BC, British Columbia; EC_{50}, half maximal effective concentration; PDA, potato dextrose agar.

Pink snow mold and Microdochium patch (also known as Fusarium patch) are common diseases of turfgrass caused by the fungus *Microdochium nivale* (formerly known as *Fusarium nivale*). Microdochium patch occurs in the fall and spring when conditions are cool and wet, giving rise to small, irregularly shaped patches or diffused blighting, whereas pink snow mold grows under snow cover during the winter months, producing distinctly circular patches. Damage caused by these diseases can be extensive if left untreated. Most golf courses spray curative and preventative fungicides in an attempt to control *M. nivale* and other pathogenic fungi, leading to selective pressure for isolates for decreased sensitivity to these fungicides.

The first dicarboximide fungicide, iprodione, was introduced in 1974 by Rhône-Poulenc in an effort to control *Botrytis cinerea* (Morton and Staub, 2008). Dicarboximide fungicides have since been widely used to control a variety of ascomycetous pathogens, including *M. nivale*. Due to their widespread use, resistance to the dicarboximide group of fungicides has developed in many different fungal populations (FRAC, 2013), including *B. cinerea* (Grabke et al., 2014).

Experiments with the fungi *B. cinerea*, *Alternaria alternata*, and *Neurospora crassa* have shown that dicarboximide fungicides affect osmoregulation and that resistance can be conferred by mutation at a single locus (Beever, 1983; Cui et al., 2002; Oshima et al., 2002; Dry et al., 2004; Tanaka and Izumitsu, 2010). Iprodione,

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which is the only dicarboximide registered for use in Canada, has been used to control *M. nivale* on turf since 1987 (Health Canada, 2016). To date, there has not been a report of field resistance to iprodione in *M. nivale* populations in Canada, although there have been instances of field resistance elsewhere. The first report was by Chastagner and Vassey (1982), who documented the presence of iprodione-tolerant *M. nivale* isolates where Rhône-Poulenc had previously performed turf field trials with iprodione (Seattle, WA). A second report was by Pennucci et al. (1990), who collected iprodione-tolerant *M. nivale* from several turf locations in New Zealand.

In 2014, a superintendent of a Victoria, British Columbia (BC), golf course observed that applications of iprodione used to control Microdochium patch had been less successful than in previous years. He was concerned that frequent use of iprodione, biweekly applications throughout the cool wet season (10–15 applications yr^{-1}) for at least the last 15 yr, was leading to resistance issues. In fall 2015, when the issue emerged again, he sent samples of turfgrass to the University of Guelph, from which we isolated *M. nivale*. The purpose of this research was to assess the level of iprodione resistance of these *M. nivale* isolates and to compare these levels with *M. nivale* isolates from our worldwide reference collection.

MATERIALS AND METHODS

Microdochium nivale Isolates

In October 2015, grass samples of *M. nivale* were collected by a Golf Course Superintendent in Victoria, BC, with at least five leaves per patch, as little soil as possible, and placed and wrapped in regular printing paper. Samples arrived 3 d later in sealed envelopes that contained grass blades taken from three locations on the golf course (USGA putting greens). *Microdochium nivale* was isolated from samples of *Poa annua* L. by placing individual leaves in diluted bleach (1% sodium hypochlorite) for 30 s, rinsing in sterile distilled water for 10 s, and placing on 9-cm-diameter Petri dishes containing 10 mL autoclaved potato dextrose agar (PDA, Difco Laboratories). Isolates were grown on PDA (39 g L^{-1}) at 20°C for 2 to 5 d with continuous overhead fluorescent lighting. Isolates were then visually compared to known *M. nivale* and later confirmed by the production of pink-orange sporodochia in culture. A single isolate was retained per patch (Table 1). 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. Isolates from our worldwide collection with incompletely documented exposure levels to iprodione were selected from our laboratory stock collection as comparisons. Many of these were collected from the Guelph Turfgrass Institute from areas with little or no fungicide exposure over the last 20 yr.

Preparation of Iprodione-Amended Media

Iprodione (23.3%, Bayer Crop Science) 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 calculated final concentration of acetone (0.10% v/v). Acetone at this concentration did not inhibit growth of *M. nivale* (data not shown). Fungicide-amended PDA was added to 9-cm-diameter plates in 10-mL aliquots and allowed to set.

Iprodione Full Range Sensitivity Testing

A total of 21 isolates were tested for sensitivity to iprodione using an agar plug assay described by Hsiang et al. (1997). Agar plugs 5 mm in diameter were taken from the growing edge of active mycelia and placed with hyphae face down onto the center of 9-cm-diameter plates where the PDA had been amended with four different concentrations of iprodione: 0, 1, 10, and 100 μg mL^{-1} with three replications per isolate by concentration combination. Plates were incubated in a growth chamber at 22°C with constant overhead fluorescent lighting. Two diameter measurements (90° from each other) were taken at 48 and 96 h after plating. This experiment was repeated three times.

Iprodione Discriminatory Testing

Based on results of the full range concentration tests, and previous literature on iprodione resistance (Chastagner and Vassey 1982; Pennucci et al., 1990), a concentration of 10 μg mL^{-1} for iprodione was chosen as the discriminatory concentration for further testing. This discriminatory concentration was chosen by analyzing the data to see at which point presumed sensitive isolates would be fully inhibited, whereas isolates showing reduced sensitivity would still show growth. From the laboratory stock collections, 105 isolates of *M. nivale* were selected for discriminatory testing. Isolates were revived from long-term storage tubes onto antibiotic-amended PDA (0.2 mg L^{-1} streptomycin, 0.2 mg L^{-1} tetracycline). Iprodione-amended PDA plates were prepared at the discriminatory concentration (10 μg mL^{-1}) in addition to unamended PDA plates. To reduce waste, the solid medium in the 9-cm-diameter plates were cut using six blades mounted on a 9-cm-diameter plexiglass holder. Agar was then removed, leaving 1-cm-wide strips, following Hsiang et al. (1997). Three technical replicates of each isolate were grown on the discriminatory concentration by placing 5-mm plugs onto the center of each strip with hyphae face down, with another three replicates on PDA as controls. Plates were incubated in a growth chamber at 22°C with constant overhead fluorescent lighting. For each agar strip, two radial measurements were taken after 48 and 96 h of incubation.

Statistical Analyses

Iprodione Full Range Sensitivity Testing

For the multiple concentration fungicide plate assays, only the growth that occurred between 48 and 96 h was used for analysis, since the first 48 h of growth 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 unamended medium)] × 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%). Probit transformation is

Table 1. Origin and host species of *Microdochium nivale* isolates used in full range iprodione sensitivity assay.

Isolate	Origin†	Host species
BCMN01	A golf course, center green, Victoria, BC, Canada	*Poa annua*
BCMN02	A golf course, center green, Victoria, BC, Canada	*Poa annua*
BCMN03	A golf course, center green, Victoria, BC, Canada	*Poa annua*
BCMN04	A golf course, green #7, Victoria, BC, Canada	*Poa annua*
BCMN05	A golf course, green #7, Victoria, BC, Canada	*Poa annua*
BCMN06	A golf course, green #7, Victoria, BC, Canada	*Poa annua*
BCMN07	A golf course, green #17, Victoria, BC, Canada	*Poa annua*
BCMN08	A golf course, green #17, Victoria, BC, Canada	*Poa annua*
BCMN09	A golf course, center green, Victoria, BC, Canada	*Poa annua*
gticb178	GTI, pathology green, Guelph, ON, Canada	*Poa annua*
gticb179	GTI, pathology green, Guelph, ON, Canada	*Poa annua*
gticb37	GTI, roadside of upper green, Guelph, ON, Canada	*Poa pratensis*
gticb99	GTI, native green, Guelph, ON, Canada	*Poa annua/Agrostis stolonifera*
gtikb55	GTI, Guelph, ON, Canada	*Poa pratensis*
itatrit6	Medicina, Italy	*Triticum turgidum*
ukcb82	United Kingdom	*Agrostis stolonifera*
jpncb408	Kitasato University Farm, Yagumo, Hokkaido, Japan	*Poa annua/Agrostis stolonifera*
jpntrit07	Upland Agriculture Research Center, Memuro, Hokkaido, Japan	*Triticum* sp.
otttrit62	Ottawa Experimental Farm, Ottawa, ON, Canada	*Triticum* sp.
gercb101	St. Leon-Rot Golf Club, Baden-Württemberg, Germany	*Poa annua/Agrostis stolonifera*
altacb01	Edmonton, AB, Canada	*Poa annua/Agrostis stolonifera*

† GTI, Guelph Turfgrass Institute.

used to straighten the dosage response curve and allows for more accurate estimations of EC_{50} values than untransformed data (Sokal and Rohlf, 1981). Examples of the SAS program statements are available on request from the corresponding author. This experiment was repeated three times and mean EC_{50} values, as well as standard error values, were calculated.

Iprodione Discriminatory Concentration Testing

For discriminatory concentration testing, a mean was calculated for PDA growth rate from the various replicates. This mean was used as the denominator to calculate proportion of growth on the discriminatory concentration (10 μg mL^{-1}) where the 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 the Fisher's protected LSD ($p = 0.05$).

RESULTS

Iprodione Sensitivity Testing

The isolates used for the multiple concentration sensitivity testing are shown in Table 1. The calculated EC_{50} values ranged from 1.2 to 40.8 μg mL^{-1} (Fig. 1). The isolates from BC showed an EC_{50} range of 14.2 to 40.8 μg mL^{-1} with an average EC_{50} of 24.2 μg mL^{-1}. In comparison, the other isolates, which were from Ontario (Canada), Europe, and one each from Japan and Alberta (Canada), showed an EC_{50} range of 1.2 to 4.3 μg mL^{-1} with a mean of 1.9 μg mL^{-1}. The BC isolates as a group had statistically significantly ($p = 0.05$) higher EC_{50} values than the other isolates as a group according to an ANOVA test.

Iprodione Discriminatory Testing

A response curve (Fig. 2) was plotted to represent the growth of select isolates on the different concentrations of iprodione tested. This curve (Fig. 2) plus the bar graph depicting EC_{50} values (Fig. 1) led us to conclude that 10 μg mL^{-1} could be used as a discriminatory concentration. We then tested over 100 isolates (Table 2) on this discriminatory concentration and obtained the proportional growth relative to unamended PDA.

In a distribution graph of proportional growth rates on 10 μg mL^{-1} iprodione (Fig. 3), all the BC isolates (9) were between 0.6 and 1.0 proportional growth, whereas for the other 117 isolates, half were in the lowest category (0–0.1 proportional growth). There were a few isolates from the non-BC collection that also showed very high proportional growth on the discriminatory concentration: PGF2, WF127, PR-E5, and OTT36. The four non-BC isolates (PGF2, WF127, PR-E5, and OTT36) that showed indications of resistance in the discriminatory testing were tested along with the other members of their groups (26, which were all sensitive) for their growth rate on unamended PDA. These data were subjected to ANOVA, and the significant ($p = 0.05$) treatment effect showed that the average growth rate of the non-BC resistant isolates at 5.0 mm d^{-1} was significantly less than the sensitive isolates at 6.5 mm d^{-1}. Similarly, when all BC isolates were pooled as one population, all non-BC isolates were pooled as a second population, and the data were subjected to ANOVA with means separated by LSD, the mean daily growth rate for BC isolates was 6.4 mm d^{-1} and for non-BC isolates was 7.0 mm d^{-1} with an LSD ($p = 0.05$) of 0.5.

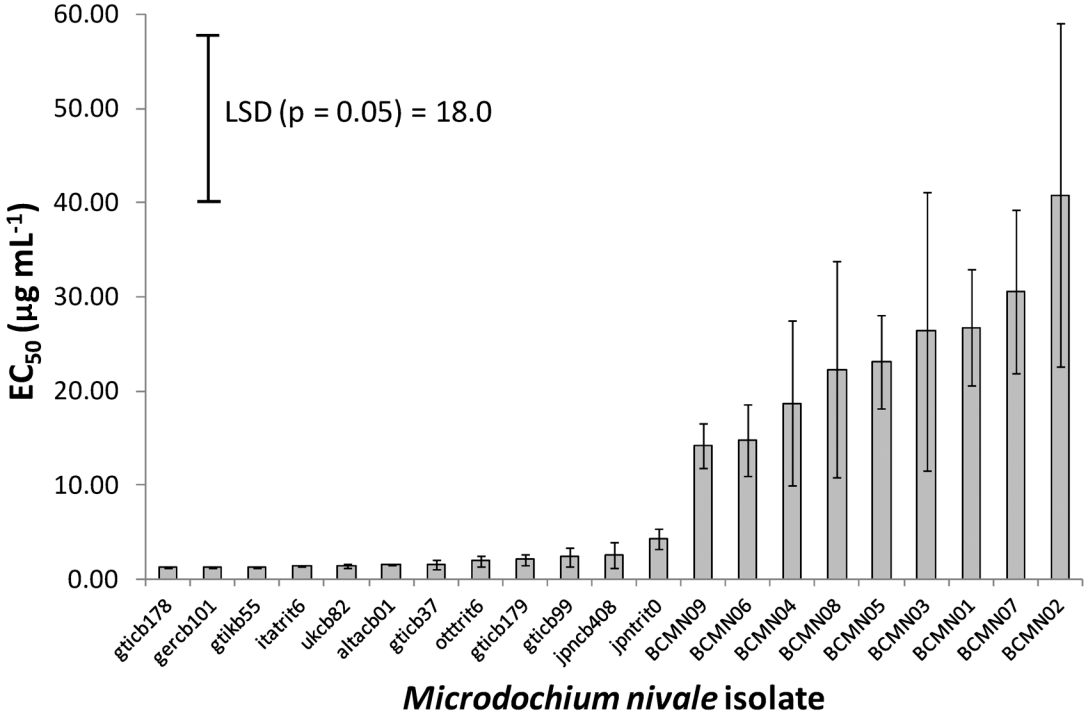

Fig. 1. Effective concentration (EC_{50}, µg mL^{-1}) of iprodione required to inhibit *Microdochium nivale* mycelial growth on amended potato dextrose agar plates after 2 d of incubation at 22°C, calculated using PROBIT analysis. The bar heights represent means from three repeat experiments, and standard error bars are shown.

DISCUSSION

The west coast of BC is subject to strong Microdochium patch disease pressure for more than half of the year. Recently, BC superintendents have suspected that they have fungicide resistance issues, since applications of some fungicides seem to provide much shorter intervals of control (or none at all) than in previous years. The results of this work demonstrate reduced sensitivity to iprodione in *M. nivale* isolates from this golf course in Victoria, BC, compared with other isolates from around the world, with a population resistance factor (EC_{50} resistant/EC_{50} sensitive) of 24.2/1.9 = 12.7.

This level of resistance found in the BC *M. nivale* isolates indicates greatly reduced sensitivity. However, this level has not resulted in reported full-blown field resistance, since iprodione applications are still effective, but some superintendents have reported decreased duration of efficacy. The isolates of *M. nivale* tested here showed similar iprodione sensitivity results to those tested by Chastagner and Vassey (1982) and Pennucci et al. (1990). The EC_{50} values for our isolates with reduced sensitivity ranged between 14.2 and 40.8 µg mL^{-1}, whereas Pennucci et al. (1990) reported a range of 14.1 to 54 µg mL^{-1}. Pennucci et al. (1990) also

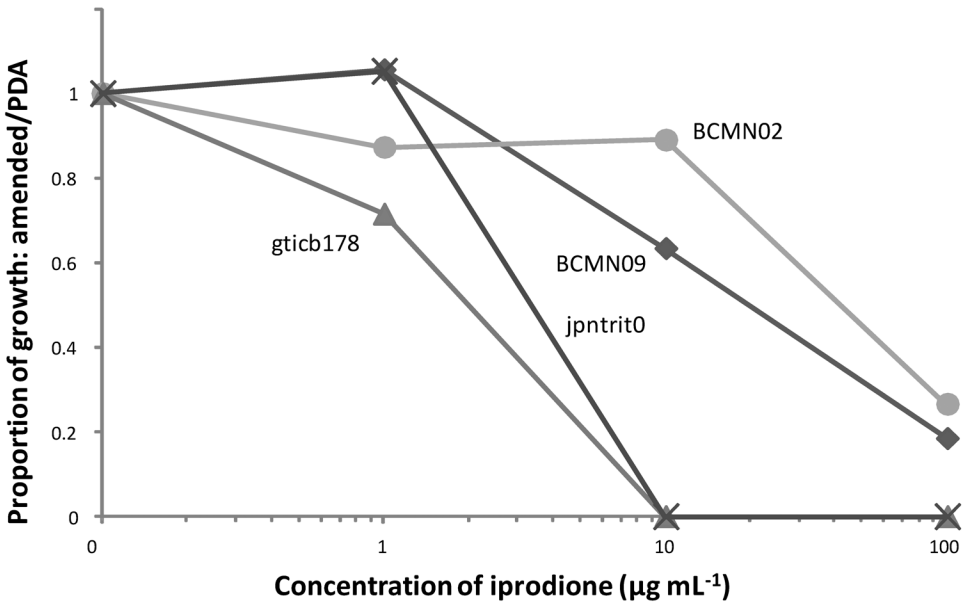

Fig. 2. Dose response curves of four select *Microdochium nivale* isolates grown on potato dextrose agar (PDA) media containing 0, 1, 10, or 100 µg mL^{-1} of iprodione.

Table 2. Population, host species, and number of *Microdochium nivale* isolates from each population used in iprodione discriminatory concentration (10 μg mL^{-1} iprodione) testing.

Population	Origin†	Host	Isolates
KBNG	GTI, near native green, Guelph, ON, Canada	*Poa pratensis*	5
KBBW	GTI, between research greens, Guelph, ON, Canada	*Poa pratensis*	5
KBPG	GTI, between green and road, Guelph, ON, Canada	*Poa pratensis*	6
KBRD	GTI, roadside, Guelph, ON, Canada	*Poa pratensis*	5
NGC	GTI, native green, Guelph, ON, Canada	*Poa annua/Agrostis stolonifera*	5
NG02	GTI, native green, Guelph, ON, Canada	*Poa annua/Agrostis stolonifera*	4
NG03	GTI, native green, Guelph, ON, Canada	*Poa annua/Agrostis stolonifera*	5
OTT	Ottawa Experimental Farm, Ottawa, ON, Canada	*Triticum* sp.	5
OTT03	Ottawa Experimental Farm, Ottawa ON, Canada	*Triticum* sp.	5
PGCB	GTI, pathology green, Guelph, ON, Canada	*Agrostis stolonifera*	4
PG	GTI, pathology green, Guelph, ON, Canada	*Agrostis stolonifera*	5
PR03	GTI, roadway, Guelph, ON, Canada	*Lolium perenne*	6
PR-E	GTI, roadway, Guelph, ON, Canada	*Lolium perenne*	5
RD02	GTI, roadway, Guelph, ON, Canada	*Lolium perenne*	4
RDKB	GTI, roadside, Guelph, ON, Canada	*Poa pratensis*	4
RD	GTI, Guelph, ON, Canada	*Lolium perenne*	2
WF1	Highway 131, near Atwood, ON, Canada	*Triticum* sp.	4
WF2	Along Highway 86 near Listowel, ON, Canada	*Triticum* sp.	5
GH	Graham Hall, Guelph, ON, Canada	*Poa pratensis*	4
PGF	GTI, pathology green fringe, Guelph, ON, Canada	*Poa pratensis*	8
UPR	GTI, roadside of upper green, Guelph, ON, Canada	*Poa pratensis*	6
UPH	GTI, hillside of upper green, Guelph, ON, Canada	*Poa pratensis*	3
Total			105

† GTI, Guelph Turfgrass Institute.

calculated a resistance factor of 12 for their resistant isolates and stated that "Fusarium patch symptoms (on the course greens) were common despite, in some instances, the regular and repeated use of iprodione." Whereas Chastagner and Vassey (1982) did not report EC_{50} values, they reported that iprodione failed to control Fusarium patch on two golf greens. Resseler and Buchenauer (1988a) were able to generate iprodione-resistant mutants in vitro with resistance factors of 20 to 200, but these isolates were not normal and had sensitivity to osmotic pressures, unlike sensitive wild-type isolates (Resseler and Buchenauer, 1988b).

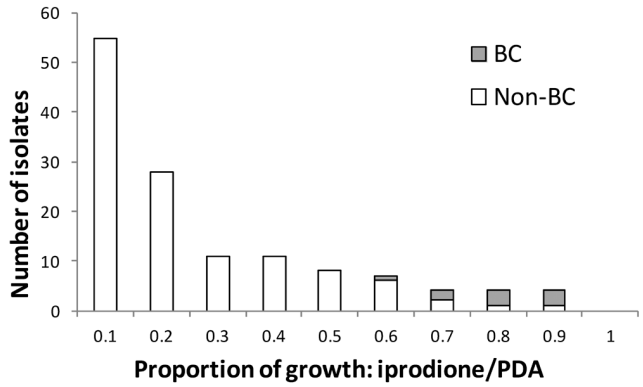

Fig. 3. Iprodione sensitivity of over 100 isolates of *Microdochium nivale* on potato dextrose agar (PDA) amended with 10 μg mL^{-1} compared with growth on PDA. Chi-squared test ($p < 0.001$) indicated that the proportion of British Columbia (BC) isolates with >50% relative growth was significantly higher than the proportion for non-BC isolates.

This implies that resistant mutants generated in vitro may not have the fitness and in situ survival characteristics of field-generated mutants.

The iprodione-resistant *M. nivale* isolates tested showed some reduction in fitness compared with sensitive isolates. In growth tests on nonamended PDA, the non-BC resistant isolates grew more slowly than sensitive isolates from the same populations (5.0 vs. 6.5 mm d^{-1}), with a 76% growth rate compared with the sensitive isolates. Similarly, the resistant BC isolates showed slower growth rates (6.4 mm d^{-1}) at 91% of the growth rate of the non-BC isolates (7.0 mm d^{-1}). This is in line with previous research, which found that isolates of *Botrytis* species with decreased sensitivity to iprodione grew at a slower rate than sensitive isolates (Hsiang and Chastagner, 1991), and that sclerotia of iprodione-resistant isolates of *Botrytis elliptica* showed lower survival rates than sensitive isolates (Hsiang and Chastagner, 1992). Raposo et al. (2000) reported decreased sclerotia survival for iprodione-resistant *Botrytis cinerea* isolates compared with sclerotia of sensitive isolates; however, they did not see differences in mycelial growth.

Instances of field resistance to iprodione have been reported in other economically important plant pathogen species. *Sclerotinia homoeocarpa*, another important pathogen of turf, has had EC_{50} values of iprodione-field-resistant isolates reported at 381 (Detweiler et al., 1983) and 360 μg mL^{-1} (Bishop et al., 2008). The widely studied

species *B. cinerea* has had EC_{50} values for iprodione-resistant isolates reported several times: 4.3 to 13.9 μg mL^{-1} (LaMondia and Douglas, 1997), 1.58 to 3.01 μg mL^{-1} (Raposo et al., 2000), and 16.1 to 96.1 μg mL^{-1} (Weber, 2011). Another widespread species, *Alternaria alternata*, has had EC_{50} values of iprodione-field-resistant isolates reported at 290 (Hutton, 1988) and 280 μg mL^{-1} (Solel et al., 1996). The *M. nivale* isolates from BC showed an EC_{50} range of 14.2 to 40.8 μg mL^{-1} with an average EC_{50} of 24.2 μg mL^{-1}, which is comparable with the studies cited above.

More work is required to fully elucidate the extent of iprodione resistance (and resistance to other fungicides) in coastal BC *M. nivale* populations and in areas where disease caused by *M. nivale* is prominent throughout the year. Future work will examine isolates from other turfgrass locations and investigate a greater number of isolates. Experiments will also be conducted to examine the fitness and pathogenicity of the iprodione-resistant isolates in the absence of fungicide selection pressure in the laboratory and field.

Conflict of Interest

The author declares there is no conflict of interest.

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