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Resistance to the demethylation-inhibiting fungicide propiconazole in Canadian populations of *Microdochium nivale*

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Abstract

Turfgrass managers have anecdotally reported decreased efficacy of DMI fungicides for the control of *Microdochium* patch and pink snow mold at golf courses in British Columbia, Canada. Isolates of *Microdochium nivale* from these locations, along with isolates collected in Ontario, Canada, were tested for their sensitivity to the fungicide propiconazole [1-([2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl)-1,2,4-triazole]. Ontario isolates (47) had values for effective concentration causing 50% growth inhibition (EC_{50}) ranging from <0.001 to 0.89 μg ml^{-1}. In comparison, British Columbia isolates (50) had an EC_{50} range of 0.02 to 8.7 μg ml^{-1}. Sensitivity testing with a discriminatory concentration (0.1 μg ml^{-1}) of a larger set of isolates revealed that 24% of Ontario isolates (43 of 181) and 77% of British Columbia isolates (55 of 71) exhibited resistance to propiconazole (>50% growth on 0.1 μg ml^{-1} compared to non-amended media). Because of the cool, wet climate of coastal British Columbia, turfgrass managers use more applications of fungicides annually, including propiconazole, to control diseases caused by *M. nivale*, and this has resulted in a greater proportion of isolates being resistant to propiconazole. In contrast, Ontario has a less favorable climate for these diseases, with accordingly fewer fungicide applications directed toward this pathogen and hence less risk of fungicide resistance developing.

1 | INTRODUCTION

The fungal plant pathogen *Microdochium nivale* is the causal organism of two economically important diseases of turfgrass: *Microdochium* patch and pink snow mold. *Microdochium* patch (also called *Fusarium* patch) develops during cool, wet weather, usually in spring and fall, and is characterized by small, irregularly shaped patches of blighted grass, whereas pink snow mold develops under snow cover and produces distinctly circular patches of bleached grass (Smiley et al., 2005). In consistently cool, wet temperate climates, such as those in northwestern Europe and the northwestern coast of North America, *M. nivale* can cause severe damage if left untreated (Smiley et al., 2005). The primary method of disease control for most turfgrass managers is the application of preventative and curative fungicides, which creates a strong selection pressure for decreased sensitivity to the fungicide families applied.

In Canada, various demethylation-inhibiting (DMI) fungicides are registered for the control of turfgrass diseases, including difenoconazole, metconazole, myclobutanil, propiconazole, tebuconazole, and triticonazole. The DMIs are a subgroup of the sterol biosynthesis inhibitors, which comprises dozens of structurally diverse compounds. The DMIs have been registered for use on turfgrass in Canada since

Abbreviations: DMI, demethylation-inhibiting; EC_{50}, effective concentration causing 50% growth inhibition; PDA, potato dextrose agar; RF, resistance factor.

1994 and are widely used for the control of various fungal pathogens, including *M. nivale*. The development of fungicide resistance is a well-documented phenomenon, and resistance to the DMI fungicides has been reported in over 40 plant pathogens, with 28 of those species having confirmed field resistance (Fungicide Resistance Action Committee, 2020).

Previously, a small set of isolates collected from Victoria, BC, was found to be resistant to the dicarboximide fungicide iprodione (Gourlie & Hsiang, 2017). Based on anecdotal reports from turfgrass managers across southwestern British Columbia, it was suspected that isolates of *M. nivale* may also have developed resistance to the DMI fungicide propiconazole [1-([2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl)-1,2,4-triazole]. The purpose of this work was to collect a larger set of samples from southwestern BC, as well as locations in southern Ontario and to assess them for their sensitivity to propiconazole.

Core Ideas

- *Microdochium nivale* disease is common in cool, wet climates in Canada.
- *Microdochium nivale* collected from turfgrasses is becoming less sensitive to propiconazole.
- This is the first report of *M. nivale* resistance to propiconazole.

2 | METHODS AND MATERIALS

2.1 | Sample collection, fungal isolation, and storage

In April 2016, turfgrass samples exhibiting symptoms of *Microdochium* patch were collected from three locations in Victoria, BC, two golf courses and a practice green near Victoria Harbour. These locations were approximately 15 to 20 km from each other. In November 2016, May 2017, and December 2017, samples were collected from Vancouver, BC. Only at one of these locations did the turf manager suspect that they had resistance to DMI fungicides. In March and April 2017, four golf courses in Ontario were also sampled. For the Ontario locations, samples were taken from at least five sites (e.g., greens and fairways) at each golf course. None of these locations reported resistance to DMI fungicides. Samples were kept at 4 ˚C and processed within 2 wk.

Individual grass blades were surface-sterilized by dipping them in 75% ethanol for 5 to 10 s, placed in 1% sodium hypochlorite for 1 to 2 min, and rinsed with sterile water. Sterilized leaves were placed in petri plates containing 10 ml of autoclaved 2% potato dextrose agar (PDA) amended with the antibiotic's streptomycin sulfate and tetracycline hydrochloride, both at 0.2 μg ml^{-1}, and incubated at 20˚C with fluorescent lighting. Subcultures on PDA were visually compared to known *M. nivale* cultures, especially for the presence of pink-orange sporodochia, and a single isolate was retained per sample. Select isolates from the local lab collection were also included in the sensitivity tests.

2.2 | Full concentration range testing

Propiconazole in the form of Banner Maxx (14.3% propiconazole) was diluted in 100% acetone (6.99 μl Banner Maxx in 9.93 ml acetone), for a stock concentration of 100 μg ml^{-1}. The stock solution was added to molten PDA (55 ˚C) to obtain final propiconazole concentrations of 0, 0.01, 0.1, and 1 μg ml^{-1} while maintaining an equal final concentration of acetone (0.10% v/v). Propiconazole-amended PDA was added to 9-cm-diameter plates in 12-ml aliquots and allowed to set. The solid medium was cut with six blades mounted on a 9-cm-diameter aluminum holder, and excess agar was then removed, leaving three 1-cm-wide agar strips in each plate following Hsiang et al. (1997).

A random subset of isolates from British Columbia (50) and Ontario (47) were selected for testing with the full range of concentrations. Agar plugs, 5 mm in diameter, were taken from the growing edge of active mycelia and placed hyphal side down at the middle of the agar strips. Three replicates were used per isolate per concentration, with each replicate grown on a separate plate. Plates were incubated at 20 ˚C, and radial growth measurements were taken at 48 and 96 h after plating. The inhibition data were analyzed with SAS Proc PROBIT. Probit transformation was used to straighten the dosage response curves and allows for more accurate estimations of effective concentration causing 50% growth inhibition (EC_{50}) than untransformed data (Sokal & Rohlf, 1981). Full concentration range experiments were conducted at least two times for each isolate. Resistance factors (RFs) for each isolate that was deemed resistant (EC_{50}: ≥0.1 μg ml^{-1}) were calculated by dividing their EC_{50} value by the average EC_{50} of the sensitive isolates (EC_{50}: <0.1 μg ml^{-1}).

2.3 | Discriminatory concentration testing

Dose–response curves were generated for isolates tested at the four propiconazole concentrations by plotting daily growth against the increasing concentrations of propiconazole. The

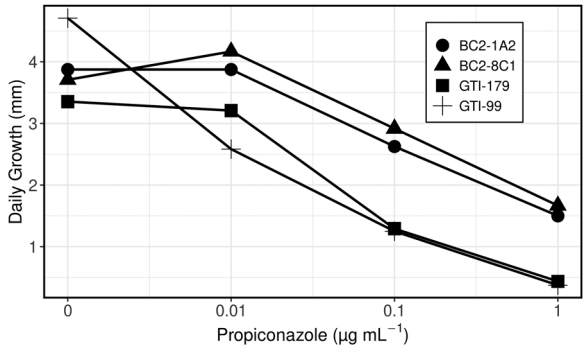

FIGURE 1 Examples of dose–response curves of select isolates, growth rate per day (mm) of each isolate of the full range of propiconazole concentrations (0, 0.01, 0.1, and 1 µg ml^{-1})

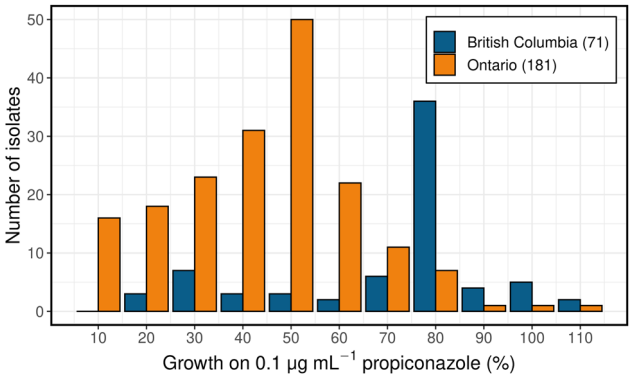

FIGURE 2 Distribution of isolates from Ontario (light orange) and British Columbia (dark blue), which were grouped into percentage growth bands (e.g., 0–10%, 11–20%, etc.), on the discriminatory concentration of propiconazole (0.1 µg mL^{-1}), where growth on nonamended potato dextrose agar was used as the denominator. The total number of isolates included in the discriminatory tests is in parentheses

response curves were used to determine the discriminatory concentration to distinguish resistant and sensitive isolates. Fungicide-amended plates at the discriminatory concentration (0.1 µg ml^{-1}) were prepared in addition to nonamended PDA plates. Fungal plating, growth conditions, and measurements proceeded in a similar manner to the full concentration range testing above. Means were calculated for the growth of each isolate on nonamended PDA. This mean was used as the denominator to calculate the percentage of growth on the discriminatory concentration, where individual measurements on propiconazole-amended media (0.1 µg ml^{-1}) were used as the numerator. These data were subjected to ANOVA in SAS PROC GLM. The means were then separated by Fisher's protected LSD ($p = .05$). The discriminatory tests were repeated twice per isolate, and isolates with a percentage growth >50% on the discriminatory concentration were deemed to be resistant to propiconazole. Isolates were grouped in 10-percentile bands of growth (e.g., 0–10, 11–20, 21–30%, etc.) to aid in visualizing the distributions.

3 | RESULTS

In total, 71 isolates of *M. nivale* were successfully recovered from the four locations in British Columbia and 67 isolates were recovered from the four Ontario locations. An additional 114 Ontario isolates from the lab collection obtained within the last 10 yr were revived for inclusion in propiconazole sensitivity testing. Most of the isolates were from creeping bentgrass (*Agrostis stolonifera* L.) or annual bluegrass (*Poa annua* L.). A dose–response curve is shown for four isolates (Figure 1) illustrating the differences between two resistant isolates (BC2-1A2 and BC2-BC1) and two sensitive isolates (GTI-179 and GTI-99).

The results from the full concentration range testing showed that the Ontario isolates (47) had an EC_{50} range of <0.001 to 0.89 µg ml^{-1}, with resistant isolates (EC_{50}: >0.1 µg ml^{-1}) having an average EC_{50} of 0.344 µg ml^{-1} and an RF of 7.9. In comparison, British Columbia isolates (50) had an EC_{50} range of 0.02 to 8.7 µg ml^{-1}, with resistant isolates having an average EC_{50} of 0.791 µg mL^{-1} and a RF of 20.8.

When tested at the discriminatory concentration, 43 of the 181 isolates (24%) from Ontario were found to have resistance to propiconazole (>50% growth on 0.1 µg ml^{-1}). In contrast, of the 71 isolates tested from British Columbia, 55 (77%) exhibited resistance to propiconazole. The British Columbia samples, as a group, had a statistically significantly ($p < .05$) higher proportion of propiconazole-resistant isolates than the Ontario samples (Figure 2). The mean growth rate of all isolates from Ontario on 0 ug ml^{-1} was 6.7 mm/day, and the rate in those from British Columbia was 6.5 mm/day, with no significant difference (LSD = 0.3).

4 | DISCUSSION

The climate of coastal British Columbia creates high disease pressure for *Microdochium* patch for most of the year. In recent years, some turfgrass managers in British Columbia have suspected that they are dealing with fungicide resistance issues, with several anecdotally reporting that applications of propiconazole appeared to provide shorter intervals of control relative to previous years. The results of the sensitivity testing presented here support those observations, with many isolates collected from southwestern British Columbia exhibiting significant resistance to propiconazole.

Field resistance to DMI fungicides has been documented in at least 28 different species (Fungicide Resistance Action Committee, 2020). However, there have been no confirmed reports of DMI resistance in field isolates of *M. nivale*.

Cristani and Gambogi (1993) were able to generate DMI-tolerant *M. nivale* in the lab by using UV radiation. The small number of DMI-insensitive isolates they were able to generate exhibited low to moderate insensitivity to a variety of DMI fungicides, with RFs ranging from 1.1 to 12.5. In comparison, the propiconazole-resistant isolates collected from British Columbia had an average RF of 20.8 and resistant isolates from Ontario had an average RF of 7.9. The EC_{50} values and RFs of propiconazole-resistant isolates from the locations in Ontario and British Columbia were similar to those reported for other fungal species showing DMI resistance (Köller & Wubben, 1988; Golembiewski, 1995; Hsiang et al., 1997). However, a few isolates with very high RF values in British Columbia (RF > 200) are concerning and could indicate the beginning of serious disease control problems.

This is the first lab-verified case of DMI resistance in *M. nivale* worldwide, although field trials are still necessary to confirm whether the observed resistance translates to control failure in the field (as noted anecdotally by turfgrass managers in British Columbia). Most locations in British Columbia and all locations in Ontario provided records of fungicide use for *M. nivale* (data not shown), which supported the hypothesis that higher usage of propiconazole was associated with resistance. These results highlight the need for turfgrass managers to adhere to strong fungicide resistance management practices whenever possible. Future research can be conducted to determine if there are fitness costs such as reduced virulence associated with resistance and to determine the genetic basis of propiconazole resistance in *M. nivale*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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