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Abstract

Monilinia vaccinii-corymbosi (Reade) Honey (*M.vc*), the causal agent of Monilinia blight of wild blueberry, is controlled primarily by fungicide applications. Demethylation-inhibiting fungicides (DMIs) have been used for over 30 years for Monilinia blight control due to flexibility of use (i.e., ability to use after an infection period) and disease control effectiveness and consistency. In the present study, the sensitivity of ten *M.vc* isolates to three DMIs- propiconazole, difenoconazole and prothioconazole-desthio were evaluated *in vitro* by a mycelial growth inhibition assay. In addition, four field trials were conducted during two crop seasons: 2012 and 2013, to examine the efficacy of these DMIs to control Monilinia blight. All the tested DMIs were effective in inhibiting mycelial growth of *M.vc* isolates, although the mean EC_{50} values differed significantly. In field experiments, three of four trials had significant treatment effect on disease incidence and severity of vegetative buds. Prothioconazole-desthio and propiconazole provided consistent control against Monilinia blight. Conversely, difenoconazole was effective in *in vitro* analysis, but did not demonstrate satisfactory Monilinia blight control in all field trials. In the 2012 trials, both prothioconazole-desthio and propiconazole reduced disease incidence of vegetative buds by 100% compared to the untreated control. Prothioconazole-desthio reduced disease development in 2013 with 94 and 99.8% less incidence, and 75 and 99.5% less severity. Similarly, propiconazole also reduced incidence of vegetative buds by 88% and 99.8%, and severity by 54% and 99.7%. No phytotoxic symptoms were observed in any of the field trials. The results of the study serve as a benchmark to monitor shifts in *M.vc* sensitivity to these fungicides in the future.

Additional Keywords: Monilinia blight, lowbush blueberry, fungicide sensitivity, propiconazole, prothioconazole-desthio, difenoconazole

Introduction

Monilinia blight, caused by *Monilinia vaccinii-corymbosi* (Reade) Honey (*M.vc*), is an important yield-limiting, fungal disease of wild blueberries. The disease has two infection phases with- the primary infection resulting in blighting of emerging vegetative and floral buds and the secondary phase resulting in infection of flowers and developing berries producing pseudosclerotia (mummy berries) (Hildebrand et al., 1995). Together, the primary and secondary infection processes cause losses in leaf and floral tissue, yield potential and berry quality. Control measures such as intense and uniform burning can be effective in destroying mummy berries (Delbridge and Hildebrand, 1995), however, sequential burning has a high cost and reduces the organic matter content in the soil. Annis and Yarborough (2014) stated that efficient harvest techniques reduces the number of infected berries dropping to the ground. Although, these measures reduce the

number of infected berries left in the field, they may not give sufficient control of disease (Guo, 2016). Effective management of the disease relies heavily on the use of fungicide applications.

Demethylation inhibitors (DMIs) compose one of the most important group of fungicides used for Monilinia blight control in Canada. DMIs specifically target the demethylation process at C-14α demethylase and disrupt fungal sterol biosynthesis (Köller, 1988). The *Monilinia vaccinii-corymbosi* populations in commercial wild blueberry fields in Nova Scotia have been exposed to DMI fungicides for approximately 30 years since the first usage of triforine in the 1980s (Lockhart et.al., 1983). It was replaced by propiconazole in the 1990s, and the wild blueberry producers have been reliant on propiconazole for Monilinia blight control for past 20 years. Despite a history of success in using propiconazole, inconsistent efficacy has been observed in research trials conducted in Nova Scotia in 2007, 2008, 2010 (Percival and Beaton, 2012) and 2017. Extensive and consecutive use of a specific fungicide can lead to a sensitivity shift toward resistant populations of the pathogen to the fungicide (Brent and Hollomon, 2007). Furthermore, cross-resistance can occur between fungicides that belong to the same chemical group or have the same mode of action.

Consequently, studies completed by Percival and Beaton (2012) reported prothioconazole-desthio as an effective Monilinia blight control product, with the additional benefit of no residues detected in harvested or processed berries. The importance of assessing other DMIs for Monilinia blight became more apparent with propiconazole being declared a human health risk by the European Union (WTO, 2018). Since propiconazole has been extensively used for Monilinia blight control and site- specific fungicides are prone to resistance development, assessing the sensitivity of *M.vc* to the most commonly used DMI is crucial for Monilinia blight management. This study was conducted to determine the sensitivity of *M.vc* isolates to DMI group fungicides (propiconazole, prothioconazole-desthio and difenoconazole) and to investigate the ability of these fungicides to control Monilinia blight in commercial wild blueberry fields.

Materials and Methods

Monilinia vaccinii-corymbosi isolates

Isolates were collected from mummy berries and Monilinia blighted shoots from five commercial wild blueberry fields in Nova Scotia during 2011 to 2013. Mummy berries and Monilinia blighted shoots were surface sterilized and blocks of white medulla cut from the center of mummy berries and pieces of blighted leaves were inoculated onto potato dextrose agar (PDA) plates amended with antibiotics (0.5mg mL^{-1} streptomycin sulfate, 0.5mg mL^{-1} penicillin) to suppress bacterial growth. Petri dishes were incubated at 22 ± 2 °C for further mycelial growth.

Determination of fungicide sensitivity

The fungicides used in this study were the pure technical grade active ingredients: propiconazole (Syngenta Honeywood Research Farm, Platisville, ON, CA), prothioconazole-desthio (Bayer Crop Science, MO, US) and difenoconazole (Syngenta Honeywood Research Farm, Platisville, ON, CA). Stock solutions/suspensions of fungicides were prepared by dissolving active ingredients in acetone on the day of the experiment. Autoclaved PDA was amended with 0, 0.005, 0.01, 0.05, 0.1 μg mL^{-1} propiconazole and difenoconazole and 0, 0.001, 0.002, 0.003, 0.004 μg mL^{-1} prothioconazole-desthio. The control medium was not amended with fungicides. In all cases, including the control plates, the final acetone concentration was 0.1 % (v/v). Tests for each isolate were replicated three times per concentration of each fungicide.

Sensitivity of 10 isolates was estimated based on mycelium growth inhibition assay (Golembiewski et al., 1995; Hu et al., 2011; Wise et al., 2011). For each isolate, a 4mm- diameter mycelial plug was cut from the edge of a 10-day-old colony grown on PDA medium and placed upside down on fungicide- amended and un-amended media. The plates were incubated at 22 ± 2 °C in the dark. After 7 days, the colony diameter was measured in two perpendicular directions, and the diameter of the mycelial plug was subtracted before calculating the mean diameter of the colony. For each concentration/fungicide, the EC_{50} value (effective concentration that reduced mycelial growth by 50%) was calculated by regressing the relative growth (RG: colony diameter on fungicide-amended medium divided by colony diameter on unamended medium × 100) against the $\log_{10}$ fungicide concentration. As per the reports of Thompson and Annis (2014), mean EC_{50} value for baseline isolates from unmanaged fields of Maine to propiconazole is 0.016 g μg mL^{-1}. Since there were no previous references of *M.vc* to difenoconazole and prothioconazole-desthio, EC_{50} values lower than the EC_{50} found for propiconazole (0.016 μg mL^{-1}, (Thompson and Annis, 2014) were considered "sensitive" (S); EC_{50} values ranging from 0.016 to 1.0 μg mL^{-1} were considered "moderately sensitive" (MS); and EC_{50} values above 1.0 μg mL^{-1} were considered "non-sensitive" (NS). The variation factor (VF) to fungicides was calculated as the highest EC_{50} value divided by the lowest EC_{50} value, in order to evaluate the extent of variability in fungicide sensitivity among the populations.

Field Experiments

Field trials were conducted during 2012 and 2013 to determine the efficacy of the three DMIs against natural infections caused by *M.vc*. Experiments were performed in the cropping phase of production in four commercial wild blueberry fields. Two trials were located at Kemptown, Nova Scotia (NS) (coordinates = 45° 30´ N, 63° 07´ W) and Aulac, New Brunswick (NB) (coordinates = 45° 53´ N, 64° 16´ W) in 2012. The other two field trials were located at Mt. Thom, NS (coordinates = 45° 29´ N, 62° 59´ W) and Farmington, NS (coordinates = 45° 34´ N, 63° 53´ W) in 2013. The trials were in a randomized complete block design with five replications. Each plot was 4 × 6 m with 2m buffers between plots.

DMI treatments consisted of application of Proline® 480SC (a.i. prothioconazole) at 300 mL product·ha^{-1}, Tilt® 250E (a.i. propiconazole) at 500 mL product·ha^{-1}, and Inspire® (a.i. difenoconazole) at 80 g product·ha^{-1}. A 2000 series Watchdog® weather station (Spectrum Technologies, Inc. Plainfield, IL, US) was set up in the center of each trial to monitor air temperature, relative humidity, wind speed and direction, and leaf wetness every minute. Fungicides were sprayed two times. The first application was done when the field reached to 40 – 50 % V2 and/or F2 stage (V2: 2 - 5 mm green leaf tissue emerged, F2: floral bud scales separating). The second application was done 7 to 9 days after the first application. The timing was decided by consulting with the Monilinia forecasting system (which uses temperature, wetness duration, spore dispersal, and developing stage of vegetative and/or floral buds), which was available at lowbush blueberry blog (provided by Perennia, Truro, NS, CA). In 2012, fungicide applications were made on May 2 and May 11 at the Kemptown site and on April 30 and May 8 at the Aulac site. In 2013, fungicide applications performed on May 8 and May 17 at the Mt. Thom site and on May 7 and May 14 at the Farmington site. Each fungicide was mixed with distilled water and was applied using a Bell spray Inc. ® hand held sprayer with a 2-m CO_2 propelled boom and 4 TeeJet Visiflow 8003VS nozzles. The sprayer pressure was 32 PSI (220 kPa). The nozzle discharge rate was 12.5 mL·s^{-1} and application ground speed was approximately 1.19 m·s^{-1}.

Disease assessment

Disease incidence and severity were visually assessed on fifteen randomly selected stems per plot using a line transect (samples were collected every 4.5 m along the transect). Since symptoms of Monilinia blight do not show until 10 to 17 days after the susceptible buds were infected by the ascospores (Delbridge and Hildebrand, 1995), first assessments occurred prior to the second fungicide applications, which were 7 to 9 days after first fungicide application. Second assessment occurred within 2 weeks after the second fungicide applications.

Disease incidence was determined by percentage of floral buds and/or vegetative buds per stem with visual symptoms of Monilinia blight. While, severity was determined by percentage of infected area per flower or leaf with visual symptoms of Monilinia blight within a stem. The suppression efficacy of fungicides was determined by comparing the disease incidence and severity of wild blueberry plants sprayed with fungicides to the control trials (without fungicide). Phytotoxicity was assessed visually by examining the floral/vegetative buds of each stem for phytotoxic symptoms. The impact of the treatments on yield potential was determined by examining parameters including stem length, number of vegetative nodes, floral nodes, set fruit (adequately pollinated and fertilized marketable blueberries) and, pinheads (small under-formed and unmarketable blueberries).

In August, berries were harvested from the field. A forty-tine, commercial wild blueberry hand rake was used to harvest berries from four randomly selected 1-m^2 quadrats in each plot. Harvested berry yield was recorded using a bench scale.

Data Analysis

For *in vitro* tests, analysis of variance was completed using the PROC Mixed procedure of SAS (version 9.3, SAS institute, Inc., Cary, NC), using a factorial design of isolates and active ingredients, with the replicate as the blocking factor. Orthogonal contrasts and Tukey's mean comparison test were used. The PROC CORR procedure of SAS was used for correlation analysis. For *in vivo* tests, analysis of variance for all the data collected from the experiment was completed using the PROC MIXED procedures of SAS (version 9.3, SAS institute, Inc., Cary, NC). LSD was used for multiple means comparison at the level of $\alpha = 0.05$.

Results and Discussion

Sensitivity of *Monilinia vaccinii-corymbosi* to DMI fungicides

DMI fungicides are the dominant fungicide products for Monilinia blight control in wild blueberry production in Nova Scotia due to: their disease control efficiency, ease of use, low cost, and economic benefits. However, there is concern that the *M.vc* populations in commercial wild blueberry fields in Nova Scotia may have developed reduced sensitivity to propiconazole because of their extensive and consecutive usage for past 20 years. In the present study, none of the tested isolates exhibited reduced sensitivity, although, the mean EC_{50} value differed significantly among each of the tested fungicides (Table 1). Prothioconazole-desthio was the most effective inhibitor with a mean EC_{50} of 0.00086 $\mu g \cdot mL^{-1}$. Difenoconazole was found to be more effective in inhibiting mycelial growth on PDA medium than propiconazole.

Although, propiconazole maintained its overall effectiveness against *M.vc* isolates, inconsistency existed among the isolates. This might be due to a relatively wider range observed in propiconazole EC_{50} value than prothioconazole-desthio and difenoconazole (Table 1). This inconsistency could be attributed to its erratic efficacy, as observed in the field research trials.

Thompson and Annis (2014) found that isolates of *M. vaccinii-corymbosi* from unmanaged areas has EC_{50} value of 0.016 $\mu g \cdot mL^{-1}$ for propiconazole. In our analysis, the mean propiconazole EC_{50} value was 0.012 $\mu g \cdot mL^{-1}$, which was lower than the baseline value, which indicates the isolates are sensitive to propiconazole. Similarly, the isolates were not exposed to prothioconazole-desthio and difenoconazole before the year 2014; consequently, data were not available for comparisons. Thus, EC_{50} results of prothioconazole-desthio and difenoconazole provided valuable information concerning the normal variation in fungicide sensitivity of *M.vc*, which can be used as a baseline for further sensitivity analysis.

Table 1. Inhibitory effect of each active ingredient on mycelial growth of ten *M. vaccinii-corymbosi* isolates from commercial wild blueberry fields in Nova Scotia and their comparison using Tukey's LSD

	EC_{50} values ($\mu g \cdot mL^{-1}$)[a]		
Active ingredient	Range	Mean[b]	Variation factor[c]
Propiconazole	0.008-0.016	0.012 a	2
Difenoconazole	0.006-0.0083	0.0068 b	1.4
Prothioconazole-desthio	0.00076-0.0012	0.00086 c	1.6

[a] EC_{50} value is the effective concentration that inhibit mycelial growth by 50%

[b] Means that share the same letter is not significant different from each other. Proc Mixed procedure was used for analysis of variance (ANOVA)

[c] Variation factor = highest EC_{50} value divided by lowest EC_{50} value

It is well documented that different DMIs can show distinct but consistent cross-resistance patterns (Hildebrand et al., 1988; Köller & Wubben, 1989; Kendall et al., 1993). Cross-resistance is generally accepted to be present among DMI fungicides active against the same fungus (Fungicide Resistance Action Committee, 2015) because of their unique mode of action. A study by Holb and Schnabel (2007) observed cross-resistance among triazoles by noting their effects on *M. fructicola* on peach. In this study, propiconazole EC_{50} values were significantly correlated with difenoconazole EC_{50} values and prothioconazole-desthio EC_{50} values at the level of α=0.05 (Table-2). A high correlation between propiconazole and prothioconazole-desthio was found (r = 0.73). However, the correlation between propiconazole and difenoconazole was very weak (r = 0.39) Correlation implies the possible emergence of cross-resistance among these DMIs. Development of reduced sensitivity or resistance of *M. vaccinii-corymbosi* to propiconazole would have an impact on control efficacy of prothioconazole and difenoconazole. A moderate cross-resistance between propiconazole and difenoconazole has been reported for *Alternaria alternata* isolates on pistachio (Avenot et al., 2016). As per Thomas et al. (2012), cross-resistance among DMI fungicides is not universal. Several studies also documented the lack of cross-resistance among DMIs (Leroux et al., 2000; Thomas et al., 2012). Thus, we cannot assume the existence of a positive cross-resistance between fungicides with a similar mode of action. Moreover, the sample size used in this study was relatively small. A larger sample size may have provided better correlation results.

Table 2. Pearson correlation coefficient (*r*) values of EC_{50} values for propiconazole, difenoconazole and prothioconazole-desthio

Active ingredient	Difienoconazole		Prothioconazole-desthio	
	r	*P*-value	*r*	*P*-value
Propiconazole	0.39	0.038	0.73	<0.001
Difenoconazole	-	-	0.17	0.4

Effect of DMI fungicides on Monilinia blight control on field conditions

Monilinia blight incidence was observed to be higher on leaf tissues than floral tissues in all trials in 2012 and 2013. There was a significant interaction among fungicide treatment and field trials. This significance led us to carry out one-way ANOVA for each of the field trials. In the field trials of this study, vegetative and floral buds did not show any Monilinia blight symptoms after the first fungicide application. Relatively dry weather at the field trials in 2012 and 2013 might have contributed to low disease pressure, which was in contrast to severe Monilinia blight pressure in 2009 (D. Percival, personal communication, Dalhousie University, NS, CA).

Similarly, in the four field experiments, only three fields demonstrated significant treatment effects (Table-3). In the trials conducted in 2012, the Aulac trial did not demonstrate significant treatment effects. However, the disease incidence and severity of vegetative buds in the Kemptown trial ranged from 0 % to 0.3 % and 0 % to 1.01 % respectively. Even though, the fungicides did not show a suppressive effect on disease incidence, they significantly lowered disease severity by 100 %.

In 2013, trials conducted in Farmington and Mt. Thom, the Monilinia blight disease pressure was found to be higher than 2012. After the second fungicide application, in the Farmington trial, prothioconazole-desthio, propiconazole and difenoconazole lowered disease incidence of vegetative buds by 94 %, 88% and 27 % and disease severity by 75 %, 52 % and 10 % respectively. In the Mt. Thom trial, compared to untreated control, prothioconazole-desthio and propiconazole significantly decreased disease incidence and disease severity of vegetative buds by 100 %. However, difenoconazole lowered disease incidence and severity by 89 % and 86 % respectively. No significant treatment effect was observed on the physical development of wild blueberry plants or berry yield after the first and second fungicide applications in all trials. This might be due to the low disease incidence on floral buds as observed in all field trials (data not shown).

Table 3. Influence of DMI fungicides on the suppression of Monilinia blight on vegetative buds on trials with significant treatment effects after second fungicide application

Treatment	2012/Kemptown		2013/Farmington		2013/Mt.Thom	
	DI[a] (%)	DS[b] (%)	DI[a] (%)	DS[b] (%)	DI[a] (%)	DS[b] (%)
Untreated	0.3	1.01	4.18	15.4	1.22	6.18
Proline® 480SC	0	0	0.24	3.87	0.003	0.03
Tilt® 250E	0	0	0.51	7.33	0.003	0.02
Inspire®	0.07	0.23	3.07	13.8	0.14	0.89
ANOVA results[c]	NS	*P*=0.04	*P*=0.02	*P*=0.048	*P*=0.01	*P*=0.02

[a] DI (Disease incidence) = 0 to 100% where 0 = no vegetative buds affected and 100 = all vegetative buds are affected with at least one lesion.

[b] DS (Disease severity) = 0 to 100% where 0 = no disease and 100 = one whole vegetative bud is affected

[c] Analysis of variance (ANOVA) results refer to treatment effects that were either not significant (NS) or significant at $p<0.05$. Mean separation was completed using LSD multiple means comparison test procedure ($\acute{\alpha}=0.05$).

DMIs represent one of the largest groups of systemic fungicides that have been used to control agriculturally important fungal pathogens (Zhan et al., 2006). In the present study, prothioconazole-desthio and propiconazole provided consistent control against Monilinia blight. Among these, prothioconazole-desthio was the most effective fungicide product that showed significant reduction in disease incidence and severity of vegetative buds for all trials (Table-3). These are similar to the results observed in field research trials conducted in 2009 (Percival and Beaton, 2012). However, it should be noted that difenoconazole did not suppress Monilinia blight in the field trials, but was effective in *in vitro* inhibition of mycelial growth. Despite long-term use, *M.vc* populations are still sensitive to propiconazole.

Conclusion

According to the results from the *in vitro* sensitivity study of *M.vc* isolates and *in vivo* fungicide efficacy examination for Monilinia blight control, the *M. vaccinii-corymbosi* populations affecting lowbush blueberry had not developed reduced sensitivity or resistance to the DMIs used in this study and this class of fungicides remains the most effective. Besides difenoconazole, the *in vivo* results were consistent with *in vitro* results that prothioconazole-desthio and propiconazole had higher control efficacy against Monilinia blight in field trials. The information presented in the study can be used to evaluate population shifts in fungicide sensitivity in subsequent years.

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