# Distribution of Myclobutanil Fungicide Sensitivities among Populations of *Venturia inaequalis*, the Causal Agent of Apple Scab, in Ontario

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**Abstract**

**Sensitivity of five populations of *V. inaequalis*, collected from four geographical locations in Ontario, to myclobutanil, a demethylation inhibitor fungicide, was investigated. Sensitivities of isolates to fungicide were determined by $ED_{50}$ values and a sensitivity test based on the relative growth (mean colony diameter on media amended with discriminatory myclobutanil doses per mean colony diameter on unameded media x 100) of mycelial colonies of a baseline population at a single discriminatory dose (0.1 µg/ml). Both assays were highly correlated. The wild-type population had a baseline sensitivity (mean $ED_{50}$) of 0.074 µg/ml of myclobutanil and showed lognormal distributions to myclobutanil. The mean $ED_{50}$ values were 0.114, 0.074, 0.076, 0.071 and 0.581 µg/ml of myclobutanil for orchards 1, 2, 3, 4, and 5, respectively. With the exception of the orchard 5, all populations showed lognormal distributions to myclobutanil. Mean isolate sensitivities between baseline population and populations sampled from orchards that have never been or rarely treated with myclobutanil were not significantly different (P = 0.20). Isolates from one experimental orchard, orchard 5, that was treated with myclobutanil showed tolerance to the fungicide and had significantly different mean isolate sensitivities than the baseline population ( *P* = 0.03). The resistance factor for orchard 5 was 7.8 (mean $ED_{50}$ of resistant population/ mean $ED_{50}$ of the baseline population), while resistance factors for four other orchards ranged from 0.9 to 1.5 for myclobutanil. Although fungicide resistance to myclobutanil in *V. inaequalis* was not observed in the commercial orchards, a shift in fungicide sensitivities was observed in an an experimental orchard.**

## INTRODUCTION

Apple scab caused by *Venturia inaequalis* (Cooke) G. Wint. is an important disease world wide, especially during the years of above-average rainfall. Following the reports of practical resistance, defined as diminished levels of disease control caused by selection of resistant isolates, of benomyl in *V. inaequalis* in apple orchards in Ontario in the late 1970's, the use of the fungicide was either reduced or restricted and new fungicides have been introduced to control apple scab in North America.

Fungicides in the class of sterol demethylation inhibitors (DMI), identified as potent inhibitors of the C-14 demethylation of 24-methylenedihydrolanosterol, a precursor of fungal sterol biosynthesis, have broad spectrum of fungicidal activity and have been used to control apple scab. The DMIs were registered for apple scab control in 1989 in the USA. First report of practical resistance in *V. inaequalis* to DMI was reported in an apple orchard in Michigan in 1997 (Koller et al., 1997).

Prior to its introduction in commercial orchards in Canada, DMI resistance in *V. inaequalis* was observed under high selection pressure with DMI fungicides in experimental orchards both in Nova Scotia in 1988 (Hildebrand et al., 1988) and in British Columbia in 1989 (Scholberg et al., 1989). In Canada, myclobutanil was introduced in 1992 for apple scab control. Although there has been no report of reduction of apple scab control due to resistance to DMI in Ontario since the introduction of myclobutanil, the reports of DMI resistance in commercial orchards in the US introduces

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concerns about the development of resistance to these materials in Canada. The objective of this study is to determine sensitivity of isolates of *V. inaequalis* to DMI fungicide, myclobutanil, in different orchards in Ontario and also to determine the baseline sensitivity of the fungus to myclobutanil.

## METHODS

### Origin of Isolates of *V. Inaequalis*

Wild-type population of *V. inaequalis*, collected in 1999 from two crab apple trees, that has never been treated with fungicides, on the Niagara Escarpment in St. Catharines, Ontario. Isolates of *V. inaequalis* from the five orchards in Ontario were colleted during 1998-2000. Orchards 1 to 4 were commercial orchards: orchards 1 and 3 have never been exposed to myclobutanil, Orchard 2 had one DMI application in early season for a three years and the past spray record for the Orchard 3 was not available. The Orchard 5 was an experimental orchard that has been exposed twice to myclobutanil. All isolates were single spored from scab infected apple leaves and stored at 4 °C for long term storage. Two DMI-sensitive isolates, Race 1, and 95-02, which have never been exposed to DMI fungicides, and two DMI-tolerant isolates, 95-15 and 95-20, were used as reference isolates.

### Determination of Fungicide Sensitivity

Myclobutanil, the fungicide used in the study was obtained from Rohm and Haas Canada, Inc., Westhill, Ont. The isolates were assayed on concentrations, 0.03, 0.05, 0.07; and 0.1 to 4 µg/ml in increments of 0.1 µg/ml, of myclobutanil at 22 ∀ 2 °C. Relative growth (RG), determined with three replicates per isolate, was defined as the diameter of a mycelial colony on myclobutanil-amended medium divided by the diameter on unamended medium x 100. All assays were repeated at least once.

### Data Analysis

The $ED_{50}$ values were calculated by regressing RG against the log of the myclobutanil concentrations. Frequency distributions of log transformed $ED_{50}$ values determined for individual populations were analysed according to the univariate procedures of SigmaStat. Means of RG values were compared according to the nonparametric Kolmogorov-Smirnov test of SigmaStat (Sigma Stat 2.0 for Windows, SPSS Science, Chicago, IL).

## RESULTS AND DISCUSSION

There was no increase in DMI resistance in *V. inaequalis* in commercial apple orchards, which ranged from orchards that have never been exposed to myclobutanil and to an orchard which was exposed to a single application per year for a period of five years, in Ontario. However, the use of the myclobutanil in the experimental orchard, Orchard 5, was correlated with the reduced sensitivities. Higher frequency of isolates with RG values >80 were detected in orchard 5 than the baseline populations. According to the Koller et al., (1997) RG = 80 distinguished between DMI-sensitive and DMI-resistant phenotypes. Frequency shifts observed in the population from orchard 5 may have been caused by the selection of such isolates in response to the use of myclobutanil.

### Sensitivity of Wild-Type Isolates of *V. Iinaequalis* to Myclobutanil

Baseline sensitivity of the wild-type population of *V. inaequalis*, that was not exposed to any DMI fungicides, ranged between 0.005 - 0.485 µg/ml with a mean $ED_{50}$ of 0.074 µg/ml of myclobutanil. Based on the inhibitory effect of myclobutanil on mycelial growth, sensitivities ($ED_{50}$ values) were determined (Fig 2). The frequency distribution of $ED_{50}$ values was lognormal ($P$ = 05) according to Kolmogorov-Smirnov statistics for the wild-type population. A single myclobutanil dose, 0.1 µg/ml, was derived from a concentration close to the mean $ED_{50}$ value. This discriminatory dose represents a

quantitative measure of isolate sensitivities and include a precise measure of myclobutanil sensitivities. Similar discriminatory doses were reported (Koller., 1997). The RG of the baseline population and two reference DMI-sensitive isolates were not significantly different while significant differences were observed between baseline population and DMI-tolerant reference isolates (Table 1).

**Comparison of $ED_{50}$ Values and Relative Growth**

Two fungicide sensitivity measures, $ED_{50}$ values and RG were compared. In this study, sensitivity of all of the isolates of *V. inaequalis* to myclobutanil have been determined as $ED_{50}$ values. In addition, a simplified sensitivity test, RG of mycelial colonies at a single discriminatory dose (0.1 µg/ml) was derived based on the $ED_{50}$ values of isolates collected from wild type populations in Ontario. This discriminatory dose was similar to that proposed by Koller et al., 1991. Both myclobutanil sensitivity measures, $ED_{50}$ values and RG at a single discriminatory dose were highly correlated.

**Sensitivity of Five Populations *V. Inaequalis* toMyclobutanil**

Five populations of *V. inaequalis* were tested for their RG on a discriminatory dose of 0.1 µg/ml. The mean RG at 0.1 µg/ml of myclobutanil was 46.1, 56.9, 42.4, 40.3, and 73.59 for orchards 1, 2, 3, 4, and 5, respectively (Table 1). The mean RG for the wild type population was 34.4. There was no significant difference in mean RG between orchards 1, 3 , 4 and baseline populations ($P = 0.20$) and two sensitive isolates, Race 1 and 95-02. Myclobutanil sensitivity distribution followed a lognormal distribution pattern for orchards 1 to 4.

Significant difference was observed between orchards 2, 5 and baseline populations ($P = 0.03$). There was no significant difference in the RG between orchard 5 and the two DMI-tolerant reference isolates, 95-15, and 95-20. A significantly higher $ED_{50}$ values of populations from orchard 5, an experimental orchard which was exposed to myclobutanil, than the mean of the four commercial orchards, and baseline population suggests that the use of the DMI fungicide in some instances increases the frequencies of *V. inaequalis* phenotypes with substantially less sensitivity than the baseline population mean. Quantitative changes in increase in resistance to myclobutanil in the experimental orchard is consistent with other studies (Braun and McRae, 1992 and Koller et al., 1997). In contrary, no resistance was observed after 12 years of extensive DMI application in an orchard in the US (Smith et al., 1991).

**Sample Size**

Samples size used to detect sensitivities of pathogens to different DMI fungicides in the previous studies varied. A comparison of $ED_{50}$ values of a sample size of 13 isolates was very close to 300 baseline isolates (Koller, 1991). Smith et al., (1991) found that a sample size of 50 isolates could detect 1.6 -fold and higher difference between any sensitivities of any two populations of *V. inaequalis*. Isolates ranging from 9 to 15 were used to determine the sensitivity of populations of *V. inaequalis* to myclobutanil (Braun and McRae, 1992). The sample size of populations in this study ranged between 15 to 68 isolates of *V. inaequalis* and the baseline sensitivity for myclobutanil was determined based on the $ED_{50}$ values for the 54 isolates (Table 1).

In conclusion, the reduced sensitivities in the experimental orchard 5, indicates that there is a shift in sensitivities and therefore, there is need for resistance monitoring in orchards that use DMIs. Fungicide monitoring based on either $ED_{50}$ values or RG on a single discriminatory dose of the fungicide is very labour intensive and alternative tools such as molecular probes, which can detect the region of the genome of the fungus that is responsible for DMI resistance, are needed for accurate and rapid monitoring of fungicide resistance in populations of *V. inaequalis*. Correlating resistance monitoring and pathogen's shift in sensitivity to a given fungicide's diminished control will enable the field practitioners to use appropriate control measures for apple scab.

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## Tables

Table. 1. Sensitivity distribution of *Venturia inaequalis* to a demethylation inhibitor fungicide myclobutanil.

| Sampling site | *n* | $ED_{50}$ (Φg/ml) | | Variation in population | | Normality test (Kolmorgorov-Simmov; $P = 0.05$) |
|---|---|---|---|---|---|---|
| | | Range | Mean | Mean relative growth[a] | Resistance factor[b] | |
| Location A (wild-type) | 54 | 0.005 - 0.385 | 0.074 | 34.4 | - | Passed |
| Orchard 1 | 15 | 0.011 - 0.246 | 0.114 | 46.1 | 1.5 | Passed |
| Orchard 2 | 16 | 0.005 - 0.185 | 0.076 | 56.1 | 1.0 | Passed |
| Orchard 3 | 28 | 0.012 - 0.141 | 0.074 | 42.5 | | Passed |
| Orchard 4 | 15 | 0.006 - 0.132 | 0.071 | 40.3 | 0.9 | Passed |
| Orchard 5 | 68 | 0.006 - 1.82 | 0.581 | 73.6 | 7.8 | Failed |
| Race1, S [c] | 1 | 0.027 | - | 32.9 | - | - |
| 95-02 SI, S[c] | 1 | 0.086 | - | 36.1 | - | - |
| 95-15 SI, T[d] | 1 | 1.222 | - | 73.9 | - | - |
| 95-20 SI T[d] | 1 | 1.100 | - | 99.9 | - | - |

[a]Relative growth defined as mean colony diameter on media amended with discriminatory myclobutanil doses per mean colony diameter on unamended media x 100.
[b]Mean $ED_{50}$ of resistant population/ mean $ED_{50}$ of the baseline population;
[c] S= Sensitive; [d] T = Tolerant

## Figures

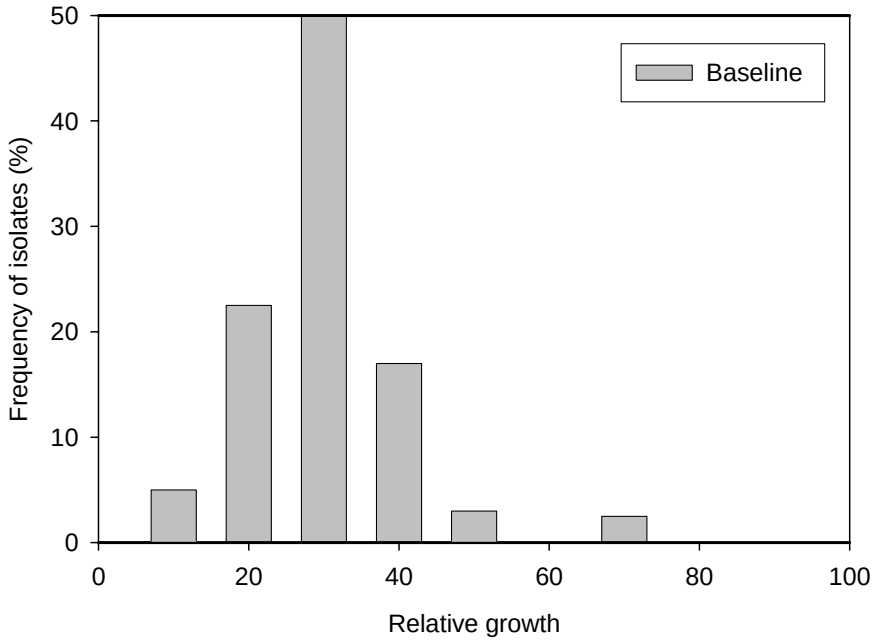


Fig. 1. Myclobutanil sensitivity distributions of monoconidial isolates of wild-type population of *Venturia inaequalis*.

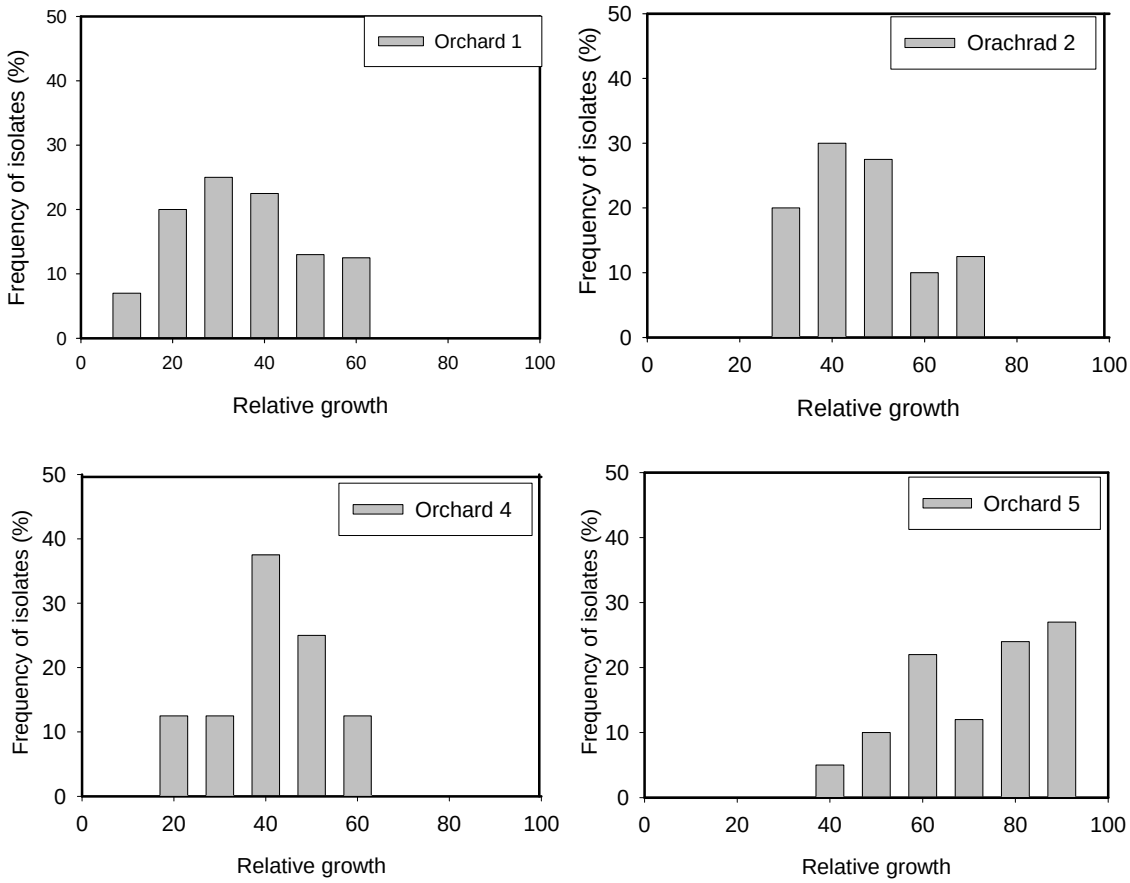


Fig. 2. Frequency distribution based on relative growth of individual isolates of *Venturia inaequalis*, on myclobutanil amended medium (0.1 µg/ml). Orchards 1, 2, 4 and 5 are presented. Relative growth is defined as as mean colony diameter on media amended with discriminatory myclobutanil doses per mean colony diameter on unameded media x 100.