REDUCED SENSITIVITY OF *PERONOSPORA TABACINA*, CAUSAL AGENT OF TOBACCO BLUE MOLD, TO DIMETHOMORPH FUNGICIDE IN CONNECTICUT

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Tobacco blue mold, caused by *Peronospora tabacina,* may be difficult to manage in commercial tobacco crops despite repeated dimethomorph (DMM) fungicide applications. To investigate whether this could be due to reduced efficacy, we compared fungicide efficacy in replicated field trials in certain years from 2002 to 2011 and in in vitro assays to evaluate sporangial germination, infection, disease, and sporulation. In 2002 and 2004, DMM fungicide was as efficacious against blue mold as DMM plus mancozeb. In 2009 and 2011, however, DMM was intermediate to mancozeb and untreated controls. In 2002, sporangial germination on 1.2 or 12 mg/L DMM-amended media was 87.0 and 9.1%, respectively, of germination on 0 mg/L media. No germination occurred at 120 mg/L DMM. In 2011, normalized sporangial germination on 1.2, 12, and 120 mg/L DMM-amended media was 86.2, 12.3, and 3.0%, respectively. There were no significant differences in germination between 2002 and 2011. In 2002, researchers in North Carolina, using detached leaf assays, determined that the baseline sensitivity to DMM was less than 1 mg/L, with no sporulation observed. In 2011, using the same assay, we determined that 37% of leaf disks floating on 1.2 mg/L DMM were diseased, with both sporangiophores and sporangia produced. On the basis of these data, efficacy of DMM against *P. tabacina* appears to have been partially reduced over time.

Additional key words: blue mold, disease control, fungicide, *Nicotiana tabacum*, *Peronospora tabacina*, resistance, tobacco

INTRODUCTION

Blue mold of tobacco (*Nicotiana tabacum*, L.), caused by the oomycete pathogen *Peronospora tabacina* Adam (*Peronospora hyoscyami* de Bary), is an economically devastating disease of the cigar wrapper tobacco types (*Nicotiana tabacum* L.) grown in the Connecticut River Valley of Connecticut and Massachusetts. An isolate of *P. tabacina* capable of causing disease under warm conditions in the field resulted in a national pandemic in 1979 that caused more than $250 million in losses (10). After 1980, the disease was effectively controlled by widespread use of metalaxyl fungicide. However, metalaxyl-resistant *P. tabacina* was initially reported in Mexico in 1984 (19) and was subsequently detected in the United States in 1991. Metalaxyl-resistant *P. tabacina* became widespread and predominant over time and was eventually introduced into Connecticut and Massachusetts in 1996 where it has either overwintered or been reintroduced each year since (8). The shade-grown and broadleaf cigar wrapper tobacco types grown in Connecticut and Massachusetts both require unblemished leaves to be marketable. As a result, tens of millions of dollars of losses have been sustained in this small production area over the last 16 years, despite best efforts to control the disease.

Blue mold is currently managed by repeated fungicide applications throughout the season. Dimethomorph DMM, azoxystrobin, and mancozeb fungicides are labeled for use on shade tobacco; only DMM and mancozeb are used for blue mold control on broadleaf tobacco.

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Because the pathogen is an obligate parasite, researchers in North Carolina established the baseline sensitivity to DMM of a wide variety of geographical isolates of *P. tabacina* and tested for the development of insensitivity over time using a detached leaf assay (15,16). No DMM insensitivity was reported over that time period. Over the last few years, however, growers in Connecticut have been concerned about perceived reduced fungicide efficacy resulting in more severe blue mold than expected under certain conditions. The objectives of this research were to review historical fungicide efficacy data from replicated field plots and to conduct in vitro tests to evaluate the sensitivity of Connecticut and Massachusetts isolates of *P. tabacina* to the carboxylic acid amide (CAA) fungicide DMM.

MATERIALS AND METHODS

The obligate nature of *P. tabacina* prohibited typical fungicide sensitivity assays, so we evaluated historical data from field plots and sporangial germination in vitro as well as conducting the leaf disk bioassay used to establish baseline sensitivity of the pathogen to the fungicide.

Field experiments to evaluate DMM efficacy. To evaluate possible changes in *P. tabacina* sensitivity to DMM, we compared results from replicated fungicide trials conducted in 2002 and 2004 with those conducted in 2009 and 2011. Experiments in these years were selected because they evaluated DMM alone as one of the treatments tested for blue mold efficacy. All field experiments were conducted in a shade tent at the Connecticut Agricultural Experiment Station Valley Laboratory Research Farm in Windsor, CT. Shade-grown tobacco in 5- by 5-m plots consisting of 4 planted rows were fertilized annually with cottonseed meal

based 10–8–10 (approximately 168 kg/ha) before planting in early June. Mefenoxam (Ridomil Gold EC at 1.2 liters/ha) and chlorpyriphos (Lorsban 4E at 7 liters/ha) were lightly harrowed in at the same time before planting to control root rot and cutworms, respectively. Plots were planted with blue-mold-susceptible shade tobacco cultivar 8212 transplants in early June in 4 rows (15 plants per row 30 cm apart within rows with 39 cm between rows). Plots were side-dressed with approximately 168 kg/ha 10–8–10 and cultivated in mid-June for a total of 336 kg N/ha. Pendimethalin (Prowl 3.3 E at 2.9 liters/ha) was applied as a lay-by directed spray using a 8004E nozzle at 175 kPa in late June to control weeds. Plants were hand suckered, tied at the end of June, and wrapped in early and mid-July. Fungicide sprays were applied to the two inside plot rows using hand pump backpack sprayers (Solo, Newport News, VA) at 175 to 200 kPa in 225 liters/ha (first spray date), 450 liters/ha (second spray), and 900 liters/ha (sprays 3 through 6) to achieve coverage of spray rows. The 2 outside rows were unsprayed borders in each plot. Treatments were arranged in a randomized block design with 4 replicate plots of each treatment. Fungicides were applied at 14-day intervals starting 3 weeks after transplanting for a total of 6 applications.

Materials tested for efficacy against blue mold. Dimethomorph plus mancozeb (Acrobat MZ, BASF Corp., Research Triangle Park, NC); DMM (Forum, BASF Corp., Research Triangle Park, NC); mancozeb (Dithane DF Rainshield, Dow AgroSciences, Indianapolis, IN); and azoxystrobin (Quadris SC, Syngenta Crop Protection, Inc., Greensboro, NC) were evaluated for blue mold management in shade-grown tobacco field plots. Acrobat MZ was applied to appropriate plots at rates of 224 g/ha at the first and last spray dates and at 448 g/ha for the other application dates. Forum was applied at rates of 440 ml/ha at the first spray date and at 585 ml/ha for all other sprays. Dithane DF was applied at a rate of 1.1 kg/ha when used as a mixing partner for appropriate Forum applications. Quadris was applied at a rate of 585 ml/ha at all applications. Ripe leaves were picked, 3 leaves from each plant, and blue mold lesions counted on picked leaves at weekly intervals, typically starting in mid-July, for all plants in the 2 spray rows. The number of lesions per plot on picked leaves and number of healthy leaves (no visible lesions) per plot were recorded for each picking. Border row plants were not picked and moderate to severe disease levels occurred in borders of all plots. Data were tested for normality and analyzed by analysis of variance and means were separated by Fisher's LSD Multiple Comparison test.

Sporangial germination. The effects of DMM on sporangial germination were determined in the first 3 weeks of September in 2002 and 2011. A volume of 0.1 ml of sterile distilled water or 1.2, 12.0, 120.0, or 1,200.0 mg/L DMM in sterile distilled water was placed on the surface of a 9-cm-diam petri dish containing water agar and spread with a sterile glass rod. Fresh *P. tabacina* sporangia were washed from newly sporulating lesions collected before 9:00 AM. One drop of a sporangial suspension containing approximately 500 sporangia was placed in the center of each plate. After 24 hours, plates were observed under a microscope and the numbers of germinated sporangia of the first 100 observed were recorded. Sporangia were considered germinated if the germ tube was longer than the sporangia. There were 3 replicates of each fungicide concentration and the experiment was performed 3 times in each year. Data were analyzed separately by logistic regression and analysis of deviance, using the normalized sporangial germination as the dependent variable and fungicide concentration and year as independent variables. The deviance from the full regression model (including intercept, concentration, and year) was compared with regression models lacking concentration, year effect, or both (the null model) to calculate 1- and 2-degree-of-freedom chi-square statistics to test significance of these effects (1).

Leaf disk bioassay. The effects of DMM on sporangial germination, leaf disk infection, disease development, and sporulation were determined in the first 3 weeks of September in 2011 using a leaf disk assay. Ten 1.4-cm-diam leaf disks from greenhouse-grown 8212 shade tobacco plants were submerged to completely wet, then floated abaxial side up in 20 ml of 0, 1.2, or 12 mg/L DMM in a 9-cm-diam petri dish in 2011. After the surface dried, a single drop containing approximately 1,500 sporangia of *P. tabacina* was placed on each disk. Disks were incubated in a growth chamber in the light for 12 hours at 21°C and in the dark for 12 hours at 18°C. After 1 week, the diameter of the diseased area of the leaf disk was determined and disks were observed for sporulation. The experiment was performed twice and data were compared with published results from North Carolina (15) using Fisher's Exact Test.

RESULTS

Field experiments to evaluate DMM efficacy. Field fungicide evaluations from both 2002 and 2004 demonstrated that DMM alone significantly reduced blue mold compared with the untreated controls and was as efficacious in reducing the number of lesions as DMM plus additional mancozeb (formulated as Acrobat MZ or Forum plus Dithane DF; $P = 0.05$; Table 1). In 2002 and 2004, DMM alone reduced the number of blue mold lesions compared with untreated controls by 80 to 93%, respectively. In 2009 and 2011, disease severity in DMM-alone plots was not significantly different from the untreated control plots, and although not different, mancozeb alone was numerically superior to DMM alone. In 2009 and 2011, DMM alone resulted in 65% and less than 25% reduction in lesions, respectively. In comparison, mancozeb resulted in 95% control in 2009 and 49% control of blue mold in 2011.

Sporangial germination. The effects of DMM on sporangial germination were determined in the first week of September in 2002 and 2011. Normalized sporangial germination in the 2 years was similar at 1.2 and 12.0 mg/L DMM, but a difference observed

Table 1. Efficacy of fungicides against the number of blue mold lesions caused by *Peronospora tabacina* on shade-grown wrapper tobacco in Windsor, CT.

Treatment[a]	2002[b]	2004	2009	2011
Untreated control (UTC)	883[c] c	878 b	40 b	1328 b
Dimethomorph (DMM)	174 b	60 a	14 ab	999 ab
DMM plus mancozeb	165 b	70 a		
Mancozeb			2 a	672 a
Azoxystrobin	63 a		0 a	677 a

[a] Dimethomorph formulated as Forum, mancozeb formulated as Dithane DF Rainshield, and azoxystrobin formulated as Quadris. Dimethomorph plus mancozeb was formulated as Acrobat MZ or Forum plus Dithane DF.
[b] Number of blue mold lesions per plot through mid- to late August in 2002, 2004, 2009, and 2011.
[c] Numbers within columns followed by a different letter are significantly different at P = 0.05 (analyzed by analysis of variance; means were separated by Fisher's LSD Multiple Comparison test).

between 2002 and 2011 was the low level of germination observed at 120 and 1,200 mg/L in 2011 (Table 2). There were no significant differences between years (P = 0.05).

Leaf disk bioassay. The effects of DMM on disease development and sporulation were determined using a leaf disk assay conducted in 2011. Seven days after inoculation, all tobacco leaf disks floating on water alone (0 mg/L DMM) were diseased (Figure 1A). The visible lesion on each disk averaged 74.8% of the disk and ranged from 40 to 90% of the disk area. Sporangiophores and sporangia occurred on 100% of the leaf disks. Up to 75% of the tobacco leaf disks floating on 1.2 mg/L DMM had visible lesions in the leaf tissue (Figure 1B). Lesion area averaged 14.1% of the disk area and ranged from 0 to 35%. Sporangiophores and sporangia were observed on 37% of the leaf disks with visible lesions. No disease or sporulation occurred on disks floating on 12 mg/L DMM (Figure 1C). The experiment was repeated with similar results and combined data for number of diseased leaf disks or sporulation on leaf disks at 1.2 mg/L DMM in Connecticut in 2011 were different from 1998 North Carolina results (1.0 mg/L DMM) using Fischer's Exact Test ($P = 0.16 \times 10^{-20}$ and $P = 0.5 \times 10^{-6}$, respectively).

Table 2. Percent germination of *Peronospora tabacina* sporangia on water agar media amended with different concentrations of dimethomorph in 2002 and 2011.

Dimethomorph (mg/L)	2002	2011
1.2	86.0[a]	86.2
12.0	9.1	12.3
120.0	0.0	3.0
1,200.0	0.0	0.5

[a] Experiments were conducted using fresh sporangia from shade-grown wrapper tobacco collected from Windsor, CT. There were 3 replicates of each fungicide concentration and the experiment was performed 3 times in each year. Data were normalized as a percentage of germination on nonamended water agar. Data were analyzed separately by logistic regression and analysis of deviance, using the normalized sporangial germination as the dependent variable and fungicide concentration and year as independent variables. The deviance from the full regression model (including intercept, concentration, and year) was compared with regression models lacking concentration, year effect, or both (the null model) to calculate 1- and 2-degrees-of-freedom chi-square statistics to test significance of these effects (1).

DISCUSSION

This research was initiated as a result of commercial grower concerns about potential reduced efficacy of DMM fungicide against *P. tabacina*. Blue mold has been present in Connecticut each year since 1996 (8) and grower experience with the disease has benefitted from a wide range of weather and other conditions. The unexpectedly high levels of blue mold experienced in commercial tobacco crops, despite repeated fungicide applications, during the 2010 and 2011 seasons may have been due to multiple causes: different isolates varying in virulence may have been present; spray timing and coverage issues may affect disease; weather conditions each year and immediately after sprays may vary; and additional factors such as crop maturity and nutrient status may also affect disease severity. Finally, DMM efficacy may have been reduced because of the presence of a DMM-insensitive isolate of *P. tabacina*.

To investigate whether a DMM-insensitive isolate of the obligate pathogen was indeed present, we first reviewed data concerning the efficacy of DMM fungicide in replicated field trials conducted in certain years from 2002 to 2011 when DMM was used alone or in comparison with, or in combination with, mancozeb. We also conducted in vitro assays to evaluate sporangial germination, infection, and sporulation. Our review of data from field plots mirrored grower accounts and demonstrated apparent reduced efficacy of DMM in relation to untreated controls and to mancozeb in replicated field plots between 2002 and 2004 as compared with 2009 to 2011. As a protectant fungicide, mancozeb had previously been consistently less efficacious than DMM (9,14).

Carboxylic acid amide fungicides such as DMM have been shown to affect multiple stages of oomycete pathogenesis at different concentrations. Dimethomorph has translaminar and local systemic activity against germination and mycelial growth of oomycete plant pathogens, and effectively inhibited infection and subsequent sporulation (3). Keinath (6) concluded that the half-maximal effective concentration (EC_{50}) for *Phytophthora capsici* zoospore germination was much lower than for mycelial growth or zoospore production, which was 0.07, 0.19, and 0.63 mg/L, respectively. In

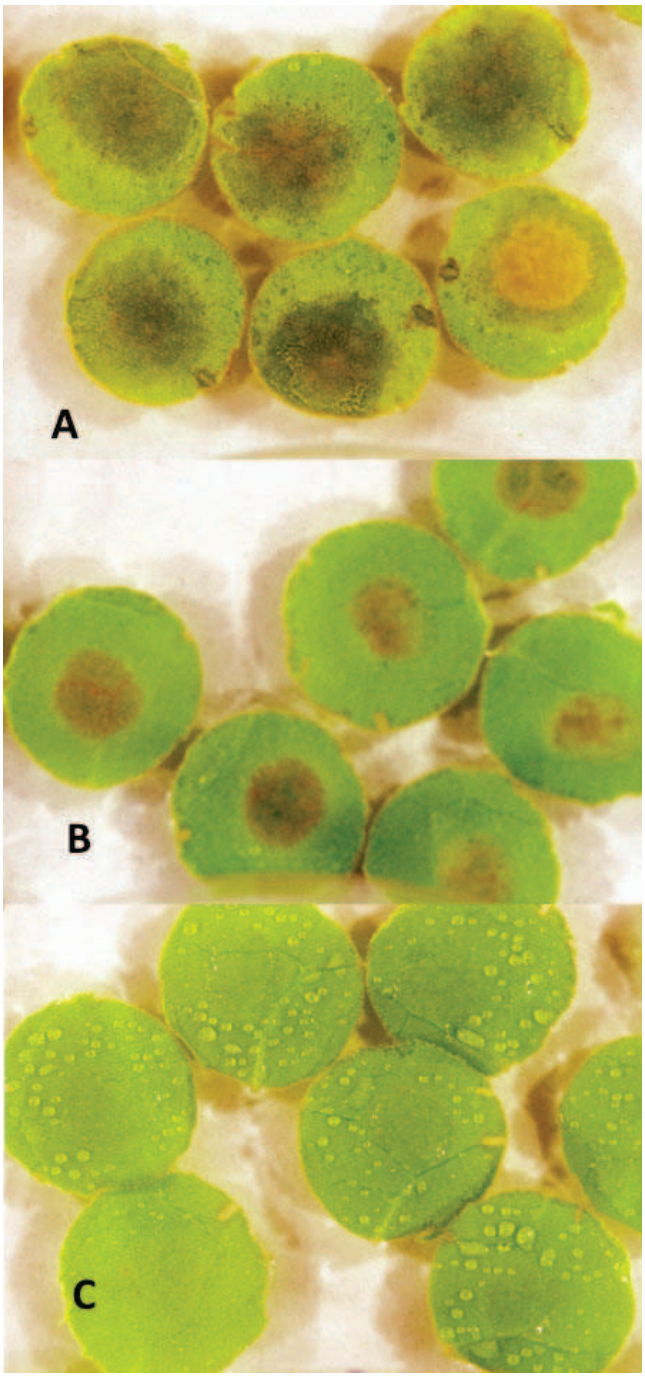

Figure 1. Disease development 7 days after inoculation with *Peronospora tabacina* on detached tobacco leaf disks floating on A) 0 mg/L DMM, B) 1.2 mg/L DMM, or C) 12.0 mg/L DMM.

contrast, Mathuron and Porchas (11) determined that for 3 different *Phytophthora* species the EC_{50} for germination (3.3–7.2 mg/L) was higher than for mycelial growth (0.1–0.4 mg/L) or sporangia production (less than 1.0 mg/L for all spp.).

Our data demonstrated that the EC_{50} for sporangial germination of *Peronospora tabacina* was between 1.2 and 12.0 mg/L DMM and was not different between 2002 and 2011. Inhibition of *P. tabacina* sporangial germination by DMM likely requires higher concentrations than for other impacts on pathogenesis in tobacco, as we observed significant germination and germ tube growth at 12 mg/L, whereas mycelial growth through leaf tissue resulting in visible disease and sporulation of the pathogen are completely suppressed in the leaf disk bioassay at that concentration. The low levels of germination that we observed at DMM concentrations greater than 12 mg/L in 2011 may have been due to sporangia that initiated germination before exposure to DMM. The effect of DMM on germination of encysted zoospores (cystospores) of other oomycetes was only active within the first hour of the germination process; germination may not be stopped or inhibited if it had already started before the introduction of the fungicide (4). Our sporangia were field-collected; it is possible that some germination may have been initiated before collection.

Before this report, tobacco leaf disk assays were conducted in North Carolina with isolates of *P. tabacina* from different locations, including Connecticut, until as late as 2006. Researchers consistently concluded that infection and growth of the pathogen through leaf tissue and sporulation were inhibited by less than 1 mg/L DMM in a leaf disk assay (15,16). This baseline sensitivity was developed to allow monitoring for potential shifts in sensitivity in the future. Our leaf disk assay results from 2011 demonstrated reduced DMM sensitivity of isolates from Connecticut and Massachusetts, as we observed both leaf disk infection and lesion development in the tissue and the development of sporangiophores and sporangia on the disks. *Peronospora tabacina* isolates from Connecticut and Massachusetts can germinate, infect, and produce sporangia on leaf disks exposed to DMM at concentrations above the baseline sensitivity previously determined in North Carolina. Although lesion size and the amount of sporangia present were reduced compared with disks in the absence of fungicide, this capacity to overcome the previously defined baseline clearly demonstrates an important shift in sensitivity to DMM, and was consistent with the reduced efficacy observed in field experiments. The mechanism of reduced sensitivity of *P. tabacina* to DMM is unknown; however, it appears to affect a later stage of pathogenesis than sporangial germination.

Naturally occurring DMM insensitivity has not been demonstrated for some oomycetes, such as *Phytophthora infestans*, but has been reported to exist naturally in the downy mildews *Plasmopora viticola* (2,13) and *Pseudoperonospora cubensis* (12). Researchers have developed CAA fungicide insensitivity in mutants and have determined that the effect of insensitivity to CAA fungicides is relatively low in comparison with phenylamide fungicides such as metalaxyl. Chabane et al. (2) developed ultraviolet (UV)-induced mutants of *Phytophthora parasitica* with insensitivity to DMM or metalaxyl. Resistance factors (the ratio of EC_{50} for mutant vs. wild type) exceeded 100 for metalaxyl insensitivity but were less than 25 for DMM insensitivity. Chemical mutagenesis resulted in moderately DMM-insensitive mutants of *Phytophthora capsici*

(maximum resistance factor less than 25; 20). Laboratory selection for DMM-resistant mutants by exposure to DMM in vitro resulted in stable isolates insensitive to reduced exposures to DMM. Isolates remained pathogenic and disease control of moderately resistant isolates was reduced, especially at lower fungicide concentrations (18). Ethidium bromide/UV mutagenesis was used to generate DMM-insensitive mutants of *P. infestans*, which were then repeatedly cultured on DMM-amended media (17). Even after the 10th subculture, resistance factors were low in comparison with phenylamide fungicides and all isolates exhibited reduced fitness, indicating a relatively low probability of field resistance to DMM (7). Rubin et al. (13) also failed to produce stable and fit isolates with insensitivity to CAA fungicides in *P. infestans*.

Gisi et al. (5) investigated the inheritance of insensitivity to CAA fungicides in *Plasmopora viticola* and concluded that fungicide insensitivity was recessive and likely conditioned by 2 nuclear genes, exhibiting cross-resistance with all CAA fungicides. The recessive nature of inheritance would slow the development of field resistance to CAA fungicides, keeping sensitive individuals in the population.

It is apparent from these studies that insensitivity to CAA fungicides is possible in a wide range of oomycetes, and currently exists in some field populations. However, the resistance factors observed were lower than for other fungicides such as phenylamides, consistent with our results, and the apparent multigenic nature of inheritance (5). Furthermore, reduced fitness would slow and moderate the shift to insensitivity, resulting in a moderate risk classification rather than a more dramatic loss of efficacy, especially where FRAC (Fungicide Resistance Action Committee) recommendations are followed. We report here that reduced sensitivity to DMM has been observed for the first time in *P. tabacina* causing blue mold of tobacco. This reduced sensitivity did not result in a complete loss of efficacy but requires higher levels of fungicide to achieve disease control. Further study and monitoring to follow the development and persistence of field resistance and changes in baseline sensitivity over time are warranted.

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