Sublethal effects of the methyl benzimidazole carbamate “fungicide” thiophanate-methyl applied to prevent *Diplodia* shoot blight of pines

by Denise R. Smith¹ and Glen R. Stanosz¹

ABSTRACT

Shoot blight, canker, and collar rot diseases caused by the fungus *Diplodia sapinea* damage red pine (*Pinus resinosa*) seedlings in forest tree nurseries in the northcentral USA. Application of fungicides, including the methyl benzimidazole carbamate chemical thiophanate-methyl (TM), have been used to reduce disease incidence. But because *Diplodia sapinea* is known to persist on or in asymptomatic seedlings in sprayed beds and to subsequently proliferate and kill outplanted seedlings, we tested the effects of various concentrations of TM on *D. sapinea* *in vitro*. Mycelial growth on potato dextrose agar (PDA) was greatly inhibited at concentrations of $\geq 1 \mu\text{g TM ml}^{-1}$. However, frequencies of conidial germination on water agar (WA) were not reduced even at $1000 \mu\text{g TM ml}^{-1}$ (compared to those on non-amended WA). Although exposure to concentrations $\geq 1 \mu\text{g TM ml}^{-1}$ in WA resulted in deformed germ tubes and hyphae, colonies resulting from germination and growth for four hours on WA amended with up to $1000 \mu\text{g TM ml}^{-1}$ resumed normal mycelial growth when transferred to non-amended PDA. The observed fungistatic, rather than fungicidal, effects of TM might help explain persistence of *D. sapinea* on or in asymptomatic nursery seedlings treated with TM or similar chemicals.

Keywords: *Diplodia sapinea*, thiophanate-methyl, benzimidazole carbamate, fungistatic, fungicide resistance

RÉSUMÉ

La flétrissure du feuillage, le chancre ainsi que la pourriture du collet que provoque le champignon *Diplodia sapinea* endommagent les semis de pin rouge (*Pinus resinosa*) dans les pépinières du Centre-Nord des É.-U. On a appliqué différents fongicides comme le méthyle benzimidazole carbamate appelé thiophanate-méthyle (TM) pour réduire l'incidence de la maladie. Mais comme le *Diplodia sapinea* peut demeurer la surface ou à l'intérieur des semis asymptomatiques dans les platebandes vaporisées avec du TM pour ensuite proliférer et tuer les semis une fois mis en terre, nous avons testé *in vitro* les effets de diverses concentrations de TM sur *D. sapinea*. Il est apparu d'abord qu'une concentration de TM $\geq 1 \mu\text{g ml}^{-1}$ limite considérablement la croissance du mycélium sur de la gélose dextrosée à la pomme de terre (PDA). Toutefois, les fréquences de germination des conidies sur de l'eau gelosée (WA) n'ont pas chuté, même à $1000 \mu\text{g TM ml}^{-1}$ (comparativement à celles sur de WA non traitée). Bien que l'exposition à des concentrations $\geq 1 \mu\text{g TM ml}^{-1}$ dans de WA ait provoqué une déformation du tube germinatif et des hyphes, les colonies formées après germination et quatre heures de croissance sur WA traité avec des doses aussi fortes que $1000 \mu\text{g TM ml}^{-1}$ ont repris leur croissance mycélienne lorsque transférées sur de PDA non traitée. Les effets fongistatiques et non fongicides constatés avec le TM pourraient aider à comprendre pourquoi *D. sapinea* arrive à se maintenir à la surface ou à l'intérieur des semis asymptomatiques traités avec du TM ou d'autres produits chimiques en pépinière.

Mots-clés : *Diplodia sapinea*, thiophanate- methyl, benzimidazole carbamate, fongistatique, résistance aux fongicides

Introduction

The fungal pathogen *Diplodia sapinea* (Fr.) Fuckel (syn. *D. pinea*, *Sphaeropsis sapinea*) has caused severe damage to red pine (*Pinus resinosa* Aiton) seedlings in forest tree nurseries in the northcentral United States (Palmer and Nicholls 1985). Sources of primary inoculum have included diseased trees in adjacent windbreaks from which conidia are disseminated. Diseased seedlings bear conidia on killed needles and stems

to initiate secondary cycles of disease within nursery beds. Shoot blight, cankers, and collar rot can develop quickly following infection, killing seedlings or otherwise rendering them nonmerchantable.

Managers have long relied on application of chemical sprays at regular intervals to reduce disease incidence in nurseries. Stanosz and Smith (1996) demonstrated that thiophanate-methyl (TM) was among the most effective of mate-

¹Department of Forest and Wildlife Ecology, University of Wisconsin-Madison, Madison, WI 53706, USA Correspondence: gstanosz@wisc.edu

rials for preventing Diplodia shoot blight in beds of 1-0 and 2-0 red pine seedlings. TM is a methyl benzimidazole carbamate (MBC), one of a group of chemicals that interferes with fungal beta-tubulin assembly in mitosis (Fungicide Resistance Action Committee; (FRAC) 2017). However, development of Diplodia collar rot on recently outplanted seedlings (Stanosz and Cummings Carlson 1996) led to the discovery that fungicide-treated red pine seedlings may frequently acquire virulent strains of *D. sapinea*, but remain asymptomatic in the nursery (Stanosz *et al.* 1997; Stanosz *et al.* 2005). It was additionally demonstrated that host water stress can result in release of this latently persisting pathogen from quiescence with rapid development of disease (Stanosz *et al.* 2001).

Knowledge that *D. sapinea* can persist on or in fungicide-treated red pine nursery seedlings prompted an investigation of the *in vitro* effects of TM on this fungus. Mycelial growth, germination of conidia, hyphal morphology, and survival were assessed on media amended with various amounts of this chemical.

Materials and Methods

Mycelial growth from inoculum plugs

Two *D. sapinea* isolates (128, from Grant Co., WI; 411, from Clearwater Co., MN) that had been obtained from red pines by M. Palmer, USDA Forest Service, were used in all experiments. For experiment 1, Difco potato dextrose agar (PDA; Becton, Dickson and Company, Franklin Lake, NJ) was either not amended or amended after autoclaving to achieve concentrations of 0.01, 0.1, 1, 10, 100, and 1000 µg TM ml⁻¹. The source of TM used in all experiments was Cleary's 3336 WP (Cleary Chemical Corporation, Dayton, New Jersey; 50% active ingredient). Plugs (4-mm-diameter) used as inoculum were cut from margins of actively growing colonies in 90-mm-diameter Petri plates containing 20 ml PDA. Inoculum plugs were placed mycelium-side-down in centers of 90-mm-diameter plates containing 20 ml of PDA amended with TM. Three plates were used for each isolate-TM concentration combination, in each of two separate trials. Plates were sealed and incubated in the dark at 24 °C until mycelium neared the edge of the medium or for seven days. Colony diameters were measured along two perpendicular axes and daily diameter growth calculated by dividing the average of the two diameter measurements (minus the inoculum plug diameter) by the number of days incubated.

Brief exposure to TM during and after conidial germination

Conidial germination and hyphal morphology in a brief period immediately following germination in the presence of TM were observed in experiment 2a. Conidia of each isolate were extracted in sterile water from pycnidia produced on autoclaved red pine needles placed on 2% water agar (WA, Fisher Scientific, Pittsburgh, PA) in plates that had been inoculated and incubated for 14-21 days at 24 °C under white and black lights. In this experiment and experiments described below, conidial suspensions were adjusted to $\geq 10^4$ spores ml⁻¹. Two-percent WA was either not amended or amended to achieve concentrations of 0.01, 0.1, 1, and 5 µg TM ml⁻¹. Conidial suspensions were spread on the surface of the medium in each of three 60-mm-diameter Petri plates per isolate-TM concentration combination, each containing 10

ml of medium. Plates were sealed, incubated in the dark at 24 °C for 4 hr, and then the medium surface was sprayed with 0.1 mg/L thimerosal to halt hyphal growth. Germination (+/-) of 100 conidia per plate was assessed by viewing the conidia directly on the medium surface using a light microscope at 100 x. Images for characterization of the morphology of hyphae produced by ten arbitrarily selected germinated conidia per plate were obtained using a digital camera mounted on a microscope. For each of these ten conidia, the total hyphal length (all branches summed) and farthest hyphal extent (distance from center of a conidium to farthest tip of a hypha), were measured using Assess v2.0 software (APS Press, St. Paul, MN, USA), and the number of hyphal apices per germinated conidium was counted.

Experiment 2b investigated the effects of exposure to a range of greater TM concentrations in the brief period during and immediately following germination. Suspensions of conidia of each isolate were produced as described above. Two-percent WA was either not amended or amended to achieve concentrations of 10, 100, and 1000 µg TM ml⁻¹. Conidial suspensions were spread on the surface of the medium in one 90-mm-diameter Petri plate containing 20 ml of the medium for each isolate-TM concentration combination in each of two separate trials. Plates were sealed and incubated in the dark at 24 °C for 4 hr, and then germination (+/-) of 100 conidia per plate was assessed by viewing conidia directly on the medium surface using a light microscope at 100 x.

At this time, ten arbitrarily selected germinated conidia of each isolate-TM concentration combination were picked off the medium and transferred to the centers of individual 90-mm-diameter plates containing 20 ml PDA. Plates were sealed and incubated in the dark at 24 °C until the mycelium neared the edge of the medium or for six days. Diameters of resulting colonies were measured and daily growth calculated as described for experiment 1.

Prolonged exposure to a high concentration of TM after conidial germination

The effects on hyphal morphology from prolonged exposure to a high concentration of TM were studied in experiment 3a. Suspensions of conidia of the two isolates were produced as described above. Two-percent WA was either not amended or amended to achieve a concentration of 1000 µg TM ml⁻¹. Conidial suspensions of each isolate were spread on the surface of the medium in each of three 60-mm-diameter Petri plates per isolate for the non-amended WA and six plates per isolate for the TM-amended WA, each containing 10 ml of medium. The plates were sealed and incubated in the dark at 24 °C for 4 hrs for the non-amended WA and for three of the 1000 µg TM ml⁻¹ WA. Incubation of the other three plates with 1000 µg TM ml⁻¹ in WA continued in the same conditions for seven days. At the end of the respective incubation periods, each culture was sprayed using an atomizer with 0.1 mg l⁻¹ thimerosal to stop hyphal growth. Images for characterization of the morphology of hyphae produced by ten arbitrarily selected germinated conidia per plate were obtained and data collected from these images as described above.

In experiment 3b, the effects of a high concentration of TM on both conidial germination and the vitality of incipient colonies after prolonged exposure were investigated. Suspensions of conidia of each of the two isolates used previously

were produced as described above. Two-percent WA was either not amended or amended to achieve a concentration of 1000 µg TM ml⁻¹. Conidial suspensions of each isolate were spread separately on the surface of the medium in two 90-mm-diameter Petri plates of the non-amended WA and two plates of the TM-amended WA, each containing 20 ml of medium. Plates were then sealed and incubated in the dark at 24 °C. Germination (+/-) of 100 conidia per non-amended WA plate was assessed by viewing conidia directly on the medium surface after 4 hr using a light microscope at 100 x. At this time ten arbitrarily selected germinated conidia were picked off the medium on each of the two non-amended WA plates per isolate and transferred to the centers of individual 90-mm-diameter plates containing 20 ml PDA. Plates to which these transfers had been made were then sealed and incubated in the dark at 24 °C. When mycelium neared the edge of the PDA medium, colony diameters were measured and daily diameter growth calculated as described above. Germination (+/-) of 100 conidia per TM-amended WA plate was assessed by viewing conidia directly on the medium surface after seven days. At this time, ten arbitrarily selected germinated conidia of each isolate were picked off the medium on each of the two TM-amended WA plates per isolate and transferred to centers of individual 90-mm-diameter plates containing 20 ml PDA to assess viability. Plates to which these transfers had been made were then sealed and incubated seven more days in the dark at 24 °C, and then examined.

Statistical analyses

Preliminary data analyses revealed no consistent significant effects attributable to the two isolates, different trials, or individual plates (for example, plates from which plugs or conidia

were transferred) on responses in any experiment. Therefore, respective data were pooled for analyses. Diameter growth from plugs data (experiment 1) were compared using the Kruskal-Wallis test for equality of medians. Percentages of conidial germination (experiments 2a, 2b, and 3b) were converted to proportions and the arcsine of the square root transformation was applied before analysis using one-way ANOVA. Hyphal morphology data (experiments 2a, 3a) also were analyzed using one-way ANOVA. Where appropriate, Tukey simultaneous tests were performed. All analyses were performed using MINITAB statistical software release 14.

Results

Mycelial growth from inoculum plugs

The influence of TM on mean daily diameter growth of *D. sapinea* colonies that developed from transferred mycelial plugs in experiment 1 depended on concentration (Table 1). Colonies produced from plugs transferred to PDA amended with 0.01, and 0.10 µg TM ml⁻¹ grew at rates similar to that of colonies on non-amended PDA. In contrast, daily mycelial growth was negligible on PDA with 1, 10, 100, and 1000 µg TM ml⁻¹. Results of the Kruskal-Wallis test for equality of media indicated a significant effect of concentration at $p < 0.01$.

Brief exposure to TM during and after conidial germination

Relatively low concentrations of TM in WA (up to 5 µg ml⁻¹) in experiment 2a did not affect frequency of germination of *D. sapinea* conidia, but did affect morphology of hyphae produced during the 4-hr period of exposure (Table 2). Germination ranged from 83.5% to 81.2% at TM concentrations of 0 to 5 µg TM ml⁻¹, respectively. However, mean total hyphal length was approximately one-third less and mean farthest hyphal extent was nearly three-quarters less on WA amended with 5 µg TM ml⁻¹, compared to results on non-amended WA (Fig.1). These differences can be attributed to the much more frequent branching of hyphae on TM-amended WA, with an average of 5.7 hyphal apices on WA amended with 5 µg TM ml⁻¹, compared to 1.1 on non-amended WA.

Table 1. Mean^a daily diameter growth (mm) of *Diplodia sapinea* from mycelial plugs at various concentrations of thiophanate-methyl in potato dextrose agar (PDA)

	Thiophanate-methyl concentration µg ml ⁻¹ in PDA							<i>p</i> value ^a
	0	0.01	0.10	1	10	100	1000	
Daily diameter growth	23.4	22.9	24.2	0.7	0.6	0.5	0.3	< 0.01

^aValues are means for three plates of each of two isolates in each of two trials

^bValue of *p* from Kruskal-Wallis test for equality of medians

Table 2. Germination of *Diplodia sapinea* conidia and morphology of hyphae produced during 4-hr incubation on water agar amended with low concentrations of thiophanate-methyl (TM) (experiment 2a)

TM µg ml ⁻¹	Germination % mean ^a (range)	Total hyphal length, mm mean ^b (range)	Farthest hyphal extent, mm mean ^b (range)	No. hyphal apices mean ^b (range)
0	83.5 (82-87)	0.75b (0.45-1.18)	0.47b (0.21-0.78)	1.1c (1-2)
0.01	81.3 (78-84)	0.73b (0.35-1.06)	0.47b (0.19-0.69)	1.5c (1-4)
0.1	82.5 (81-84)	0.88a (0.40-1.36)	0.53a (0.21-0.87)	2.4b (1-5)
1	83.0 (82-85)	0.38c (0.21-0.65)	0.14c (0.07-0.25)	4.5a (1-8)
5	81.2 (76-85)	0.47c (0.15-0.91)	0.12c (0.08-0.20)	5.7a (1-11)
<i>p</i> value ^c	0.29	< 0.01	< 0.01	< 0.01

^aGermination percentages are means for 100 conidia for each of two isolates on each of three plates. Data were analyzed after applying the arcsine of the square root transformation to proportions

^bValues of total hyphal length, farthest hyphal extent, and no. of hyphal apices are means for ten germinated conidia of each of two isolates on each of three plates

^cValues of *p* from one-way ANOVA for each response. Means within a column followed by different lower case letters were significantly different at $p \leq 0.05$ using Tukey simultaneous tests

Fig. 1. Hyphal growth from conidia of *Diplodia sapinea* isolate 128 after 4 hours incubation on **(A)** non-amended water agar or **(B)** water agar amended with 5 µg thiophanate-methyl ml⁻¹

Table 3. Morphology of *Diplodia sapinea* hyphae produced from germinated conidia during 4 hr or 7 days incubation on water agar amended with high concentration of thiophanate methyl (TM) (experiment 3a)

TM µg/ml	Incubation period	Total hyphal length, mm mean ^a (range)	Farthest hyphal extent, mm mean ^a (range)	No. hyphal apices mean ^a (range)
0	4 hr	0.53 (0.17-0.90) a	0.38 (0.10-0.60) a	1.13 (1-2) a
1000	4 hr	0.09 (0.04-0.37) bA	0.04 (0.02-0.11) bA	2.82 (1-5) bA
1000	7 days	0.93 (0.27-1.66) B	0.19 (0.08-0.21) B	8.67 (4-13) B

^aValues are means for ten germinated conidia of each of two isolates on each of three plates. One-way ANOVA indicated that means within a column followed by different lower case letters (0 or 1000 µg TM ml⁻¹ after 4 hr) or different upper case letters (1000 µg TM ml⁻¹ after 4 hr or 7 days) were different at $p < 0.01$

Exposure to even higher concentrations of TM for a brief period had little effect on either percentage of conidia that germinated or subsequent colony diameter growth in experiment 2b. Mean frequencies of conidial germination in this experiment ranged from 72.8% on non-amended WA to 66.0% on WA with 1000 µg TM ml⁻¹ ($p = 0.88$). Diameter growth of *D. sapinea* colonies developing from conidia germinated on non-amended or TM-amended WA and then after 4 hr transferred to PDA in experiment 2b differed only slightly. Mean daily diameter growth was greatest (15.9 mm) for conidia transferred from non-amended plates and 15.4, 14.7, and 14.1 mm for conidia transferred from plates with WA amended with 10, 100, and 1000 µg TM ml⁻¹, respectively. The slightly lower means of daily diameter growth of colonies for the two greatest concentrations of TM were strongly influenced by one relatively slow-growing colony for each. One-way analysis of variance indicated only weak support for significance of this effect ($p = 0.06$, $n=11$ to 19).

Extended exposure to a high concentration of TM after conidial germination

Exposure to a relatively high concentration of TM in WA (1000 µg TM ml⁻¹) in experiment 3a did not quickly halt hyphal development, but did markedly affect hyphal morphology (Table 3). After four hours incubation on WA amended with 1000 µg TM ml⁻¹ mean total hyphal length and mean farthest hyphal extent were much less than on non-amended WA. At the same time, numbers of hyphal apices were more than double on this TM-amended medium compared to the non-amended medium. Greater means of total hyphal length, farthest hyphal extent, and number of hyphal apices after seven days on this high concentration TM-amended medium (compared to these means on the same medium after just four hours), indicates that colonies contin-

ued to grow for at least some period of time on WA with 1000 µg TM ml⁻¹.

In experiment 3b, 1000 µg TM ml⁻¹ in WA did not affect frequency of conidial germination, but was lethal after longer incubation. Percentages of conidia which had germinated were similar on non-amended WA (mean=87, range=85-89) and 1000 µg TM ml⁻¹ WA (mean=88, range=81-92). Colonies developed from all conidia germinated on non-amended WA and then transferred to PDA. Mean daily radial growth of these colonies was 16.2 mm (range=15.9-16.8). No subsequent colony development occurred when conidia germinated and maintained for seven days on WA amended with 1000 µg TM ml⁻¹ were transferred to PDA.

Discussion

"A fungicide is a specific type of pesticide that controls fungal disease by specifically inhibiting or killing the fungus causing the disease" (McGrath 2004). In annual crop production, fungicide efficacy may be reflected by a reduction in disease incidence and severity below a threshold of unacceptable aesthetic damage or yield reduction at time of harvest. Success in reforestation programs, however, is dependent on seedling survival and growth after outplanting. Therefore, the possibility that a chemical is merely fungistatic and that the targeted pathogen survives chemical treatments to be disseminated on seedlings and later proliferates to cause disease must be considered in nursery production of tree seedlings.

The extreme inhibition of *D. sapinea* mycelial growth *in vitro* by as little as 1 µg TM ml⁻¹ is consistent with sensitivity of related fungi (i.e., family Botryosphaeriaceae) to this chemical. For example, Amponsah *et al.* (2012) reported mean effective TM concentrations that reduced mycelial growth by 50% (EC₅₀) were 1.079 µg ml⁻¹ for three isolates each of grapevine pathogens *Neofusicoccum australe* (Slippers, Crous

& M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, 1.237 $\mu\text{g ml}^{-1}$ for *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, and 0.485 $\mu\text{g ml}^{-1}$ for *Diplodia mutila* (Fr.) Mont. Similarly, Cavalcante *et al.* (2014) classified isolates of *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. from papaya with a range of EC_{50} values from 0.88 to 5.78 $\mu\text{g ml}^{-1}$ as sensitive to TM. Study of another MBC fungicide, carbendazim, revealed a mean EC_{50} of just 0.02 $\mu\text{g ml}^{-1}$ for multiple isolates of *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *Diplodia seriata* De Not., and *L. theobromae* (Pitt *et al.* 2012). Suppression of mycelial growth of botryosphaeriaceous fungi explains the apparent effectiveness of these chemicals in preventing Diplodia shoot blight of red pine seedlings in the nursery, and similar diseases of other woody plants.

Reported TM effects on *in vitro* germination of conidia of botryosphaeriaceous fungi vary widely, and differences may in part reflect methods. Compared to non-amended medium, Li *et al.* (1995) reported 78.2% suppression of conidial germination of a *L. theobromae* isolate on a medium including 30 $\mu\text{g TM ml}^{-1}$. However, in that study germination was assessed after just 12 hr. Mean EC_{50} values, based on conidial germination after 24 hours incubation on TM-amended medium were 5.09, 2.41, and 0.29 $\mu\text{g TM ml}^{-1}$, respectively, for *N. australe*, *N. luteum*, and *D. mutila*. Our use of an extended incubation period (i.e., seven days) allowed discovery of relative insensitivity of *D. sapinea* conidial germination, which was similar at concentrations of TM from $\leq 5 \mu\text{g ml}^{-1}$ to even 1000 $\mu\text{g ml}^{-1}$.

Morphological alterations similar to those exhibited by *D. sapinea* hyphae exposed to TM have been associated with exposure to MBC chemicals for a small but diverse group of ascomycete plant pathogens. In the study of Li *et al.* (1995), germ tubes of botryosphaeriaceous species on TM-amended medium “became twisted and formed more branches than usual.” Other reports of deformed germ tubes mention *Sclerotinia fructicola* (G. Winter) Rehm (Penrose and Koffman 1977), *Botrytis cinerea* Pers. (Nakata *et al.* 1987), *Cercospora beticola* Sacc. (Nakata *et al.* 1987; Solel 1970), and *Verticillium theobromae* (Turconi) E.W. Mason & S. Hughes (Igeleke and Ayanru 2007). In addition, Richmond and Pring (1971) described and illustrated similar morphological effects of exposure to the MBC chemical benomyl. At concentrations of 0.1 to 0.3 $\mu\text{g ml}^{-1}$, germ tubes from *Botrytis fabae* Sardiña conidia were “swollen and distorted” and “branched more frequently than those of normal conidia.”

Richmond and Pring (1971) also noted that effects of an MBC fungicide may be sublethal and reversible, as we have observed. In that study, normal *B. fabae* hyphal growth occurred after transfer of conidia germinated in the presence of benomyl to fungicide-free medium. Similar tolerance of *D. sapinea* to brief (i.e., 4 hr) exposure to TM was demonstrated by resumption of normal growth when *D. sapinea* conidia were transferred from plates with up to 1000 $\mu\text{g TM ml}^{-1}$ to non-amended medium. Although exposure to a high concentration of TM was eventually lethal to *D. sapinea*, substantial increases in total hyphal length, farthest hyphal extent, and numbers of hyphal apices during the 7-day incubation (compared to 4 hr) on medium with 1000 $\mu\text{g TM ml}^{-1}$ further demonstrates tolerance to TM.

The sublethal effects of TM on *D. sapinea* are subjects for further research. However, the behavior of *D. sapinea* does not appear to be consistent with fungicide “resistance” as commonly described. Mycelial growth of *D. sapinea* was greatly inhibited at just 1 $\mu\text{g TM ml}^{-1}$. In contrast, mycelial growth of isolates of the melon wilt pathogen *Fusarium oxysporum* f. sp. *niveum* and papaya stem rot pathogen *Lasiodiplodia theobromae* that were characterized as resistant to TM were not greatly inhibited at this relatively low concentration of the chemical (Petkar *et al.* 2017; Cavalcante *et al.* 2014). Also, as used by FRAC (2017), fungicide resistance “refers to an acquired, heritable reduction in sensitivity of a fungus to a specific anti-fungal agent.” An acquired, heritable reduction in sensitivity to TM has been explained in other fungi by occurrence of a beta-tubulin gene mutation that confers benzimidazole resistance (e.g., Petkar *et al.* 2017), but this has not yet been explored in *D. sapinea*. Study of the mechanisms by which *D. sapinea* maintains ability to germinate and survive in the presence of TM and later resume growth in absence of this chemical, may contribute to improvements in management of the diseases it causes.

Acknowledgements

The authors thank Sydney Everhart and Hanna Stevenson who contributed to preliminary experiments as undergraduate researchers. The provision of isolates used in these studies by former USDA Forest Service scientist Marguerita A. Palmer, and her contributions to our understanding of Diplodia shoot blight and canker diseases of pines are gratefully acknowledged.

References

- Amponsah, N.T., E. Jones, H.J. Ridgway and M.V. Jaspers. 2012. Evaluation of fungicides for the management of Botryosphaeria dieback diseases of grapevines. *Pest Manage. Sci.* 68(5): 676–683. doi:10.1002/ps.2309.
- Cavalcante, R.D., W.G. Lima, R.B. Martins, J.M. Tovar-Pedraza, S.J. Michereff and M.P.S. Câmara. 2014. Thiophanate-methyl sensitivity and fitness in *Lasiodiplodia theobromae* populations from papaya in Brazil. *Eur. J. Plant Pathol.* 140(2): 251–259. doi:10.1007/s10658-014-0459-9.
- FRAC. 2017. (Fungicide Resistance Action Committee). FRAC Code List 2017: Fungicides sorted by mode of action (including FRAC Code numbering). <http://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2017-final.pdf>. Accessed August 22, 2017. Fungicide Resistance Action Committee, 2014. <http://www.frac.info/resistance-overview/what-is-resistance->, Accessed August 10, 2017. <http://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2017-final.pdf>.
- Igeleke, C.L. and D.K.G. Ayanru. 2007. Evaluation of fungicides on growth and conidial germination of *Verticillium theobromae* isolated from plantain. *J. Appl. Sci.* 7(4): 531–535. doi:10.3923/jas.2007.531.535.
- Li, H.Y., R.B. Cao and Y.T. Mu. 1995. *In vitro* inhibition of *Botryosphaeria dothidea* and *Lasiodiplodia theobromae*, and chemical control of gummosis disease of Japanese apricot and peach trees in Zhejiang Province, China. *Crop Prot.* 14(3): 187–191. doi:10.1016/0261-2194(95)00011-A.
- McGrath, M.T. 2004. What are Fungicides? (online). The Plant Health Instructor. doi: 10.1094/PHI-I-2004-0825-01. Updated 2016. <https://www.apsnet.org/edcenter/intropp/topics/Pages/Fungicides.aspx>.

Nakata, A., S. Sano, S. Hashimoto, K. Hayakawa, H. Nishikawa and Y. Yasuda. 1987. Negatively correlated cross-resistance to N-phenylformamidoximes in benzimidazole-resistant phytopathogenic fungi. *Ann. Phytopathol. Soc. Jpn.* 53(5): 659–662. doi:10.3186/jjphytopath.53.659.

Palmer, M.A. and T.H. Nicholls. 1985. Shoot blight and collar rot of *Pinus resinosa* caused by *Sphaeropsis sapinea* in forest tree nurseries. *Plant Dis.* 69(9): 739–740. doi:10.1094/PD-69-739.

Penrose, L.J. and W. Koffman. 1977. Tolerance of *Sclerotinia fructicola* to benzimidazole fungicides and control of the fungus. *Phytopathol. Z.* 88(2): 153–164. doi:10.1111/j.1439-0434.1977.tb03961.x.

Petkar, A., D.B. Langston, J.W. Buck, K.L. Stevenson and P. Ji. 2017. Sensitivity of *Fusarium oxysporum* f. sp. *niveum* to prothioconazole and thiophanate-methyl and gene mutation conferring resistance to thiophanate-methyl. *Plant Dis.* 101(2): 366–371. doi:10.1094/PDIS-09-16-1236-RE.

Pitt, W.M., M.R. Sosnowski, R. Huang, Y. Qui, C.C. Steel and S. Savacchia. 2012. Evaluation of fungicides for the management of Botryosphaeria canker of grapevines. *Plant Dis.* 96(9): 1303–1308. doi:10.1094/PDIS-11-11-0998-RE.

Richmond, D.V. and R.J. Pring. 1971. The effect of benomyl on the fine structure of *Botrytis fabae*. *J. Gen. Microbiol.* 66(1): 79–94. doi:10.1099/00221287-66-1-79.

Solel, Z. 1970. The systemic fungicidal effect of benzimidazole derivatives and thiophanate against *Cercospora* leaf spot of sugarbeet. *Phytopathology* 60(8): 1186–1190. doi:10.1094/Phyto-60-1186.

Stanosz, G.R. and J. Cummings Carlson. 1996. Association of mortality of recently planted seedlings and established saplings in red pine plantations with *Sphaeropsis* collar rot. *Plant Dis.* 80(7): 750–753. doi:10.1094/PD-80-0750.

Stanosz, G.R. and D.R. Smith. 1996. Evaluation of fungicides for control of *Sphaeropsis* shoot blight of red pine nursery seedlings. *Can. J. For. Res.* 26(3): 492–497. doi:10.1139/x26-055.

Stanosz, G.R., D.R. Smith and J.S. Albers. 2005. Surveys for asymptomatic persistence of *Sphaeropsis sapinea* on or in stems of red pine seedlings from seven Great Lakes region nurseries. *For. Pathol.* 35(4): 233–244. doi:10.1111/j.1439-0329.2005.00407.x.

Stanosz, G.R., D.R. Smith, M.A. Guthmiller and J.C. Stanosz. 1997. Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. *Mycologia* 89(4): 525–530. doi:10.2307/3760986.

Stanosz, G.R., J.T. Blodgett, D.R. Smith and E.L. Kruger. 2001. Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. *New Phytol.* 149(3): 531–538. doi:10.1046/j.1469-8137.2001.00052.x.