Ex 1A 2

The Effects of Thiabendazole on *Fusarium subglutinans*, the Causal Agent of Pitch Canker of Loblolly Pine

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ABSTRACT

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Effects of thiabendazole on growth of *Fusarium subglutinans* in culture and on pitch canker symptom expression on artificially inoculated loblolly pine seedlings were studied. The efficacy and duration of efficacy of various concentrations of thiabendazole on natural infection of loblolly pine seedlings in the field were also investigated. Growth of the fungus in vitro was suppressed at 0.1 μg a.i. ml of thiabendazole and completely inhibited at 1.0 μg a.i./ml. Thiabendazole treatment prevented symptom expression and recovery of *F. subglutinans* from artificially inoculated loblolly pine seedlings under greenhouse conditions. A negative linear relationship existed between natural infection of loblolly pine seedlings in the field and thiabendazole concentration, with 21.3 g a.i. L resulting in the lowest pitch canker incidence. Thiabendazole at 14.2 g a.i. L controlled pitch canker for 9 wk; higher concentrations were necessary for extended control, however.

Pitch canker, caused by *Fusarium subglutinans* (Wollenw. & Reink.) Nelson, Toussoun, & Marasas (= *F. moniliforme* Sheld. var. *subglutinans* Wollenw. & Reink.) (15), was recognized as economically important on slash pine (*Pinus elliottii* Engelm. var. *elliottii*) in Florida in 1974 (5) and on loblolly pine (*P. taeda* L.) in North Carolina in 1983 (12). *F. subglutinans* causes a disease complex (10) and can infect and damage virtually all vegetative and reproductive parts of most southern pine species (4,10,13) throughout the life of these hosts (1,6).

Disease incidence is relatively low on plantation-grown loblolly pine in eastern North Carolina but can exceed 75% in localized areas. The disease results in dieback of terminal and lateral shoots, which can affect over 50% of the live crown of some trees and result in high levels of mortality under certain environmental conditions (Runion, *unpublished*). At present, no means of disease control exist, although proper silvicultural management of the host can reduce the economic impact of the disease and the potential of using genetic resistance is being investigated (10).

Thiabendazole [2-(4-thiazolyl)benzimidazole] (9) is a systemic (8,11) and residual (20) fungicide. It can control pineapple fruit rot, caused by *F. m.* var. *subglutinans* (7); mimosa wilt, caused by *F. oxysporum* Schlecht. (16); and Dutch elm disease, caused by *Ceratocystis ulmi* (Buism.) C. Moreau (18,19). Its efficacy for the control of pitch canker of loblolly pine has not been investigated, however.

This paper reports on: 1) the efficacy of thiabendazole in suppressing growth of *F. subglutinans* in culture, 2) the efficacy of various concentrations of thiabendazole in controlling the disease on artificially inoculated loblolly pine seedlings in the greenhouse and on naturally infected seedlings in the field, and 3) the duration of efficacy of these concentrations in controlling the disease in the field.

MATERIALS AND METHODS

In vitro test. Difco potato-dextrose agar (PDA) was prepared and 100-ml aliquots dispensed into 250-ml flasks. The medium was sterilized by autoclaving for 15 min at 121 C, allowed to cool to 50 C, and amended with thiabendazole (Mertect 340F) at 0, 0.1, 1.0, 10, or 100 μg a.i./ml. Then, 10 ml of each amended medium was dispensed into each of 10 sterile plastic petri plates (87 × 15 mm).

F. subglutinans was isolated from an infected 7-yr-old loblolly pine in Beaufort County, NC. A single spore isolate of the fungus was obtained on PDA and used in all tests. A 5-mm-diameter agar plug from 14-day-old mycelium was placed in the center of each plate, and the plates were incubated under ambient laboratory conditions (approximately 20 C). Average radial growth of the fungus was determined after 14 days by measuring from the outer edge of the agar plug to the outer edge of the fungus colony in four directions. The experiment was repeated three times.

Greenhouse inoculation. Seedlings of four half-sib loblolly pine families were grown in plastic window boxes (30 × 11 × 10 cm) that contained a 3:1:1 (v/v) mixture of steam-treated sandy loam soil, sand, and peat moss at 20 seedlings per box. Three-month-old seedlings were inoculated with an aqueous spore suspension (50,000 microconidia per milliliter) of *F. subglutinans* obtained from the isolate described previously. A 5-cm^3 syringe with a 21-gauge needle was used to create a small wound approximately 2 cm below the growing terminal of each seedling. A small droplet of inoculum (approximately 5 μl) was injected into each wound.

Four boxes of seedlings of each half-sib family were sprayed to runoff with an

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aqueous solution of 7.1, 14.2, or 28.4 g a.i./L of thiabendazole at 0, 1, 3, 5, or 7 days after inoculation. Four boxes of seedlings from each family were sprayed with sterile distilled water on each of the five spray dates and served as controls.

The number of seedlings showing symptoms (mortality of the terminal distal from the point of inoculation) was recorded at 5-day intervals after inoculation. Forty days after inoculation, a stem section (approximately 4 cm long) surrounding the inoculation wound was removed from each seedling, surface-disinfested in 1.05% NaOCl for 5 min, and placed on Nash and Snyder's Fusarium selective medium (14). Stem sections were incubated under ambient laboratory conditions (approximately 20 C) for 10 days, at which time the occurrence of *F. subglutinans* was recorded. The experiment was repeated using 5-mo-old seedlings of the same four half-sib loblolly pine families.

Rate determination. Six-month-old loblolly pine seedlings from eight half-sib families were grown in plastic window boxes as described for the greenhouse inoculation study. Seedlings from each half-sib family, not artificially wounded or inoculated, were placed within the drip line of *F. subglutinans*-infected trees in a 7-yr-old loblolly pine plantation in Beaufort County. Seedlings were sprayed to runoff with 7.1, 14.2, 21.3, or 28.4 g a.i./L of thiabendazole. Control seedlings were sprayed to runoff with distilled water.

All seedlings were exposed to natural inocula of *F. subglutinans* for 5 wk, after which a 5-cm section was cut from the terminal of each surviving seedling and processed as previously described. Presence of inoculum was verified weekly via spore trapping on Nash and Snyder's selective agar medium (14). The experiment was conducted four times from May through September 1983.

Duration of efficacy. In August 1984, 6-mo-old, window box-grown loblolly pine seedlings from the same eight half-sib families used in the rate determination study were placed within the same loblolly pine plantation used in that study. In August 1985, seedlings from these eight plus two additional half-sib loblolly pine families were placed within a 5-yr-old loblolly pine plantation in Beaufort County, which had a similar pitch canker incidence as the plantation used in 1984. Seedlings in both studies were placed in open areas in the plantations and not within the drip line of infected trees.

In both years, seedlings were sprayed to runoff with 14.2, 21.3, or 28.4 g a.i./L of thiabendazole and exposed to natural inocula of *F. subglutinans* for 3, 6, 9, or 12 wk. Control seedlings were sprayed to runoff with distilled water. Presence of inoculum was verified and seedlings were processed as described for the rate determination study. All exposure period by thiabendazole concentration treatments were applied in a completely randomized design to one box (= one replication) of seedlings per half-sib family in 1984 and two boxes per family in 1985.

Data collection and analysis. In the rate determination and duration of efficacy studies, the percentage of seedlings whose terminal sections yielded *F. subglutinans* in culture was recorded and assumed to represent the percentage of seedlings infected by the fungus. Mean to variance plots for all percentage data demonstrated no heterogeneity of variance, so data were not transformed before analysis.

Data from all experiments were analyzed using the general linear models procedure (PROC GLM) of the Statistical Analysis Systems (17). In all analyses, means were considered significantly different if they differed at the $P < 0.05$ level.

Table 1. Effect of thiabendazole concentration on the percentage of loblolly pine seedlings naturally infected by *Fusarium subglutinans* for the rate determination study

Thiabendazole concentration (g a.i./L)	Percent infection[y]
0	77.8 a[z]
7.1	57.2 ab
14.2	40.8 bc
21.3	28.8 cd
28.4	13.4 d

[y] Proportion of seedlings (approximately 640) whose terminal section yielded *F. subglutinans* on selective medium. Data are averaged across all eight half-sib families from four replications.

[z] Means followed by the same letter are not significantly different ($P = 0.05$) according to contrasts conducted using the general linear models procedure of the Statistical Analysis Systems.

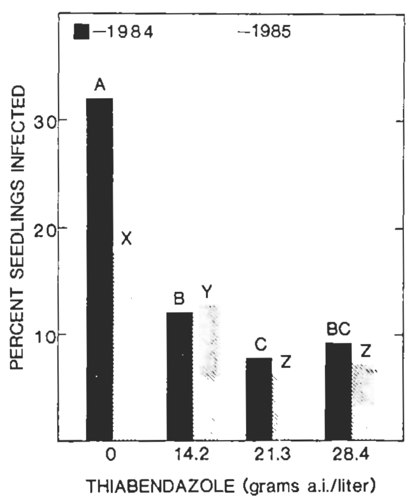

Fig. 1. Percentage of loblolly pine seedlings infected with *Fusarium subglutinans* for the various concentrations of thiabendazole for the 1984 and 1985 duration of efficacy studies. Data for both years were averaged across all half-sib families and across all four exposure periods (3, 6, 9, and 12 wk). Within a year, columns labeled with the same letter are not significantly different ($P = 0.05$) according to contrasts conducted using the general linear models procedure of the Statistical Analysis Systems.

RESULTS

In vitro test. After 14 days, *F. subglutinans* completely covered the surface of the agar medium with no thiabendazole; this represented a radial growth of 41 mm. There was no growth of *F. subglutinans* in plates containing 1.0, 10, or 100 μg a.i./ml of thiabendazole after 14 days. Radial growth of *F. subglutinans* was 3–5 mm in plates containing 0.1 μg a.i./ml of thiabendazole; this represented a suppression of over 87%.

Greenhouse inoculation. Control seedlings showed necrosis of the terminal shoot 15 days after inoculation; at day 40, 88% of these seedlings showed this symptom. Except for two seedlings sprayed with 7.1 g a.i./L of thiabendazole 7 days after inoculation, no treated seedling developed terminal necrosis by day 40. *F. subglutinans* was reisolated from all control seedlings and from the two treated seedlings with terminal necrosis but was not reisolated from tissues surrounding the point of inoculation on any other seedling.

Rate determination. A negative linear correlation existed between percent infection and thiabendazole concentration. This relationship was described by the equation: percent infection = 77.17 − 2.81 (rate), which had an R^2 value of 0.53. All concentrations tested except the 7.1-g rate resulted in significantly lower percent infection than in controls. The 28.4-g rate showed a significantly lower incidence than all concentrations except 21.3 g a.i./L (Table 1).

Differences also existed in percent infection among the half-sib loblolly pine families tested. Family 8-01 showed the highest percent infection (52.8) and family 8-61, the lowest (37.0); the other six half-sib families did not differ significantly from one or both of these extremes.

No significant interaction between thiabendazole concentration and half-sib family was observed; the various thiabendazole concentrations tested had comparable effects in controlling the disease on all half-sib families tested. The overall percent infection was much higher in the rate determination study conducted in 1983 than in either the 1984 or the 1985 duration of efficacy study.

Duration of efficacy. The overall incidence of infection for the duration of efficacy studies was higher in 1984 than in 1985. There were no differences in percent infection among the various durations of exposure to *F. subglutinans* inocula and no interactions observed between exposure period and thiabendazole concentration or between exposure period and half-sib family in 1984.

The relationship between percent infection and thiabendazole concentration for the 1984 duration of efficacy study was best described by the quadratic equation: percent infection = 32.01 − 2.05 (rate) + 0.04 (rate^2), which had an R^2 value of 0.33. All concentrations tested showed significantly lower percent infection than did controls. The 21.3-g rate provided the best control but was not significantly better than the 28.4-g rate (Fig. 1).

Differences among half-sib families were also observed in the 1984 duration of efficacy study. Family 8-01 had the highest level of infection (19.3 %); this level was significantly higher than those for families 8-31 and 8-33 (12.3 and 12.8 %, respectively). The other five half-sib families did not differ significantly from any family tested.

Differences were observed among exposure periods in the 1985 duration of efficacy study. The percentage of infected seedlings increased significantly from the 3-wk (6.3) to the 6-wk (11.5) exposure period but did not increase with additional exposure.

A negative linear relationship existed between percent infection and thiabendazole concentration for the 1985 duration study and was described by the equation: percent infection = 17.61 − 0.42 (rate), which had an R^2 value of 0.17. Infection was lower for all thiabendazole concentrations than for controls. The 21.3- and 28.4-g rates showed less infection than the 14.2-g rate but were not significantly different from each other (Fig. 1).

There were no significant differences in percent infection among the 10 half-sib families tested in the 1985 duration of efficacy study. There also were no interactions between half-sib family and exposure period or between half-sib family and thiabendazole concentration. However, there was a significant interaction of exposure period with thiabendazole concentration in the 1985 duration of efficacy study (Table 2). After 3, 6, or 9 wk of exposure to natural inocula of *F. subglutinans*, all concentrations of thiabendazole resulted in significantly lower incidence of infection than in controls. After 9 wk, the 14.2-g rate had a significantly higher incidence than did the 21.3-g rate, and after 12 wk, the 14.2-g rate was not significantly different from controls but showed higher percent infection than the 21.3- and 28.4-g rates. There were no significant differences between the 21.3- and 28.4-g rates at any exposure period.

DISCUSSION

These results indicate that thiabendazole can be an effective tool for control of pitch canker on loblolly pine seedlings in eastern North Carolina. Thiabendazole, even at the lowest concentrations tested, was effective in limiting growth of *F. subglutinans* in vitro and in preventing pitch canker symptom expression on artificially inoculated loblolly pine seedlings in the greenhouse. *F. subglutinans* can be isolated from surface-disinfested, artificially inoculated loblolly pine stems after four days (2,3). In our greenhouse study, however, thiabendazole prevented symptom expression and recovery of *F. subglutinans* from loblolly tissues even when the fungicide was applied 5 or 7 days after artificial inoculation. These data indicate that thiabendazole may act systemically in loblolly pine stem tissues. Thiabendazole does act systemically in other woody hosts and has been used as a chemotherapeutic control for *C. ulmi*, the causal agent of Dutch elm disease (18).

Significant differences in percent infection among half-sib loblolly pine families were observed in the rate determination and 1984 duration of efficacy studies, and these differences were consistent across all fungicide concentrations tested. However, relative performance of half-sib families was not consistent between the two studies. Differences in relative family performance were probably due to the interaction of host genetic differences with differences in quantity and type of wounding agents and differences in environmental conditions. The absence of family differences in the 1985 duration of efficacy study was probably due to infection incidence being too low to differentiate among half-sib families.

Differences in percent infection among families suggest that infection is influenced by host genetics and indicate the potential of breeding for resistance as a means of pitch canker control. However, additional experimentation is needed to determine the practical and economic potential of resistance breeding as a means of controlling pitch canker of loblolly pine.

Several factors, other than differences in environment among years, may have contributed to differences in percent infection among the three field studies. The frequency and extent of wounding and the quantity of inocula deposited were probably higher on the seedlings in the rate determination study, which were placed within the drip line of infected trees, than on seedlings in the duration of efficacy studies, which were placed in open areas in the plantation. The duration of efficacy studies were conducted later in the year than the rate determination study, which could also have reduced infection by altering host physiology, quantity of inocula, and quantity of insect wounding agents.

Differences in infection levels between the 1984 and 1985 duration of efficacy studies were probably due to the presence of Hurricane Diana, which skirted the North Carolina coast 9 September 1984. The hurricane probably aided in dissemination of inocula of *F. subglutinans* and created wounds that served as infection courts for the inocula.

Data from the rate determination study demonstrated a higher R^2 value than did data from the duration of efficacy studies because of the addition of exposure period as a variable in the latter studies. The quadratic relationship between percent infection and thiabendazole concentration in the 1984 duration study was due to an increase in infection from the 21.3- to the 28.4-g rate. We have observed this effect in other field studies where data suggest that higher thiabendazole rates result in larger seedlings (Runion, *unpublished*). Larger seedlings may provide more area for infection by *F. subglutinans* than seedlings receiving less thiabendazole.

There was no effect of duration of exposure to *F. subglutinans* inocula on pitch canker infection incidence and no interaction of exposure period with thiabendazole concentration in 1984. The reasons for these results are unknown, but the time of year the study was conducted may have had a negative influence on infection, as previously discussed. Hurricane Diana was probably the primary wounding agent during the 1984 study, indicating that the majority of wounds were inflicted within the first 3 wk after thiabendazole application. This could have masked other small increases in infection from other wounding agents that occurred after the hurricane. Also,

Table 2. Percentage of loblolly pine seedlings naturally infected by *F. subglutinans* for the various thiabendazole concentration by exposure period treatments for the 1985 duration of efficacy study[x]

Thiabendazole concentration (g a.i./L)	Exposure period[y]			
	3	6	9	12
0	12.3 cde[z]	22.6 a	20.0 ab	16.4 bc
14.2	5.0 fgh	10.1 def	14.3 cd	21.2 ab
21.3	4.0 gh	5.7 fgh	8.0 efgh	7.4 efgh
28.4	3.3 h	9.0 defgh	9.3 defg	6.4 fgh

[x] Data are percentage of seedlings (approximately 400) whose terminal section yielded *F. subglutinans* on selective medium. Data are averaged across all 10 half-sib families.

[y] Weeks after thiabendazole application.

[z] Means followed by the same letter are not significantly different ($P = 0.05$) according to contrasts conducted using the general linear models procedure of the Statistical Analysis Systems.

rains from the hurricane probably washed some of the fungicide off the pine seedlings, limiting the potential prophylactic protection of the pine tissue and reducing the amount of thiabendazole becoming systemic.

There was a significant increase in percent infection from the 3-wk to the 6-wk exposure period in the 1985 duration of efficacy study, with no subsequent increase in infection after 6 wk. The reason infection did not increase after 6 wk may be the time-of-year effects previously discussed. This lack of response was not due to an absence of inocula, since viable, pathogenic inocula of *F. subglutinans* were collected throughout both studies.

In 1985, there was a significant interaction of exposure period with thiabendazole concentration. Control of pitch canker on loblolly pine seedlings in the field for 9 wk was achieved with 14.2 g a.i./L of thiabendazole, whereas 21.3 or 28.4 g a.i./L provided disease control for 12 wk and would allow for a less frequent spray schedule. Since no significant differences were observed between 21.3 and 28.4 g a.i./L in all studies, the 21.3-g rate is recommended for management of pitch canker on loblolly pine seedlings in eastern North Carolina.

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