Effects of Three Fungicides on Mycelial Growth, Sclerotium Production, and Development of Fungicide-Tolerant Isolates of *Sclerotinia minor*

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ABSTRACT

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Mycelial growth of *Sclerotinia minor* was reduced in vitro on media amended with benomyl and dicloran but was much less evident on media amended with procymidone. Very little mycelium was observed on media amended with procymidone at 0.5 μg/ml and none was observed at 1 and 5 μg/ml after incubation for 96 hr. Tolerance to procymidone developed in in vitro bioassays at a frequency of 2.3% on media amended with this fungicide. Development of tolerance to benomyl and dicloran was not observed. Growth of tolerant *S. minor* isolates on media amended with procymidone at 100 μg/ml was similar to that on unamended media. Mycelial growth rates for tolerant isolates were similar to those of nontolerant isolates at 15, 18, 21, 27, and 30 C. Growth of tolerant isolates on soil plates sprayed with procymidone was similar to growth on untreated plates. Sclerotia produced by procymidone-tolerant isolates were frequently larger and fewer than those produced by isolates on unamended media. Tolerance to procymidone persisted after 10 weekly hyphal-tip transfers on unamended media. Procymidone-tolerant isolates of *S. minor* were not found in field plots treated with this fungicide.

Sclerotinia blight, caused by *Sclerotinia minor* (Jagger) Kohn (7), is a destructive disease of peanut (13,14). This disease, first observed in Virginia in 1971 (11), occurs in Virginia wherever peanuts are grown. It is also widespread in North Carolina and Oklahoma and was recently observed in Texas. Disease loss estimates based on interpretation of infrared photography indicated peanut yield losses to Sclerotinia blight exceeded 13% in Virginia in 1979 (14). Fungicides and a resistant cultivar (2) are now used in an integrated disease control program. Although not recommended for Sclerotinia blight control, benomyl suppresses disease development at 1.1 kg/ha (1). Dicloran at 5.6–11.2 kg/ha also suppresses development of this disease (1,9). The dicarboximide fungicide procymidone, however, gave almost complete control of severe Sclerotinia blight at 0.84 kg/ha (9,10).

During the past decade, much has been written about the development of tolerance by fungi to specific fungicides (3,5,6). Tolerance appears to be associated with long-term and frequent exposure of a pathogen to a fungicide (3). Species of *Sclerotinia* (4,20), *Monilinia* (15,16,18), and *Botrytis* (19) tolerant to benomyl, dicloran, and procymidone have been reported. The objectives of this study were to determine 1) the effects of these fungicides on mycelial growth of *S. minor* on amended potato-dextrose agar (PDA), 2) if tolerance to these fungicides developed under laboratory conditions, 3) the persistence of tolerance to procymidone under laboratory conditions, 4) the effects of temperature on growth of procymidone-tolerant and nontolerant isolates of *S. minor*, and 5) if tolerance to procymidone developed under field conditions.

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MATERIALS AND METHODS

In vitro fungicide bioassay. Field isolates of *S. minor* were maintained on PDA slant tubes at 21 C. Isolates of *S. minor* were grown on a 5-mm layer of PDA in 85-mm (i.d.) plastic petri plates. For the in vitro fungicide bioassay, 1-cm plugs taken from the edges of actively growing PDA cultures were inverted in the centers of PDA plates amended with benomyl (Benlate 50W), procymidone (DPX-4424 50W), and dicloran (Botran 75W) to determine the effect of each fungicide on mycelial growth and sclerotium production. Fungicides suspended in sterile distilled water were added to cooled, autoclaved PDA to give the desired concentrations of active ingredients. Plates were incubated at 21 C. In temperature studies, plates were incubated at 15, 18, 21, 27, and 30 C. Colony growth was measured at designated hourly intervals.

In vitro soil plate bioassay. Procymidone-tolerant isolates of *S. minor* from the in vitro fungicide bioassay were used in this study. A soil-cornmeal mix (5% cornmeal, w/w) was prepared using air-dried Woodstown sandy loam passed through a 10-mesh screen. Fifty grams of the mixture plus 20 ml of water were added to each 9-cm-diameter glass petri plate, autoclaved at 121 C for 1 hr, and allowed to cool to room temperature. Plugs of agar from the edges of 60-hr-old colonies of either procymidone-tolerant or nontolerant isolates of *S. minor* were inverted on the soil medium in the center of each petri plate. Plates were incubated at 21 C. After 48 hr, the soil surface was sprayed with an airbrush sprayer that delivered known quantities of procymidone in 1 ml of liquid per plate. Colony diameters were measured after 48, 96, 168, and 216 hr of incubation at 21 C. The number of sclerotia produced by *S. minor* in each plate was determined by removing a 3-cm-diameter sample of soil medium, washing the sample on a 60-mesh sieve to remove soil, and counting the trapped sclerotia under a stereoscopic microscope.

Stability of procymidone tolerance. Five nontolerant and 11 procymidone-tolerant isolates of *S. minor* were grown on unamended PDA for 1 mo at 21 C. Tolerant and nontolerant isolates obtained from the margins of 3-day-old colonies were transferred on a weekly cycle to slant tubes containing unamended PDA. After 10 successive weekly transfers, isolates were transferred to PDA containing procymidone at 10 and 100 μg/ml. Colony growth was recorded after 24, 140, and 192 hr of incubation at 21 C.

Assay for field tolerance. Isolates of *S. minor* were obtained from fields with histories of Sclerotinia blight that had been treated four times with procymidone concentrations of 28, 56, or 1.12 kg/ha. Samples from 280 peanut branches with symptoms of Sclerotinia blight were

Table 1. Mycelial growth of *Sclerotinia minor* on unamended potato-dextrose agar and on similar media amended with different concentrations of benomyl, dicloran, and procymidone

Fungicide	Concentration[a] (μg/ml)	Colony diameters (mm) after indicated hours of incubation				
		48	72	96	144	192
Unamended	···	65[b]	85	85	85	85
Benomyl	0.5	30	70	85	85	85
	1.0	20	62	85	85	85
	5.0	15	32	42	75	85
Dicloran	0.5	30	68	85	85	85
	1.0	20	42	60	85	85
	5.0	10	12	14	14	15
Procymidone	0.5	10[c]	···	10	12	15
	1.0	10	···	10	10	10
	5.0	10	···	10	10	10

[a] Concentration of active ingredients.
[b] Mean of 24 isolates from three tests with 10 plates each.
[c] A value of 10 mm (diameter of inoculum plug) indicates no growth on agar medium.

Table 2. Frequency of occurrence of isolates of *Sclerotinia minor* found to be tolerant to procymidone in in vitro bioassays on media amended with procymidone

Experiment	Procymidone concentration[a] (μg/ml)	No. of plates	Tolerance frequency[b] (%)	Tolerance stability[c] (%)
A	2.5	100	2	50
B	2.5	120	4	75
C	5.0	80	1	0
D	5.0	140	2	50
E	10.0	100	2	50
F	10.0	84	3	66

[a] Potato-dextrose agar amended with procymidone.
[b] Isolates (%) showing tolerance during initial exposure to procymidone.
[c] Procymidone-tolerant isolates (%) of *S. minor* transferred to procymidone-amended media (1 μg/ml) to determine isolates (%) maintaining tolerance.

Table 3. Mycelial growth rates of *Sclerotinia minor* isolates found to be tolerant (T) to procymidone in in vitro bioassays and nontolerant (NT) on unamended potato-dextrose agar and on similar media amended with procymidone

Isolate	Concentration[a] (μg/ml)	Colony diameter (mm) after indicated hours of incubation					Sclerotia
		72	96	168	192	240	
NT-18	0	46[b]	85	85	85	85	Yes
	1	10[c]	10	10	12	12	–[d]
	10	10	10	10	12	12	–
	100	10	10	10	12	12	–
T-18	0	50	85	85	85	85	Yes
	1	64	85	85	85	85	Yes
	10	63	83	85	85	85	Yes
	100	43	75	85	85	85	Yes
NT-24	0	76	85	85	85	85	Yes
	1	10	12	60	65	85	Yes
	10	10	10	10	10	12	–
	100	10	10	10	10	15	–
T-24	0	76	85	85	85	85	Yes
	1	84	85	85	85	85	Yes
	10	79	85	85	85	85	Yes
	100	56	74	85	85	85	Yes
NT-35	0	85	85	85	85	85	Yes
	1	10	12	12	12	12	–
	10	10	10	10	10	12	–
	100	10	10	10	10	10	–
T-35	0	74	85	85	85	85	No
	1	76	85	85	85	85	No
	10	75	85	85	85	85	No
	100	71	85	85	85	85	No

[a] Concentration of active ingredients.
[b] Mean of two tests with 10 plates each.
[c] A value of 10 mm (diameter of inoculum plug) indicates no growth on agar medium.
[d] No sclerotia were produced because mycelial growth was minimal.

surface-disinfected with 0.5% NaOCl for 3 min, placed in PDA, and incubated at 21 C. Hyphal tips of isolates were transferred to PDA slant tubes. Growth of isolates on media amended with procymidone at 1 and 10 μg/ml was determined as described previously.

RESULTS

In vitro fungicide bioassay. Mycelium of *S. minor* covered the petri plate surface after 72 hr of incubation on unamended PDA (Table 1). Most isolates growing on benomyl or dicloran at 0.5 μg/ml covered the agar surface within 96 hr. Mycelium of most isolates covered the surface of media amended with benomyl at 5 μg/ml after incubation for 192 hr. At this concentration, growth did not occur on dicloran after 192 hr. Growth of *S. minor* on media amended with procymidone at 0.5 μg/ml was almost nonexistent after 192 hr of incubation.

Sectoring of colonies of *S. minor* was often observed on media amended with benomyl or dicloran. Sectoring was not observed on procymidone-amended media. When mycelium from sectors was transferred to media containing benomyl or dicloran, only scant or no growth occurred. Occasionally, an isolate would grow on media amended with dicloran at 0.5–5 μg/ml.

Procymidone-tolerant isolates of *S. minor* developed on media containing concentrations of 0.25–10 μg/ml. Mycelial growth, which occurred in the absence of sectoring, was similar to that produced by nontolerant isolates. Visible differences between the procymidone-tolerant and nontolerant isolates were not discernible. Tolerance to procymidone occurred at a frequency of 2.3% (Table 2). About 50% of these isolates maintained their ability to grow when transferred to procymidone-amended media.

Even with procymidone at 100 μg/ml, the growth rates of tolerant isolates of *S. minor* were similar to those of nontolerant isolates growing on unamended media (Table 3). Production of sclerotia of procymidone-tolerant isolates of *S. minor* was similar in most instances to the number produced on unamended media. Sclerotia produced on procymidone-amended media, however, were often larger and fewer than those produced on unamended media. On rare occasions, some procymidone-tolerant isolates failed to produce sclerotia.

Growth rates of procymidone-tolerant and nontolerant isolates of *S. minor* on unamended media were similar at 15, 18, and 21 C (Fig. 1). Maximum growth of all isolates occurred at 21 C. At 27 C, isolates differed in their growth rates, but differences were not correlated to tolerance or nontolerance. All isolates made only a trace of growth at 30 C.

In vitro soil plate bioassay. Growth rates of procymidone-tolerant and nontolerant isolates of *S. minor* on soil

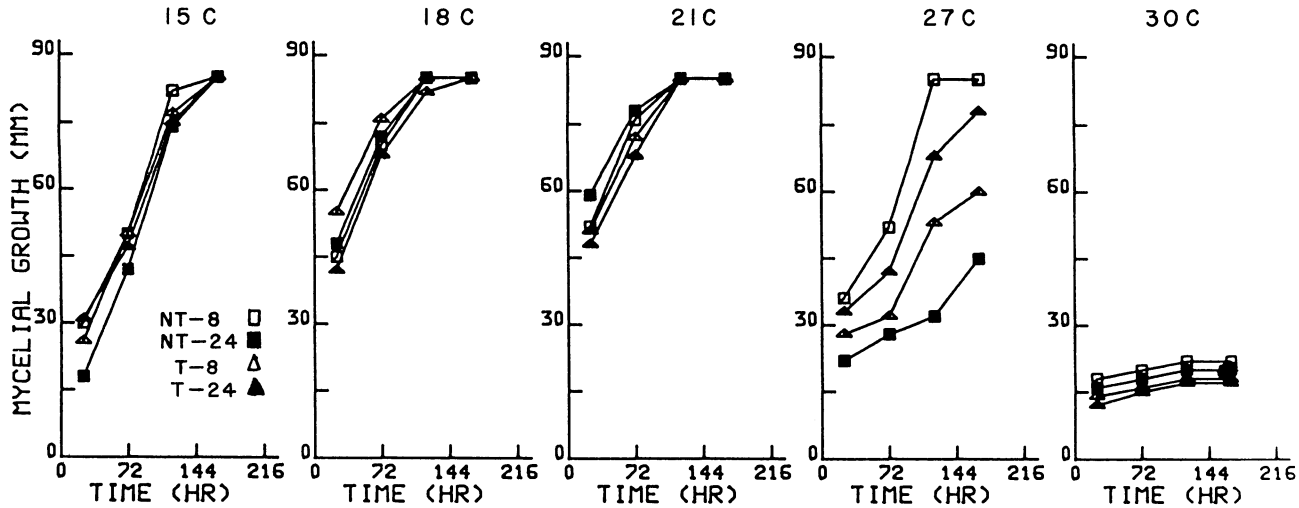

Fig. 1. Effect of temperature on growth rates of *Sclerotinia minor* isolates found to be tolerant (Δ) to procymidone in in vitro bioassays and nontolerant (□) on unamended media.

plates sprayed with procymidone (1.16 kg/ha in 186 L of water per hectare) showed marked differences between the two types of isolates (Table 4). Growth of nontolerant isolates sprayed with procymidone was nonexistent after 216 hr of incubation. Five of seven procymidone-tolerant isolates grew readily after they were sprayed with procymidone; however, sclerotium production was reduced compared with appropriate checks (Table 4).

Stability of procymidone tolerance. Tolerance to procymidone was maintained after 10 successive weekly transfers of mycelium from actively growing colonies of *S. minor* on unamended media (Fig. 2). Within 140 hr, mycelial growth of most tolerant and nontolerant isolates covered the agar surface when transferred to PDA in petri plates. When both tolerant and nontolerant isolates were transferred to media amended with procymidone at 10 μg/ml, mycelial growth was most evident in the tolerant isolates. Mycelium of nontolerant isolates was not observed until after incubation for 140 hr. Tolerant isolates grew well on media containing procymidone at rates as high as 100 μg/ml. In fact, after 192 hr of incubation, most isolates had covered the agar surface of the petri plates.

Assay of field tolerance. Isolates of *S. minor* with tolerance to procymidone were not detected under field conditions. No tolerant isolates were obtained from infected plant tissues in 280 samples of peanut branches from several locations that had received prophylactic treatments of procymidone.

Table 4. Mycelial growth rates of *Sclerotinia minor* isolates found to be tolerant (T) to procymidone in in vitro bioassays and nontolerant (NT) on soil plates after spraying with procymidone

Isolate[a]	Treatment[b]	Colony diameter (mm) after indicated hours of incubation				No. of sclerotia per plate
		48	96	168	216	
NT-17	Unsprayed	30[c]	83	85	85	731
	Procymidone	10	10	10	10	0
NT-19	Unsprayed	30	82	85	85	606
	Procymidone	10	10	10	10	0
NT-20	Unsprayed	34	83	85	85	270
	Procymidone	10	10	10	10	0
T-4	Unsprayed	28	83	85	85	299
	Procymidone	12	50	80	85	204
T-16	Unsprayed	19	57	85	85	261
	Procymidone	10	10	10	10	0
T-17	Unsprayed	17	70	85	85	107
	Procymidone	13	35	51	66	100
T-18	Unsprayed	16	31	53	63	122
	Procymidone	12	27	45	55	112
T-24	Unsprayed	14	18	32	46	196
	Procymidone	12	17	33	45	127
T-35	Unsprayed	20	44	70	83	316
	Procymidone	20	43	70	85	293
T-36	Unsprayed	21	53	67	85	0
	Procymidone	10	10	12	10	0

[a] Nontolerant (NT) isolates had not been grown on procymidone-amended media; tolerant (T) isolates were from cultures that grew readily on media amended with 50 μg/ml of procymidone.
[b] Procymidone was applied at 1.16 kg/ha in 186 L of water per hectare.
[c] Mean of two tests with 10 plates each.

DISCUSSION

Growth of mycelium of *S. minor* was suppressed on media amended with benomyl, dicloran, and particularly procymidone. Increased fungicide concentrations resulted in corresponding reductions in mycelial growth. Sectoring colonies of *S. minor* developing on media amended with either benomyl or dicloran were not tolerant to these fungicides. Tolerance to benomyl was not observed in earlier studies (12) but has been reported for other *Sclerotinia* spp. (4,20). Although tolerance to dicloran did not develop in this test, some isolates grew on low concentrations (0.25 and 0.5 μg/ml) of dicloran-amended media. Tolerance of other *Sclerotinia* spp. to dicloran has not been reported, but tolerance of isolates of *Botrytis cinerea* to dicloran has been reported (19).

The importance of fungicide tolerance and its development in fungi has recently received much attention (3,5,6,8,15–17, 19,20). In this study, procymidone tolerance was observed in vitro under controlled laboratory conditions but not under field conditions. Some procymidone-tolerant isolates of *S. minor* grew as rapidly on procymidone-amended media as nontolerant isolates grew on unamended media. Our sample of 280 infected branches may have been too small to detect procymidone-tolerant isolates of *S. minor* under field conditions. The importance of sample size in determining field tolerance was discussed by Delp (3); however, the use of other fungicides (chlorothalonil, benomyl, manzate, etc.) for control of foliar diseases of peanuts may have affected the development of procymidone-tolerant isolates of *S. minor* under field conditions. The importance of fungicide mixtures in reducing the probability of tolerance development has been discussed (3,17). The selection pressure asserted by the continuous use of a fungicide like procymidone could be averted by

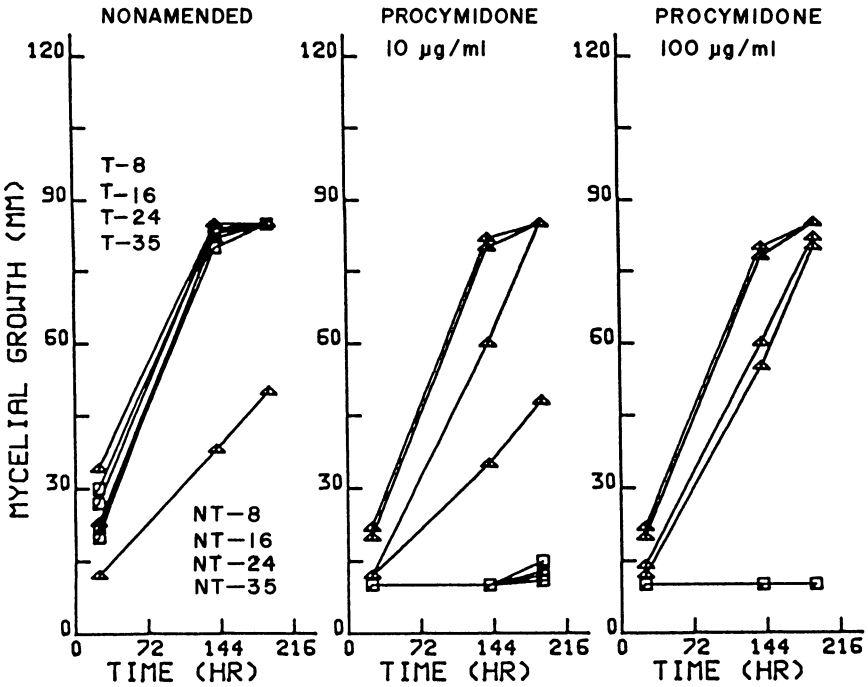

Fig. 2. Growth of *Sclerotinia minor* isolates found to be tolerant (□) to procymidone in in vitro bioassays and nontolerant (Δ) on unamended media and on media amended with 10 and 100 µg/ml of procymidone after 10 weekly transfers of mycelium to unamended media.

programs that use either mixtures or alternate scheduling of fungicides with different modes of action.

The ability of *S. minor* to retain tolerance to procymidone through 10 successive transfers of mycelial growth on unamended media is significant. When an isolate develops tolerance to procymidone, it may not revert to a nontolerant condition. The longevity of tolerant isolates under field conditions is unknown but results of this study indicate the stability of this trait. Further research will be necessary to determine if this will affect the efficacy of dicarboximide fungicides in control of *S. minor*.

With the exception of fewer and often larger sclerotia, the cultural characteristics of procymidone-tolerant and nontolerant isolates of *S. minor* are indistinguishable. Gross mycelial morphology, temperature requirements, development of sclerotial initials, and distribution pattern of sclerotia on the surface of PDA was similar for both tolerant and nontolerant isolates. Differences in colony morphology, etc., have been noted for other fungicide-tolerant fungi (15). Procymidone-tolerant isolates of *S. minor* can only be distinguished from nontolerant isolates by growing them on fungicide-amended media.

The reasons for reduced sclerotium development in some isolates of *S. minor* showing procymidone tolerance are unknown. This may indicate these tolerant isolates are less "parasitically fit" than nontolerant isolates. These isolates may be unable to exist in a field population of nontolerant isolates. They would become the dominant population under field conditions only under circumstances whereby natural populations are completely eradicated with continued use of such fungicides as procymidone. The survival "fitness" of such sclerotia has not been determined, however. If tolerant isolates do develop under field conditions and sclerotial survival is adequate, then selection pressure could shift toward improved parasitic fitness.

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