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Tolerance of *Sclerotinia minor* to Procymidone and Cross Tolerance to Other Dicarboximide Fungicides and Dicloran[1]

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ABSTRACT

Procymidone-tolerant isolates of *Sclerotinia minor* Jagger were cross-tolerant to iprodione, vinclozolin and dicloran. Hyphal growth of procymidone-tolerant isolates an agar amended with 1 to 100 μg/mL procymidone, iprodione, vinclozolin or dicloran was similar. Procymidone-tolerant isolates were tolerant to all fungicides after 10 weekly hyphal tip transfers to nonamended agar. Subsequently, cross-tolerance persisted on agar amended with either 10 μg/mL procymidone, iprodione, vinclozolin or dicloran. Most procymidone-tolerant isolates of *S. minor* were pathogenic to peanuts (*Arachis hypogaea* L.) and caused symptoms similar to those initiated by sensitive isolates.

Key Words: Fungicide resistance, sclerotinia blight, epidemiology, peanuts, *Arachis hypogaea*.

Sclerotinia blight of peanut (*Arachis hypogaea* L.), caused by *Sclerotinia minor* (Jagger) Kohn (8), is a serious problem in Virginia and North Carolina (12) and in Oklahoma (22). According to crop loss estimates based on infrared photographs, losses due to Sclerotinia blight exceeded 13% in Virginia in 1979 (15).

Specific-site inhibitor-type fungicides such as the dicarboximides often exhibit a narrow spectrum of fungicidal activity (6). Procymidone, a dicarboximide fungicide, applied broadcast over peanut foliage at 0.56 kg a.i./ha provided almost complete control of Sclerotinia blight under epiphytotic conditions (11). The efficacy of other dicarboximide fungicides including iprodione and vinclozolin against *S. minor* has recently been demonstrated (10). Dicloran, a broad spectrum fungicide indirectly related to procymidone, provides partial control of *S. minor* (1).

The tendency of specific pathogens to develop resistance to narrow spectrum fungicides such as the dicarboximides is well documented. According to Delp (4), strains of fungi tolerant to one fungicide are frequently cross-tolerant to other structurally related fungicides or to fungicides with similar modes of action. There are reports that cross-tolerance also exists between structurally unrelated fungicides (3). In these cases, both fungicides apparently act on similar sites within the fungal cell. Resistance and cross-resistance to procymidone, iprodione, vinclozolin, and dicloran has been reported with *Sclerotinia sclerotiorum* (16), *Botrytis cinerea* (9), *Penicillium expansum* (19), *Sclerotinia homoeocarpa* (5), and *Monilinia fructicola* (17,21). Recently we demonstrated that *S. minor* developed tolerance to procymidone on agar amended with various concentrations

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of this fungicide (14). This tolerance persisted through several successive generations, but isolates tolerant to *S. minor* were not found in fields showing symptoms of Sclerotinia blight and treated with procymidone (14).

The objectives of this study were to determine if isolates of *S. minor* tolerant to procymidone were cross-tolerant to iprodione and vinclozolin (fungicides directly related to procymidone) and to dicloran (a fungicide indirectly related to procymidone). The stability and pathogenicity of procymidone-tolerant isolates were also determined. Preliminary results from this study have been reported (13).

Materials and Methods

Cultures of *S. minor* used in this study were obtained from peanut plants with symptoms typical of Sclerotinia blight and from procymidone-tolerant isolates that developed on potato-dextrose agar (PDA) amended with 1 μg/mL procymidone. Hyphal tip isolates obtained from 2-day-old colonies were maintained in plastic slant tubes (90 x 15 mm) containing PDA incubated in the dark at 21 C.

In vitro fungicide bioassay. The following fungicides, including procymidone (DPX-4424 50W; E.I duPont de Nemours & Co., Wilmington, DE 19898), iprodione (Rovral 50W; Rhone-Poulenc Inc., Monmouth Junction, NJ 08852), vinclozolin (Ronilan 50W; BASF Wyandotte Corp., Parsippany, NJ 07054), and dicloran (Botran 75W; The Upjohn Company, Kalamazoo, MI 49001) were assayed in fungicide-amended PDA for effects against *S. minor*. The chemical structure of each fungicide is as follows: procymidone-[N-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide]; iprodione-[3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxomidazolidine-1-carboximide]; vinclozolin-[3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazoladine-2,4-dione]; dicloran-(2,6-dichloro-4-nitroaniline). Each formulated fungicide was suspended in sterile distilled water and added in appropriate quantities to partially cooled, autoclaved PDA. Desired concentrations of each fungicide are expressed in terms of active ingredient (μg/mL).

Isolates of *S. minor* were grown on PDA poured 5 mm deep into standard plastic petri plates having as inside diameter of 85 mm. Plates were incubated in the dark at 21 C. After 72-hr incubation, (fungal colony was ca 70 mm in diameter), plugs of mycelium (10 mm in diameter) were cut from the margins of the colony with a cork borer. These plugs were inverted in the center of plates that contained fungicide-amended PDA. Hyphal growth (diameter of colony minus 10 mm, the diameter of the inoculum plug) was measured at designated intervals.

Stability of tolerance to several fungicides. The procymidone-tolerant isolates of *S. minor* in this study were selected from previous bioassay experiments where *S. minor* was grown on PDA amended with procymidone. Mycelium from procymidone-tolerant colonies of *S. minor* was transferred to petri plates containing nonamended PDA to initiate new mycelial growth in all isolates. After incubation for 3 days, mycelial plugs (10 mm in diameter) were cut from the margin of actively growing colonies and inverted in the center of petri plates containing nonamended agar. After 10 successive weekly transfers to nonamended agar, plugs of inoculum were removed from colony margins and transferred to agar amended with 10 μg/mL procymidone, vinclozolin, iprodione and dicloran. Hypal growth was determined after 48-, 72- and 144-hr incubation at 21 C.

Pathogenicity studies. Pathogenicity studies with procymidone-tolerant and sensitive isolates of *S. minor* were conducted in a mist chamber set to maintain a 12-hr light period of 26 C and a 12-hr dark period of 21 C. Misters using deionized water were activated 15 min/hr to maintain a constant relative humidity of nearly 100%. Peanut plants (cv. Virginia 72R) grown for 6 wk in 15 cm clay pots were transferred to the mist chamber for 1 wk prior to inoculation. Sixteen procymidone-tolerant and four sensitive isolates of *S. minor* were selected for this study. Each isolate was grown on PDA. Before colony diameter approached 70 mm, mycelial plugs (5mm in diameter) were cut from the edge of actively growing colonies. Plugs were placed along the side of a branch about 2.5 cm from the surface of the soil. A superficial wound at the inoculation site was made with sandpaper. The agar plug, with mycelium in contact with the wounded tissues, was held in place with a piece of sterile cheesecloth and wooden toothpicks. Five plants were inoculated with each isolate. Check plants were also wounded but inoculated with a sterile agar plug. In two separate and replicated experiments lesion length (mm) along the inoculated branch was determined 144 and 192 hr after inoculation.

In another test, plants were inoculated and maintained in the mist chamber as previously described. However, plants were scored for disease severity by using a disease index (1 = healthy plant; 2 = slight damage with mycelium scanty; 3 = severe damage with inoculated branches flagging and mycelium abundant; and 4 = inoculated branch dead). Disease severity readings were made 13 and 17 days after inoculation.

Results

In vitro fungicide bioassay. The average growth rate of 25 procymidone-tolerant isolates of *S. minor* on PDA amended with 10 μg/mL procymidone, iprodione, vinclozolin and dicloran is given in Table 1. Isolates tolerant to procymidone were also cross-tolerant to iprodione, vinclozolin and dicloran. Some procymidone-tolerant isolates grew faster on fungicide-amended agar than on nonamended agar. A few isolates, however, grew only slightly when transferred to fungicide-amended agar. An isolate of *S. minor* that grew on procymidone-amended agar also grew on iprodione, vinclozolin, and dicloran-amended agar. If scant mycelium was produced on procymidone-amended agar, scant mycelium was likewise produced on agar amended with iprodione, vinclozolin and dicloran.

Table 1. Mean hyphal growth of 25 procymidone-tolerant isolates of *Sclerotinia minor* on potato dextrose agar amended with 10 μg/mL procymidone, iprodione, vinclozolin or dicloran.

Incubation	Hyphal growth (mm)[b]				
period(hr)[a]	Nonamended	Procymidone	Iprodione	Vinclozolin	Dicloran
24	7.5[c]	0.4	2.7	0.6	0
48	28.8	13.4	15.8	17.2	12.1
120	75.0[d]	69.3	68.4	68.1	67.6

a Plates were incubated at 21 C after transfer of inoculum plug (10 mm in diameter) to the center of the petri dish containing nonamended and fungicide-amended agar.

b Hyphal growth was determined by subtracting the width of the inoculum plug from the diameter of the fungal colony.

c Mean of hyphal growth on 10 petri plates in two experiments.

d A measurement of 75 mm indicates that hyphal growth has covered the entire surface area of the agar.

Nearly total growth inhibition was observed for two isolates of *S. minor* sensitive (S-8 and S-24) to procymidone on agar amended with 1 μg/mL procymidone, iprodione, vinclozolin and dicloran after a 72-hr incubation period (Table 2). Following 196-hr incubation, growth of *S. minor* remained scant on agar amended with 1 μg/mL procymidone, iprodione and vinclozolin. The growth of procymidone-tolerant isolates (T-8 and T-24) on 1 and 10 μg/mL procymidone, iprodione, vinclozolin, and dicloran was similar to that observed on nonamended agar. However, the growth of tolerant isolates decreased gradually with increased fungicide concentration.

Stability of tolerance to several fungicides. Procymidone-tolerant isolates of *S. minor* maintained their capacity to grow on procymidone-amended agar (10μg/mL) following 10 successive transfers to nonamended

Table 2. Hyphal growth of sensitive (S) and procymidone-tolerant (T) isolates of *Sclerotinia minor* on potato dextrose agar and on agar amended with 0, 1, 10, 100, and 1000 μg/mL procymidone, iprodione, vinclozolin, and dicloran.

Isolate	Hyphal growth after incubation at 72, 144, and 196 hr[b]														
	Procymidone (μg/mL)														
	0			1			10			100			1000		
	72	144	196[a]	72	144	196	72	144	196	72	144	196	72	144	196
S-8	48[c]	75[d]	75	0	0	4	0	0	0	0	0	0	0	0	0
T-8	38	75	75	60	75	75	62	75	75	55	75	75	38	75	75
S-24	57	75	75	0	2	12	0	0	2	0	0	0	0	0	0
T-24	62	75	75	48	75	75	38	62	75	42	52	75	2	25	35
	Iprodione (μg/mL)														
S-8	48	75	75	0	2	4	0	0	0	0	0	0	0	0	0
T-8	38	75	75	37	60	75	37	75	75	20	37	52	7	14	19
S-24	57	75	75	0	0	2	0	0	0	0	0	0	0	0	0
T-24	62	75	75	38	75	75	50	75	75	17	52	75	2	35	73
	Vinclozolin (μg/mL)														
S-8	48	75	75	0	0	2	0	0	0	0	0	0	0	0	0
T-8	38	75	75	44	75	75	36	70	75	34	72	75	8	18	48
S-24	57	75	75	0	0	2	0	0	0	0	0	0	0	0	0
T-24	62	75	75	56	75	75	36	75	75	35	75	75	5	14	19
	Dicloran (μg/mL)														
S-8	48	75	75	8	37	75	0	0	2	0	0	2	0	0	2
T-8	38	75	75	48	60	65	30	75	75	12	35	62	8	20	34
S-24	57	75	75	5	15	37	0	0	2	0	0	2	0	0	2
T-24	62	75	75	38	75	75	24	52	75	18	28	48	5	30	50

[a] Plates were incubated at 21 C after transfer of inoculum plug (10 mm in diameter) to the center of the petri dish containing nonamended and fungicide-amended agar.

[b] Hyphal growth was determined by subtracting the width of the inoculum plug from the diameter of the fungal colony.

[c] Mean of hyphal growth on 10 petri plates in two experiments.

[d] A measurement of 75 mm indicates that hyphal growth covered the entire surface area of the agar.

agar and grew similarly on agar amended with iprodione and vinclozolin (Table 3). Growth of mycelium occurred at a slower rate on dicloran-amended agar. Rates of hyphal growth between fungicide-amended agar and nonamended agar became less obvious with time.

Table 3. Variation in hyphal growth of procymidone-tolerant (T) isolates of *Sclerotinia minor* on agar amended with 10 μg/mL procymidone, iprodione, vinclozolin and dicloran after 10 weekly hyphal tip transfers to nonamended agar.

Isolate	Incubation period(hr)[a]	Hyphal growth (mm)[b]				
		Nonamended	Procymidone	Iprodione	Vinclozolin	Dicloran
T-84	48	34[c]	34	35	36	17
	72	55	65	63	65	33
	144	75[d]	75	75	75	75
T-101	48	38	29	30	28	15
	72	66	57	58	57	33
	144	75	72	74	74	72
T-62	48	38	2	2	1	4
	72	69	10	7	7	9
	144	75	50	40	58	35

[a] Plates were incubated at 21 C after transfer of inoculum plug (10 mm in diameter) to the center of the petri dish containing nonamended and fungicide-amended agar.

[b] Hyphal growth was determined by subtracting the width of the inoculum plug from the diameter of the fungal colony.

[c] Mean of hyphal growth on 10 petri plates in two experiments.

[d] A measurement of 75 mm indicates that hyphal growth covered the entire surface area of the agar.

Variation in the colony diameter of tolerant isolates of *S. minor* existed when these were transferred to fungicide-amended agar after 10 successive transfers to nonamended agar (Table 3). About 6% of the isolates, including T-84, grew more on agar amended with procymidone, iprodione or vinclozolin after a 72-hr incubation period than did the same isolate on nonamended agar. About 70% of the isolates, including T-101, grew at about the same rate as that noted on nonamended agar. However, about one-fourth of the procymidone-tolerant isolate of *S. minor* grew very slowly on agar amended with either procymidone, vinclozolin, iprodione or dicloran during the first 72-hr period.

Pathogenicity studies. Procymidone-tolerant isolates of *S. minor* were pathogenic to peanut branches and caused lesions similar to those caused by sensitive isolates (Table 4). Under favorable conditions for disease development, average lesion length was greater in plants inoculated with sensitive isolates than in plants inoculated with procymidone-tolerant isolates. However, some procymidone-tolerant isolates were more pathogenic than some sensitive isolates. Other procymidone-tolerant isolates were only mildly pathogenic. Lesion length increased with time with all isolates tested.

Table 4. Comparison of pathogenicity of procymidone-tolerant (T) and sensitive (S) isolates of *Sclerotinia minor* on Virginia 72R peanut.[a]

	Lesion length (mm)			
	Trial #1		Trial #2	
	144 hr	192 hr	144 hr	192 hr
S-163	58[b]	85	44	79
S-63	61	96	68	80
S-93	68	105	46	64
S-206	68	96	71	109
Mean	64	96	57	83
T-148	0	8	20	31
T-26	8	18	0	0
T-77	18	23	40	51
T-28	24	35	55	80
T-138	25	39	33	46
T-510	40	52	43	54
T-810	40	51	8	18
T-127	48	69	41	48
T-92	51	92	54	81
T-910	54	81	73	99
T-164	59	80	50	69
T-161	61	89	59	83
T-110	61	97	45	59
T-116	61	90	0	0
T-126	65	104	35	41
T-54	84	135	75	96
Mean	44	66	39	43

[a] Plants were maintained in a growth chamber with day and night temperatures of 26 C and 21 C, respectively, with a relative humidity of nearly 100%.

[b] Mean lesion length (mm) was determined by measuring the length of lesions on five inoculated plants.

After incubation for 13 days, the disease index for procymidone-tolerant and sensitive isolates of *S. minor* was similar. For procymidone-tolerant and sensitive isolates the disease index averaged 2.3 and 2.5, respec-

tively. However, disease progress was greatest in the sensitive isolates. Similar results were noted 17 days after inoculation.

Discussion

Resistance to procymidone, iprodione, and vinclozolin has been reported for *Sclerotinia* spp. (2,5,13,14,16,), *Botrytis cinerea* (9), *Monilinia fructicola* (17,18,21) and *Penicillium expansum* (19). Cross-resistance to some of these compounds also has been reported (17,19,21). Development of tolerance to directly and indirectly related fungicides by several genera of fungi has been established (3,7). Ritchie (18) observed differences in the growth rates of *M. fructicola* isolates sensitive to dicarboximide fungicides. Some isolates of *P. expansum* (19) tolerant to vinclozolin were not cross-tolerant to iprodione.

Our data show that procymidone-tolerant isolates of *S. minor* are cross-tolerant to iprodione and vinclozolin (dicarboximide fungicides directly related to procymidone), and to dicloran, a fungicide indirectly related to procymidone. In our studies the growth of fungicide-tolerant isolates of *S. minor* on agar amended with various concentrations (1, 10 and 100 μg/mL) of procymidone, iprodione, vinclozolin and dicloran was similar (Table 2). We found that colony growth on agar amended with dicloran was less than on agar containing either procymidone, iprodione or vinclozolin. In our studies some fungicide-sensitive isolates of *S. minor* grew equally well on fungicide-amended agar as on nonamended agar; some fungicide-sensitive isolates grew at a faster rate on fungicide-amended agar than on nonamened agar; and some fungicide-sensitive isolates grew only sparsely on both nonamended and fungicide-amended agar. Since these fungicides apparently interact with the nuclei of the fungal cell, differences in growth rate can be expected (16).

Dicarboximide-tolerant strains of several genera of fungi vary in pathogenicity as plant pathogens. Isolates of *S. homoeocarpa* (5), *P. expansum* (19) and *M. fructicola* (18) tolerant to procymidone, iprodione, and vinclozolin were mildly pathogenic. Procymidone-tolerant isolates of *S. minor* in this study varied in their ability to colonize peanut tissues. Some tolerant isolates were not pathogenic, some colonized tissues at rates similar to sensitive isolates, and some were more pathogenic than the sensitives isolates (Table 4). This range in pathogenic capabilities of fungicide-tolerant isolates of *S. minor* is important. The most persistent and/or pathogenic types might survive in nature although none were found in a few field assays (14). Sztejinberg and Jones (21) reported that isolates of *Monilinia fructicola* were readily isolated from fields treated with procymidone, iprodione, and vinclozolin. Thus, it is possible that some fungicide-tolerant isolates may be more "parasitically fit" than others. Such isolates may be able to survive in field populations of sensitive isolates. They could become dominant in fields treated continuously with dicarboximide fungicides. This could, subsequently, set the stage for an epidemic of Sclerotina blight in treated peanut fields.

The importance of fungicide mixtures in reducing the development of tolerant strains has been established (4,20). However, selection of an alternate fungicide is critical for effective control strategies. Cross-tolerance to dicloran by fungal isolates resistant to dicarboximide fungicides has been reported (17). Dicloran, a fungicide effective against *S. minor* (1) should not be used as an alternate fungicide to control Sclerotinia blight of peanut. Our data showed that isolates tolerant to procymidone were cross-tolerant to dicloran (Table 2). Therefore, the criteria for selecting alternate fungicides should eliminate fungicides with similar modes of action and favor fungicides that are structurally unrelated. That *S. minor* maintains its tolerance to procymidone, iprodione, vinclozolin and dicloran after 10 weekly transfers to nonamended agar is important (Table 3). Detweiler et al. (5) noted that *S. homoeocarpa* retained its tolerance to iprodione after seven consecutive transfers to nonamended agar over a 4-month period. Thus, it appears that when tolerance develops, it becomes a stable trait of a particular isolate. More importantly, the cross-tolerant nature of tolerant isolates is maintained over this period on nonamended agar. Although the survival rate of fungicide-tolerant isolates under field conditions is unknown, control strategies should not discard the possibility of long-term survival and its effects on disease development.

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