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In Vitro Evaluation of Fungicides Against *Colletotrichum acutatum* Isolates From Strawberry[1]

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Abstract. Benomyl, captan, chlorothalonil, thiram and vinclozolin were tested against five isolates of *Colletotrichum acutatum* from Connecticut using two techniques. Captan, thiram and chlorothalonil inhibited *C. acutatum* conidial germination around fungicide-amended disks containing 100, 100 or 500 µg/ml fungicide, respectively. All of the fungicides tested reduced *C. acutatum* colony diameter when isolates were added to fungicide-amended media. Concentrations of 1 µg/ml or greater for benomyl, 100 µg/ml or greater for captan, thiram and vinclozolin, and greater than 1,000 µg/ml for chlorothalonil reduced colony diameters by 50%. The inhibitory effects of captan, thiram and chlorothalonil were reinforced by zone of inhibition experiments. Connecticut isolates differed in sensitivity to benomyl, which inhibited growth, but did not inhibit conidial germination. Contact with up to 10,000 µg/ml benomyl for 3 days did not kill *C. acutatum* conidia, which continued growth when transferred to unamended media.

Introduction

Anthracnose diseases of strawberry *(Fragaria x ananassa* Duch.) caused by *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* Penz. & Sacc., have limited strawberry production in the southeastern United States and California (5,14). Anthracnose fungi may attack crowns, petioles, leaves, flowers, fruit trusses and fruit (9).

Control of anthracnose fruit and crown rot has been attempted through the use of fungicides, sanitation practices, and the development of plant resistance (3,8,10,12). Benomyl has been widely used for anthracnose control, but benomyl-resistant isolates of *C. fragariae* and *C. acutatum* have been reported (6,12,13,15).

Anthracnose fruit rot ranged in incidence from slight to severe in at least three counties in Connecticut in 1991 (11). All isolations from diseased fruit or stolons yielded *C. acutatum.* The objectives of this research were to determine the efficacy of various fungicides against *C. acutatum* isolates from Connecticut, and further, to determine if isolates differed in tolerance to these fungicides.

Materials and Methods

The five isolates of *C. acutatum* used in these experiments were isolated from anthracnose lesions on green or ripe strawberry fruit or stolons grown in six towns in Hartford, Tolland and New Haven Counties in CT. Cultures were maintained by serial transfer on potato-dextrose agar.

The evaluation of in vitro fungicide efficacy was done using a modified paper-disk method (12) and by incorporation of fungicide into potato dextrose agar (PDA). The fungicides evaluated were benomyl (Benlate 50WP), captan (Captan 50WP), chlorothalonil (Bravo 75W), thiram (Thiram 65WP) and vinclozolin (Ronilan 50W).

For the paper-disk method, conidia of *C. acutatum* isolates were washed from the surface of petri dish cultures using sterile distilled water, filtered through sterile cheese cloth, and 5.0 X 10^5 conidia were spread over the surface of solidified PDA in 9-cm-diameter petri plates using a bent glass rod. Fungicides were serially diluted with sterile distilled water to achieve concentrations of 10,000, 1,000, 100, 10, 1, or 0 µg ai/ml (ppm). Sterile 1.0-cm-diam. analytical paper disks were dipped into appropriate suspensions of each fungicide, blotted and placed onto the surface of PDA seeded with conidia. Cultures were incubated in the dark at 20° C for 3 days. Fungicide inhibition zones were determined by measuring the distance from the edge of the disk to the edge of fungal growth. All treatments were replicated four times and the experiment was repeated once. To determine whether benomyl, thiram and captan were fungicidal or fungistatic, the disks amended with these fungicides were lifted from the media after 3 days and momentarily touched to the surface of unamended PDA plates. Conidia transferred from these disks to PDA were incubated for 3 days at 20°C and resultant colony diameters were measured.

To determine the effect of fungicides on *C. acutatum* growth, 2 mm^2 plugs from PDA cultures were placed in the center of 9-cm-diam. petri dishes containing solidified PDA amended with 1,000, 500, 100, 10, 1, or 0 µg ai/ml of each

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fungicide. Fungicides were added to cool (48-50°C) media just prior to pouring into plates. Colony diameter was measured after 10 days of incubation at 20°C. There were two replicate plates of each isolate/fungicide concentration combination, and the experiment was repeated twice.

The effect of benomyl on *C. acutatum* spore germination and colony growth was investigated by uniformly spreading 0.5 ml of a spore suspension containing 1.0 X 10^2 spores per ml over solidified PDA amended with 1000, 500, 100, 10, 1, or 0 µg/ml benomyl. Numbers of colonies per plate were counted after 7 days incubation at 20°C and colony diameters of five randomly selected, isolated colonies were measured after 7 and 10 days. There were four replicate plates of each benomyl concentration, and the experiment was repeated once. All data were subjected to analysis of variance and means were separated by the protected LSD technique. Inhibition zone data were transformed to ranks prior to analysis (1).

Results

Captan, thiram, and chlorothalonil were effective in inhibiting *C. acutatum* conidial germination and growth around fungicide-amended disks (Table 1). Minimum concentrations of 100, 100, and 500 µg/ml were required for inhibition by captan, thiram and chlorothalonil, respectively. The inhibition zone was greater for thiram than for the other fungicides. Treatment of disks with benomyl and vinclozolin did not result in inhibition zones around amended disks at concentrations up to 10,000 µg/ml. However, the density of fungal growth was reduced around benomyl-amended disks at concentrations of 100 to 10,000 µg/ml.

Isolates of *C. acutatum* varied in the extent of inhibition for the three effective fungicides, and trends were similar for

Table 1. Disk assay for in vitro inhibition of *Colletotrichum acutatum* isolates by fungicides at concentrations of 0 to 1,000 µg/ml after 3 days at 20°C.

Concentration (µg/ml)	Zone of inhibition (mm)[z]				
	benomyl	captan	thiram	vinclozolin	chlorothalonil
1000	0.0[y]	10.0 a[x]	21.0 a	0.0	10.0 a
500	0.0	6.0 b	19.0 b	0.0	3.0 b
100	0.0	3.0 c	18.0 c	0.0	0.0 c
10	0.0	0.4 d	1.0 d	0.0	0.0 c
1	0.0	0.0 d	0.0 d	0.0	0.0 c
0	0.0	0.0 d	0.0 d	0.0	0.0 c
mean (1-1000)	0.0 a	3.3 c	9.7 d	0.0 a	2.0 b

[z] Zone measured as mm from the disk to the edge of fungal growth.
[y] Data the average of five *C. acutatum* isolates; analyzed after rank transformation
[x] Means within columns (within row for mean (1-1000)) followed by the same letter are not significantly different (ANOVA: LSD p=0.05).

Table 2. Disk assay for in vitro inhibition of five isolates of *Colletotrichum acutatum* after 3 days at 20°C.

Isolate	Zone of inhibition (mm)[z]						
	none	benomyl	captan	thiram	vinclozolin	chlorothalonil	mean
1	0.0	0.0[y]	4.1 a[x]	8.4 d	0.0	3.5 b	3.2 a
2	0.0	0.0	3.7 a	10.5 a	0.0	2.5 ab	3.3 a
3	0.0	0.0	2.8 bc	9.5 c	0.0	1.0 a	2.7 a
4	0.0	0.0	2.4 c	10.1 ab	0.0	1.9 a	2.9 a
5	0.0	0.0	3.3 ab	10.0 b	0.0	1.3 a	2.9 a

[z] Zone measured as mm from the disk to the edge of fungal growth.
[y] Data the average of all concentrations tested (1-1000 µg/ml).
[x] Means within columns followed by the same letter are not significantly different (ANOVA: LSD p=0.05).

Table 3. *Colletotrichum acutatum* colony diameter grown in potato dextrose agar amended with 0 to 1,000 µg ai per ml fungicide for 10 days at 20°C.

Concentration (µg/ml)	Colony diameter (cm)				
	benomyl	captan	thiram	vinclozolin	chlorothalonil
1000	1.8[z]a[y]	1.2 a	0.7 a	2.6 a	3.9 a
500	2.1 ab	1.3 a	0.7 a	2.8 a	4.5 b
100	2.2 ab	1.8 a	2.0 b	3.0 a	5.0 cd
10	2.4 b	5.1 b	4.6 c	3.8 b	5.4 cd
1	2.5 b	6.4 c	4.8 c	5.5 c	5.7 d
0	7.1 c	7.1 d	7.1 d	7.1 d	7.1 e

[z] Data the average of five *C. acutatum* isolates.
[y] Means within columns followed by the same letter are not significantly different (ANOVA: LSD p=0.05).

Table 4. Colony diameter of five isolates of *Colletotrichum acutatum* grown on fungicide-amended potato dextrose agar for 10 days at 20°C.

Isolate	Colony diameter (cm)					
	none	benomyl	captan	thiram	vinclozolin	chlorothalonil
1	7.4[z]a[y]	2.9 b	4.1 a	3.6 a	4.8 a	5.4 c
2	7.3 a	4.1 a	3.8 b	3.5 a	4.2 bc	5.6 b
3	6.8 b	2.9 b	3.6 bc	3.0 b	4.0 bc	4.7 d
4	7.4 a	2.7 c	4.2 a	3.6 a	4.3 b	5.9 a
5	6.5 c	2.3 d	3.4 c	2.8 c	3.3 c	4.7 d
mean	7.1 a	3.0 d	3.8 c	3.3 d	4.1 c	5.2 b

[z] Data the average of all fungicide concentrations (1-1000 µg/ml) tested.
[y] Numbers within columns (or within row for fungicide means only) followed by the same letter are not significantly different (ANOVA: LSD p=0.05).

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Table 5. The influence of benomyl-amended media on *Colletotrichum acutatum* spore germination and colony growth after 7 days at 23°C.

Benomyl Concentration (µg/ml)	Number of colonies	Colony diameter (mm)
1000	31.0 a[z]	4.9 a
100	34.3 a	7.8 ab
10	28.0 a	8.8 b
1	22.8 a	9.4 b
0	34.0 a	24.1 c

[z] Means within columns followed by the same letter are not significantly different (ANOVA: LSD p=0.05).

Table 6. Viability of *Colletotrichum acutatum* after 3 days exposure at 23°C to fungicides.

Concentration	Colony diameter (cm)		
µg/ml	benomyl	captan	thiram
10,000	1.7 a[z]	0.0 a	0.0 a
1,000	1.9 b	0.3 a	0.3 b
100	1.9 b	1.5 b	1.3 c
10	1.9 b	2.0 c	1.9 d
1	2.0 b	2.1 c	2.0 d
0	2.0 b	2.1 c	2.0 d

Contrast	Significance level
benomyl vs. captan and thiram	0.001
captan vs. thiram	NS

[z] Means within columns followed by the same letter are not significantly different (ANOVA: LSD p=0.05).

all isolates (Table 2). Six additional isolates were tested against benomyl with similar results (data not shown).

When the same fungicides were incorporated into PDA at concentrations of 0 to 1,000 µg/ml, colony diameter after 10 days at 20°C was reduced for all fungicide-amended media (Table 3). Benomyl and thiram were the most effective fungicides tested in restricting colony diameter, especially at low concentrations. Captan and vinclozolin were less efficacious but had a greater influence on colony growth than did chlorothalonil. While all isolates were inhibited by these fungicides, there was some variation in the degree of that inhibition (Table 4). One isolate, CT-2, exhibited reduced sensitivity to benomyl compared with the other isolates tested.

Benomyl concentration in agar did not affect conidial germination and number of colonies after 4 days at 23°C (Table 5). Colony diameter was reduced at all benomyl concentrations tested compared to unamended agar, and inhibition of growth was positively correlated with benomyl concentration.

Survival of *C. acutatum* conidia exposed to concentrations of 0 to 10,000 µg/ml benomyl, captan or thiram on paper disks for 3 days prior to transfer to unamended media was unaffected by benomyl, but concentrations of 1,000 or 10,000 µg/ml captan and thiram reduced viability and colony diameter (Table 6).

Discussion

Benomyl, captan and thiram fungicides were effective against *C. acutatum* in 'in vitro' tests. The effective concentrations of these fungicides varied somewhat among isolates, and with the exception of decreased sensitivity to benomyl by isolate CT-2, there was no significant pattern of variation.

Colletotrichum acutatum isolates were tested against fungicide concentrations of 0 to 1,000 µg/ml in agar and 0 - 10,000 ug/ml using the paper disk technique. The two techniques evaluated different properties of these fungicides. The paper disk method assessed the effect of the fungicide on conidial survival, germination and subsequent fungal growth. The incorporation of fungicides into agar and subsequent inoculation by a mycelial plug evaluated inhibition of fungal colony growth. Both techniques may measure fungistatic as well as fungicidal effects. Because the mode of action of benomyl has been demonstrated to repress cell division (16), the lack of an inhibition zone around paper disks with benomyl reflects the insensitivity of *C. acutatum* conidial germination to benomyl. Delp (3) demonstrated that 24 hr exposure to 20 µg/ml benomyl for 3 days did not affect the germination of *C. fragariae* isolates, compared to a complete inhibition of germination after exposure to 1.0 µg/ml captan. In our experiments, benomyl at concentrations of up to 1,000 µg/ml in agar slowed but did not stop conidial germination. While there was no clear inhibition zone around benomyl-amended disks, concentrations of 100 to 10,000 µg/ml resulted in zones of reduced densities of fungal growth.

Colletotrichum acutatum conidia in contact with paper disks containing 100 to 10,000 µg/ml thiram or captan for 3 days were killed as demonstrated by their failure to grow when transferred to unamended PDA. Conidia in contact with disks at lower concentrations were not killed. The fungistatic, rather than fungicidal, effect of benomyl was demonstrated when conidia in contact with disks containing 10,000 µg/ml benomyl for 3 days produced colonies when transferred to unamended PDA.

All five fungicides tested reduced *C. acutatum* colony diameter when isolates were added to fungicide-amended media. While statistically significant, this reduction in growth was often small. Colony diameters less than 50% of those on unamended media were obtained at concentrations of 1 µg/ml or greater for benomyl and 100 µg/ml or greater for captan, thiram and vinclozolin. Chlorothalonil at 1,000 µg/ml resulted in a 45% reduction in colony diameter. Benomyl, captan, thiram and vinclozolin at 1,000 µg/ml resulted in reductions of 75%, 83%, 90%, and 63%, respectively.

Label rates of fungicide in 1,900 liters of water per hectare (approximate amounts required for application to strawberries) may result in concentrations of up to 600 µg/ml benomyl; 3,600 µg/ml captan; 3,900 µg/ml thiram; and 900 µg/ml vinclozolin. By extrapolation, the most effective fun-

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gicides against *C. acutatum* at recommended spray concentrations were thiram, followed by captan, benomyl and vinclozolin. Benomyl was the most effective fungicide at lower concentrations of 1 to 10 µg/ml.

Benomyl fungicides have become ineffective against strawberry anthracnose in other production areas (6,7,13). Benomyl-resistant isolates of *C. fragariae* and *C. acutatum* have been defined by insensitivity to up to 5 u/ml benomyl (B. Smith pers. comm.). Previous research has shown that benomyl was not effective in reducing conidial germination, but restricted mycelial growth of *C. gloeosporioides* (6).

Isolates of *C. acutatum* from strawberry in Connecticut varied in growth rate, color (some isolates produced red pigments in culture), and sensitivity to fungicides such as benomyl. C. *acutatum* isolate CT-2 was less sensitive to a number of benomyl concentrations than were the other isolates tested. However, growth was still significantly inhibited by low concentrations (1 µg/ml) of benomyl. Dekker (2) defined acquired resistance to fungicides as decreased sensitivity exhibited by an isolate, when compared to a normally sensitive population. This isolate may be characterized as having decreased sensitivity to benomyl. The practical result of this variation on strawberry infection, and the future ability of benomyl fungicides to control anthracnose in Connecticut is currently unknown.

Fields severely affected by anthracnose in Connecticut in 1991 were not treated with benomyl, so its efficacy in the field was not determined. It appears from these data that benomyl is fungistatic rather than fungicidal to Connecticut isolates. Current IPM recommendations for one or two applications of benomyl at bloom to control gray mold may not be sufficient for anthracnose control. Howard (10) indicated that seven benomyl applications were necessary for sufficient levels to accumulate in strawberry plants to control anthracnose.

In Connecticut, benomyl fungicides have been typically applied in combination with fungicides such as captan. These fungicides, with different modes of action, have been used in an effort to prevent the development of benomyl resistance in *Botrytis cinerea.* In addition, fungicide resistance develops more slowly in pathogens with fewer infection cycles per season (4). The cool environmental conditions common in Connecticut which reduce anthracnose severity may also act to retard the development of benomyl resistance. In any case, the baseline sensitivity of *C. acutatum* populations in Connecticut needs to be further determined in order to detect shifts in sensitivity to benomyl which may occur with continued use.

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