Inhibition with Benomyl to Growth *In Vitro* of *Colletotrichum acutatum* and *C. fragariae* and Strawberry Fruit Infection by Benomyl-Resistant Isolates of *Colletotrichum acutatum*.[1]

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Abstract. The effects of benomyl fungicide on five isolates of *Colletotrichum acutatum* and two isolates of *C. fragariae* were determined *in vitro.* Benomyl did not inhibit conidial germination of any isolate at concentrations of up to 10,000 ppm. Germinated conidia in contact with benomyl-amended disks for 4 days resumed growth after transfer to unamended media. The growth of five C. *acutatum* isolates on benomyl-amended PDA (1 to 1,000 ppm) ranged from 23 to 62 percent of growth on unamended PDA. Growth of two *C. fragariae* isolates on amended media ranged from 1 to 8 percent of unamended media. Benomyl and/or captan application to detached fruit delayed development of anthracnose for 8 days after inoculation for benomyl and captan delayed development for 12 days after inoculation. Captan was more effective in delaying anthracnose development than benomyl and combining both fungicides did not increase efficacy over captan alone. *Colletotrichum* isolates were separated into three benomyl tolerance classes (sensitive, intermediate and tolerant) based on percent growth on benomyl amended agar compared to growth on unamended agar.

Introduction

Anthracnose diseases of strawberry (*Fragaria x ananassa* Duch.), caused by *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* Penz. & Sacc., can infect fruit, crowns, petioles, leaves, flowers, and fruit trusses (5,10,16). These diseases cause severe fruit rot epidemics and kill plants in production fields. *C. acutatum* has become the most important species in the northern United States, primarily as a fruit pathogen (12,19).

Anthracnose disease control has been attempted through the use of fungicides, sanitation practices, and the development of plant resistance (3,9,11,13). Benomyl fungicide has been widely used on strawberry for anthracnose and gray mold control, but benomyl-resistant isolates of *C. fragariae* and *C. acutatum* have been reported (6,13,15,17). Benomyl, captan and thiram fungicides were effective against Connecticut isolates of *C. acutatum* in in vitro tests. Although some isolates had reduced sensitivity to benomyl, the extent of resistance was not determined (12).

The objectives of this research were 1) to determine the effects of benomyl fungicide on conidial germination, survival and growth of *C. acutatum* isolates; 2) to compare Connecticut isolates of *C. acutatum* with other benomyl susceptible, intermediate or resistant isolates of *C. acutatum* and *C. fragariae;* and 3) to determine the effect of benomyl or captan fungicides alone or in combination on anthracnose fruit rot of strawberry.

Materials and Methods

The Connecticut isolates of *C. acutatum* used in these experiments were isolated from anthracnose lesions on ripe (CT-1) or green (CT-2) strawberry fruit or stolons (CT-9) grown in New Haven (CT-1), Hartford (CT-2) and Tolland (CT-9) Counties in Connecticut. Isolate CT-1 is chromogenic and may be part of a distinct group within *C. acutatum* (8). Cultures were maintained by serial transfer on potato-dextrose agar. Two *C. acutatum* isolates described as benomyl resistant (Mil-1) or intermediate (CF-167) in response to benomyl, and two *C. fragariae* isolates intermediate (CF-Card) or sensitive (La-1) to benomyl were obtained from Barbara Smith of the USDA ARS Small Fruit Research Station in Poplarville, MS.

The evaluation of *in vitro* fungicide efficacy was done using a modified paper-disk method (12,13), by incorporating benomyl fungicide into potato-dextrose agar (PDA), and by dipping detached fruit into benomyl (Benlate 50WP) or captan (Captan 50WP) fungicides prior to inoculation.

For the paper-disk method, conidia of *C. acutatum*

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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-diam. petri plates using a bent glass rod. Benomyl fungicide was serially diluted with sterile distilled water to concentrations of 10,000, 1,000, 100, 10, 1, or 0 ppm (µg ai/ml). 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 4 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. To determine whether benomyl was fungicidal or fungistatic, conidia were transferred from amended disks to new, unamended plates. To do this, the fungicide-amended disks, with adhering conidia, were lifted from the media after 4 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 growth was evaluated. Strong fungal growth over the complete area of the disk was rated as (++), growth over an incomplete portion of the disk was rated (+), and no growth was rated as (-).

To determine the effect of benomyl fungicide concentration 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 ppm (µg ai/ml) of benomyl (12). Aqueous suspensions of fungicide were added to cool (48–50°C) media just prior to pouring into plates. Colony diameters were measured after 5 days of incubation at 21°C and 7 days at 25°C. To eliminate differences in isolate growth rates, growth on benomyl-amended agar was normalized by dividing by growth on unamended agar. There were two or three replicate plates of each isolate/fungicide concentration combination, and the experiment was performed three times.

To evaluate the effects of benomyl and/or captan fungicides on fruit disease, detached green fruit ('Honeoye', approximately 2.5 cm diam., 4 to 5 g) which had not been exposed to fungicides were dipped into fungicide suspensions and inoculated with conidia of isolates CT-1, CT-9 or Mil-1. Fungicide treatments consisted of 100 ppm benomyl; 500 ppm benomyl; 500 ppm captan; 1000 ppm captan; 100 ppm benomyl plus 500 ppm captan; 500 ppm benomyl plus 500 ppm captan; and a sterile distilled water control. Berries with a flat surface suitable for drop-inoculation were selected and dipped into the appropriate fungicide treatment, placed on a moist paper towel in an open plastic bag and allowed to air dry. After drying, one drop of a 7.0 X 10^3 conidial suspension (approximately 230 conidia) of isolates CT-1, CT-9 or Mil-1 was placed on 10 berries in each fungicide treatment. Inoculum was produced from cultures grown on PDA at 25°C for 5 days. After 30 minutes, additional water was added to saturate the paper towel, and each bag that contained 10 fruit (one isolate per fungicide combination) was closed with a twist-tie. Berries were examined for anthracnose development after 8 or 12 days at 21°C and the number of symptomatic fruit was recorded. The experiment was performed twice; each experiment served as a replicate. Data were arcsine transformed to stabilize variance prior to analysis by ANOVA and linear contrasts of the means.

Results

Benomyl did not inhibit conidial germination of any of the *C. acutatum* or *C. fragariae* isolates tested (Table 1). Subsequent growth after germination was reduced in an 'inhibition zone' for *C. acutatum* isolates CT-1, CT-2, and CF-167 as well as for *C. fragariae* isolates CF-Card and La-1. CT-9 and Mil-1 were not affected by disk concentrations of up to 10,000 ppm. Germinated conidia in contact with benomyl-amended disks for 4 days resumed growth after transfer to new PDA plates (Table 1). *C. fragariae* isolate La-1 was the only isolate that grew poorly in contact with benomyl, and then only at 10,000 ppm.

The growth of *C. acutatum* on benomyl-amended agar (1 to 1,000 ppm) ranged from 23 to 62 percent of growth on unamended agar (Table 2). Isolates CT-9, CT-2 and Mil-1 were the most tolerant of benomyl, and the two *C. fragariae* isolates were the most sensitive isolates tested. Regression of normalized growth and benomyl concentration illustrated a negative relationship between concentration and growth for all isolates, but the slopes of the regression lines were low.

Benomyl application reduced fruit anthracnose for 8 days after inoculation and captan reduced disease for 12 days after inoculation (Table 3). Captan was more effective in reducing anthracnose than benomyl, and the combination of benomyl plus captan was not more effective than captan alone. The application of benomyl plus captan was more effective than benomyl alone.

Discussion

Colletotrichum isolates were tested against fungicide concentrations of 0 to 1,000 ppm in agar and 0 - 10,000 ppm 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 germination, survival, and subsequent fungal growth. The incorporation of fungicides into agar evaluated inhibition of fungal colony growth from mycelial plugs. Both techniques may measure fungistatic as well as fungicidal effects. Because the mode of action of benomyl has been demonstrated to repress cell division (2,18), the lack of a clear inhibition zone around paper disks amended with benomyl reflected the insensitivity of *C. acutatum* conidial germination to this fungicide. Delp (3) and LaMondia (12) demonstrated that exposure to 20 ppm or 10,000 ppm benomyl for 3 days did not affect the germination of *C. fragariae* iso-

Table 1. Response of *Colletotrichum acutatum* and *C. fragariae* isolates to benomyl-treated paper disks placed on agar seeded with conidia.

Isolate[z]	Benomyl Concentration (ppm) Inhibition zone (mm)[y] and growth rating of transferred conidia[x]											
	10,000		1,000		100		10		1		0	
CT-1	2.0	++	2.0	++	1.1	++	0.2	++	0.0	++	0	++
CT-2	1.0	++	0.8	++	0.6	++	0.3	++	0.0	++	0	++
CT-9	0.0	++	0.0	++	0.0	++	0.0	++	0.0	++	0	++
Mil-1	0.0	++	0.0	++	0.0	++	0.0	++	0.0	++	0	++
CF-167	0.9	++	1.2	++	0.8	++	0.2	++	0.0	++	0	++
CF-Card	2.5	++	1.9	++	1.3	++	0.4	++	0.0	++	0	++
La-1	2.5	+	2.3	++	2.0	++	0.5	++	0.0	++	0	++

[z] Isolates CT-1 through CF-167 = *C. acutatum,* isolates CF-Card and La-1 = *C. fragariae.*
[y] Conidia of all isolates germinated but subsequent growth was reduced in the inhibition zone.
[x] Growth rating of conidia in contact with the benomyl-amended disk transferred to unamended potato dextrose agar: ++ = strong growth; + = weak growth; - = no growth.

Table 2. The effect of benomyl concentration on growth of *Colletotrichum acutatum* and *C. fragariae* isolates on fungicide-amended agar.

Isolate[z]	Normalized colony growth (%) Benomyl Concentration (ppm)					Control Growth(mm)
	1000	500	100	10	1	0 ppm
CT-1	23.0	24.0	30.6	32.0	38.1	43.0
CT-2	40.2	42.8	44.6	42.3	53.5	20.4
CT-9	46.0	50.0	57.0	58.8	61.7	33.6
Mil-1	32.5	40.3	40.8	39.2	41.3	30.6
CF-167	26.3	25.7	32.9	34.6	38.1	45.5
CF-Card	1.1	2.2	1.8	4.3	7.0	52.6
La-1	2.4	2.5	2.2	3.9	8.3	54.5

Source of Variation	Prob. > F
Isolate	0.001
Concentration	0.001
Interaction	0.940

Regression of normalized growth

Isolate	Regression equation	R^2	P
CT-1	33.5 - 0.012(ppm)	0.38	0.02
CT-2	47.0 - 0.007(ppm)	0.12	0.20
CT-9	59.4 - 0.015(ppm)	0.56	0.01
Mil-1	41.2 - 0.007(ppm)	0.14	0.18
CF-167	35.0 - 0.011(ppm)	0.52	0.01
CF-Card	4.5 - 0.004(ppm)	0.22	0.08
La-1	4.9 - 0.003(ppm)	0.08	0.29

[z] Isolates CT-1 through CF-167 = *C. acutatum,* isolates CF-Card and La-1 = *C. fragariae.*

Table 3. The effect of fungicide dips on strawberry fruit infection by three isolates of *Colletotrichum acutatum* 8 and 12 days after inoculation.

Fungicide (ppm)	Isolate	Proportion of fruit with anthracnose[z] 8 days	12 days
None (0)	CT-1	0.80	0.95
	CT-9	0.80	0.85
	Mil-1	1.00	1.00
Benomyl (100)	CT-1	0.55	0.95
	CT-9	0.40	0.75
	Mil-1	0.75	1.00
Benomyl (500)	CT-1	0.45	0.85
	CT-9	0.40	0.85
	Mil-1	0.90	0.95
Captan (500)	CT-1	0.00	0.30
	CT-9	0.10	0.40
	Mil-1	0.20	0.55
Captan (1000)	CT-1	0.10	0.25
	CT-9	0.10	0.30
	Mil-1	0.05	0.25
Benomyl (100) plus Captan (500)	CT-1	0.25	0.35
	CT-9	0.20	0.45
	Mil-1	0.20	0.60
Benomyl (500) plus Captan (1000)	CT-1	0.10	0.10
	CT-9	0.10	0.45
	Mil-1	0.10	0.65

Source of Variation	Prob. > F	
Fungicide	0.001	0.001
Isolate	0.04	0.16
Interaction	0.60	0.98

Linear Contrasts	Prob. > F	
1. Benomyl vs. no fungicide	0.005	NS
2. Captan vs. no fungicide	0.001	0.001
3. Benomyl vs. captan	0.001	0.001
4. Benomyl 100ppm vs. benomyl 500ppm	NS	NS
5. Captan 500ppm vs. captan 1000ppm	NS	NS
6. Captan vs. benomyl plus captan	NS	NS

[z] Number of 10 fruit with anthracnose per experiment, means of two replicate experiments.

lates, compared to a complete inhibition of germination after exposure to 1.0 ppm captan. In our experiments, benomyl at concentrations of up to 1,000 ppm in agar slowed but did not stop conidial germination. While there was no clear inhibition zone around benomyl-amended disks, concentrations of 10 to 10,000 ppm resulted in zones of reduced densities of fungal growth.

Benomyl fungicide allows the germination of *Colletotrichum* conidia (3,6,12) and was not fungicidal for several *C. acutatum* isolates at 10,000 ppm, 16 times greater than the field application rate. Label rates of benomyl in 1,900 liters of water per hectare (approximate amounts required for application to strawberries) may result in application concentrations of up to 600 ppm benomyl (12).

Connecticut isolates of *C. acutatum* appeared to be similar in response to isolates CF-167 and Mil-1 described as intermediate and resistant to benomyl, respectively (17). All of the *C. acutatum* isolates tested were more tolerant of benomyl than the *C. fragariae* isolates CF-Card and La-1.

Colletotrichum isolates can be separated into benomyl tolerance classes by normalized growth at concentrations of 1 ppm to 500 ppm (field exposure levels). Benomyl sensitive isolates exhibit 0 to 20% growth; intermediate isolates range from 20 to 40% growth and benomyl tolerant (resistant) isolates have growth > 40% of growth on unamended media. Using this scheme, *C. fragariae* isolates CF-Card and La-1 are sensitive to benomyl, *C. acutatum* isolates CT-1 and CF-167 are intermediate in response, and *C. acutatum* isolates CT-2, CT-9, and Mil-1 are tolerant of benomyl.

Smith and Black (17) defined isolate Mil-1 as resistant, isolate La-1 as sensitive, and isolate CF-Card as intermediate. These groupings are consistent with the exception of isolate CF-Card. The difference in results may be due to the different assays used. Smith and Black evaluated growth as a visual rating of conidial suspension growth on the agar surface. In our experiments, germination and growth of isolate CF-Card around benomyl-amended disks were reduced more than growth on benomyl-amended media.

The low slope of the regression line between normalized growth and benomyl concentration for all isolates was unexpected and suggests that the sensitivity of a particular isolate is much more important than benomyl concentration.

Also of interest was the observation that benomyl resistant *C. acutatum* isolates had somewhat slower growth on unamended PDA plates than intermediate isolates. Future research should examine the fitness of benomyl resistant isolates to determine the stability of this trait in populations over time.

While captan was more effective than benomyl in reducing anthracnose of strawberry fruit, which is consistent with previous observations (1,7,12), benomyl application appeared to confer some protection to fruit up to 8 days after inoculation. Benomyl may slow the rate of growth, and presumably infection, after conidial germination, even under the favorable infection conditions that were utilized. Because the isolates that were tested continue to grow, although at a slower rate than in the absence of benomyl, the disease eventually developed and the protective effect of benomyl was not apparent 12 days after inoculation.

The level of tolerance to benomyl fungicide displayed by the *C. acutatum* isolates tested in these experiments was higher than that reported for *C. gloeosporioides* affecting mango despite repeated application of benomyl in Florida (14). Field (9,13) and laboratory (15,17) observations of benomyl resistance as well as the apparent cross-resistance with related fungicides and the widespread nature of resistance in *C. acutatum* (15) indicate that benomyl should be used sparingly and in combination with other fungicides with different modes of action (4).

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