DCNA-Benomyl Multiple Tolerance in Strains of *Botrytis cinerea*

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ABSTRACT

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DCNA (2,6-dichloro-4-nitroaniline)-benomyl multiple-tolerant strains of *Botrytis cinerea* were obtained by plating conidia derived from benomyl-tolerant isolates of *B. cinerea* onto potato-dextrose agar (PDA) plates amended with 100 μg of DCNA per milliliter. Colonies of the DCNA-benomyl multiple-tolerant strains grown on PDA were symmetrical with unusually tough mycelial mats. Growth rates of these multiple-tolerant strains on PDA were about half that of the benomyl-tolerant isolates. The ED_{50} for reduction of mycelial growth as measured by colony diameter ranged from 0.8 to 1.4 μg of DCNA per milliliter for the isolates tolerant to benomyl only but was 3.9 to 88.8 μg of DCNA per milliliter for the strains tolerant to both fungicides. The multiple-tolerant strains and benomyl-tolerant strains both were able to grow at 1,000 μg of benomyl per milliliter. Strains tolerant to both fungicides consistently produced fewer conidia than the isolates tolerant only to benomyl. Conidial germination of multiple-tolerant strains on PDA amended with either 10 μg of DCNA or 10 μg of DCNA plus 10 μg of benomyl per milliliter ranged from 48.7 to 92.3% after 12 hr at 20 ± 1 C. Germ tubes of multiple-tolerant strains, after 12 hr at 20 ± 1 C, on PDA amended with either 10 μg of DCNA or 10 μg of DCNA plus 10 μg benomyl per milliliter were less than half the length of those produced on PDA alone or PDA amended with 10 μg of benomyl per milliliter. All multiple-tolerant strains tested produced sclerotia on PDA and decayed DCNA or DCNA plus benomyl-treated peach fruit. Multiple-tolerant strains were pathogenic on tomato stems, but the lengths of stem colonized were less than that of the parent benomyl-tolerant strains.

Additional key words: Benzimidazole, Botran, cross tolerance, dichloran, resistance.

Benomyl tolerance in isolates of *Botrytis cinerea* Pers. from fields sprayed with benomyl has been common (2,7–9), but 2,6-dichloro-4-nitroaniline (DCNA)-tolerant *B. cinerea* has been reported only from laboratory tests (7,10). Neither DCNA-tolerant nor benomyl-tolerant isolates have shown tolerance to both DCNA and benomyl (6,8–10). Geeson (5) recently reported the selection of DCNA-carbendazim tolerant strains of *B. cinerea* after UV-irradiation of conidia from a DCNA-tolerant selection from a carbendazim-sensitive field isolate.

We selected DCNA-benomyl (multiple) tolerant strains of *B. cinerea* from benomyl-tolerant isolates and compared the strains tolerant to both compounds with the parent isolates that are tolerant only to benomyl for spore germination, growth, sporulation, sclerotial production, pathogenicity, and ability to tolerate the fungicides and to decay fruits treated with these fungicides.

MATERIALS AND METHODS

Selection of DCNA-benomyl multiple tolerance. Conidia from eight single-spored isolates of *B. cinerea* were tested for tolerance to DCNA. Five isolates were from tomato (3F5T, 9L8T, 6S14T, 9B3T, and 3L6T), two from strawberry (HR75T and 3-75T), and one from chrysanthemum (C75T). Botran 75W (DCNA, 2,6-dichloro-4-nitroaniline, Tuco, Division of the Upjohn Co., Kalamazoo, MI 49001) and/or Benlate 50W (benomyl, E. I. duPont de Nemours and Company, Wilmington, DE 19898) were used in all experiments. DCNA-amended and/or benomyl-amended potato-dextrose agar (PDA) were prepared by adding DCNA and/or benomyl per liter of PDA before autoclaving at 1.05 kg/cm^2 and 121 C for 15 min. For inoculation on PDA amended with 100 μg of DCNA per milliliter, sterile water was added to 10-day-old cultures of each isolate grown on PDA slants. Conidial suspensions were filtered through four layers of sterile cheesecloth. Approximately 2.0×10^7 conidia per isolate were added to each of three plates. After inoculated plates were incubated at 21 ± 2 C for 6 days, mycelium from colonies was transferred to PDA slants before testing for multiple tolerance. Four suspected multiple-tolerant strains, designated 9B3T-1T, HR75T-2T, C75T-2T, and 9L8T-1T, from the benomyl-tolerant isolates 9B3T, HR75T, C75T, and 9L8T, respectively, were tested further.

Growth of benomyl and DCNA-benomyl multiple-tolerant strains on PDA. The four suspected multiple-tolerant strains and the four benomyl-tolerant isolates from which they were derived were tested for their ability to grow on PDA, PDA amended with 10^0, 10^1, 10^2, and 10^3 μg of benomyl; 10^0, 10^1, 10^2, and 10^3 μg of DCNA; and 10^0, 10^1, 10^2, and 10^3 μg of DCNA plus 10^0, 10^1, 10^2, and 10^3 μg of benomyl per milliliter of PDA. Three plates of each test medium were centrally inoculated with a 4-mm diameter mycelial plug from the edge of a 3-day-old culture of each strain or isolate grown on PDA. Inoculated plates were incubated at 21 ± 2 C.

Sporulation. Four-milliliter diameter mycelial plugs from the edge of 3-day-old cultures of each strain or isolate were used to centrally inoculate five 60 × 15 mm plastic petri plates containing PDA. Inoculated plates were placed in an incubator at 22 ± 1 C with a 24-hr light source (one fluorescent lamp, F48T10CW, General Electric, 2,700 lux). After 12-day incubation, conidia were collected from each plate by shaking the entire colony in a sterile 250-ml flask with 50 ml of water. The conidia were counted using a hemacytometer.

Germination, germ tube growth, and sclerotial production. Conidia from the multiple-tolerant strains and the benomyl-tolerant isolates were tested for their ability to germinate on PDA, PDA amended with 10 μg of DCNA, 10 μg of benomyl, and 10 μg of DCNA plus 10 μg benomyl per milliliter. Each plate was inoculated with 2.5×10^5 conidia obtained from the sporulation tests. Inoculated plates were incubated at 20 ± 1 C in the dark. The percent germination was obtained by counting four sets of 100 conidia per test medium 12 hr after inoculation. The length of germ tubes was determined from four sets of 10 conidia per test medium.

The ability of the multiple-tolerant strains to produce sclerotia was determined by inoculating four 90-mm diameter petri plates containing PDA with 2.5×10^5 conidia from each strain or isolate.

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The inoculated plates were incubated in darkness at 20 ± 1 C. The number of sclerotia (immature and mature) produced on the surface of the agar (not those produced around the edge of the plate) was determined after 3-wk incubation.

Ability of multiple-tolerant strains to rot DCNA and/or benomyl-treated fruit. The ability of the multiple-tolerant strains to decay DCNA-treated fresh peach fruit was tested using cultivar Rio Oso Gem after a 15-min dip in water containing, per milliliter, 900 μg of DCNA, 300 μg of benomyl, or 900 μg of DCNA plus 300 μg of benomyl. After the dipped fruits were air-dried, four fruits per treatment were inoculated by placing a drop of a spore suspension (5.0×10^5 conidia per milliliter) of benomyl- and DCNA-sensitive isolate 39 from tomatoes, benomyl-tolerant isolate 9B3T or DCNA-benomyl multiple-tolerant strain 9B3T-1T on an injury made with the point of a sterile 2-mm diameter glass rod. Inoculated fruit were incubated in covered plastic boxes on wire racks over water at 20 ± 2 C. Lesion diameters were measured after 5 days. DCNA residues on treated fruits were determined after extraction in benzene using a ^{63}Ni electron capture detector-equipped gas chromatograph (3). The detector, injector, and oven temperatures were 200 C with a nitrogen flow of 40 ml/min through a 1,830 × 2 mm glass column packed with 10% OV 101 AW-DCMS treated on chromosorb W-100-120 mesh.

Pathogenicity of DCNA-benomyl multiple-tolerant and benomyl-tolerant strains on tomato stems. The pathogenicity of DCNA-benomyl multiple-tolerant strains 9B3T-1T and HR75T-2T obtained from benomyl-tolerant isolates 9B3T and HR75T on tomato stems was determined as follows: Tomato cultivar Ace 55 stems from 12-wk-old plants were harvested, deleafed, and surface-sterilized by immersion in a 1:10 dilution of 5.25% sodium hypochlorite and water for 2 min. Stem sections 8 cm long were cut from between the first and seventh true leaf (10–12 true leaves per plant). Stem sections were placed upright in plastic boxes with 2.5 cm of moist sterile sand in the bottom. Five stem sections were inoculated with 4-mm diameter mycelial plugs of each isolate or strain grown on PDA for 3 days at 21 ± 2 C. After inoculation, the plastic boxes were sealed and incubated at 21 ± 2 C for 9 days.

RESULTS

Selection of DCNA-benomyl multiple tolerance. After 6-day incubation, one colony developed from isolates 3-75T and 9L8T, two colonies developed from isolate 9B3T, three colonies developed from isolate C75T, and 13 colonies developed from isolate HR75T. Three isolates (3L6T, 6S14T, and 3F5T) failed to produce conidia able to germinate and grow on PDA amended with 100 μg DCNA per milliliter.

Growth of benomyl- and DCNA-benomyl multiple-tolerant strains on PDA. Benomyl-tolerant isolates and DCNA-benomyl multiple-tolerant strains grew on PDA amended with 10^3 μg of benomyl per milliliter. The ED_{50} for inhibition of radial growth on PDA by benomyl for these multiple-tolerant strains ranged from 416 to 751 μg of benomyl per milliliter. Only the DCNA-benomyl multiple-tolerant strains grew on PDA amended with DCNA or DCNA plus benomyl when the concentration of DCNA in the media was 10 μg/ml or greater (Table 1). Although the growth of these strains was resticted, they were able to grow on PDA amended with 10^3 μg of DCNA per milliliter.

On PDA the linear growth of three DCNA-benomyl multiple-tolerant strains was less than that of the parent benomyl-tolerant isolates (Table 2). As reported by Webster et al (10) for DCNA-tolerant strains, the colonies of the multiple-tolerant strains were surrounded by a clear halo extending about 2 mm from the margin of the colony. In addition these colonies were symmetrical with a dense, tough mat of mycelium that was appressed to the surface of the agar. To demonstrate the toughness, a 9-mm diameter cork borer affixed to the compression head of a Hunter Spring mechanical force gage (Model LKG-1, Ametek, Hunter Spring Division, Hatfield, PA 19440) mounted on a U.C. firmness tester (Western Industrial Supply, Inc., San Francisco, CA 94107) was used. The pressure required to cut through the mycelium 1.5 cm inward from the edge of the colony was measured. Increased pressure was needed to cut through the mycelial mat of the DCNA-benomyl multiple-tolerant strains compared with the benomyl-tolerant isolates from which they were obtained (Table 2).

The DCNA-benomyl multiple-tolerant strains retained their ability to grow on PDA amended with 10 μg of DCNA after six mycelial transfers onto fungicide-free PDA over an 18-mo period.

Sporulation and sclerotia production. Sporulation by the DCNA-benomyl multiple-tolerant strains was reduced compared with that of the parent benomyl-tolerant isolates. Two DCNA-benomyl multiple-tolerant strains equaled their parents in production of sclerotia. Strains HR75T-2T and C75T-2T produced sclerotia under the incubation conditions, but their parent benomyl-tolerant isolates did not (Table 2).

Germination and germ tube growth. More than 95% of the conidia of benomyl-tolerant isolates and DCNA-benomyl multiple-tolerant strains germinated after 12 hr on PDA or PDA amended with 10 μg of benomyl. Only the conidia of DCNA-benomyl multiple-tolerant strains germinated on PDA that contained 10 μg of DCNA per milliliter. The percent germination of conidia from strains HR75T-2T, 9L8T-1T, and C75T-2T on PDA amended with DCNA was less (49–79% germination) than that on PDA alone or PDA amended with benomyl. However, spore germination on PDA amended with 10 μg of DCNA or 10 μg of DCNA plus 10 μg of benomyl per milliliter for strains HR75T-2T, 9L8T-1T, and C75T-2T increased to at least 90% after 24 hr. Germinated conidia from the DCNA-benomyl multiple-tolerant strains, except 9B3T-1T on PDA and PDA amended with 10 μg of

TABLE 1. Effect of DCNA and/or benomyl on mycelial growth of fungicide tolerant *Botrytis cinerea* on potato-dextrose agar

Isolates or strains	ED_{50} (μg/ml)[a]		
	Benomyl	DCNA	DCNA and benomyl
9B3T[b]	457.8	1.3	0.8
9B3T-1T[c]	751.7	66.6	9.8
HR75T[b]	492.6	1.4	0.9
HR75T-2T[c]	617.6	88.8	61.0
C75T[b]	419.2	0.8	0.8
C75T-2T[c]	416.6	3.9	2.6
9L8T[b]	469.4	0.9	0.8
9L8T-1T[c]	644.8	55.0	7.3

[a] ED_{50} values were determined from colony diameter measurements after 60 hr at 21 ± 2 C.
[b] Benomyl-tolerant parent isolate.
[c] DCNA-benomyl multiple-tolerant daughter strain.

TABLE 2. Growth, toughness of mycelial mats, sporulation, and sclerotial production by parent benomyl-tolerant isolates and DCNA-benomyl multiple-tolerant daughter strains of *Botrytis cinerea*

Isolate or strain	Colony diameter[a] (mm)	Pressure[b] (g)	Conidia per cm²[c] (×10³)	Sclerotia per plate[d]
9B3T[e]	72.7	92.0	2,600	13.8
9B3T-1T[f]	41.0	200.5	1,200	11.6
HR75T[e]	79.0	106.0	1,900	0.0
HR75T-2T[f]	31.0	272.0	73	67.0
C75T[e]	70.7	86.0	3,000	0.0
C75T-2T[f]	67.0	171.0	290	10.3
9L8T[e]	70.7	102.0	3,300	34.5
9L8T-1T[f]	37.0	647.0	27	30.0

[a] Average of three replications after 60-hr growth on potato-dextrose agar (PDA) at 21 ± 2 C.
[b] Pressure needed to cut the surface of the colony growing on PDA at 22 ± 2 C with a 9-mm diameter cork borer 1.5 cm from the leading edge. Pressure was determined using a Hunter Spring mechanical force gage. Average of five replications per isolate or strain.
[c] Average of five replications per isolate or strain. Conidia produced on PDA after 12-day incubation at 22 ± 1 C with a 24-hr light source.
[d] Average of four replications per isolate or strain. The number of sclerotia produced on the surface of the agar after 3-wk incubation at 20 ± 1 C.
[e] Benomyl-tolerant parent isolate.
[f] DCNA-benomyl multiple-tolerant daughter strain.

benomyl per milliliter, had longer germ tubes than the parent benomyl-tolerant isolates after 12 hr (Table 3). The germ tube lengths of conidia from the DCNA-benomyl multiple-tolerant strains on PDA amended with 10 μg of DCNA or 10 μg of DCNA plus 10 μg of benomyl per milliliter were less than half those on PDA or PDA amended with 10 μg of benomyl per milliliter only (Table 3).

Ability of DCNA-benomyl multiple-tolerant strains to rot DCNA- and/or benomyl-treated fruit. Postharvest treatment of peaches with benomyl, DCNA, and DCNA plus benomyl, or DCNA and DCNA plus benomyl significantly reduced the decay caused by the benomyl-sensitive (39) and DCNA-sensitive and benomyl-tolerant isolate 9B3T of *B. cinerea*, respectively, but these treatments did not protect against the decay caused by the DCNA-benomyl tolerant strain 9B3T-1T (Table 4). The fruit dipped in DCNA had a residue of 17.4 μg of DCNA per gram; fruit dipped in DCNA-benomyl had a residue of 12.9 μg DCNA per gram.

Pathogenicity of benomyl-tolerant and DCNA-benomyl-tolerant strains. Both DCNA-benomyl multiple-tolerant strains 9B3T-1T and HR75T-2T were pathogenic on the tomato stems, but the average length of stem colonized by them was significantly smaller than those colonized by the parent benomyl-tolerant isolates (Table 5).

DISCUSSION

Strains of *B. cinerea* tolerant to both DCNA and benomyl were selected from certain benomyl-tolerant isolates under laboratory conditions. Unlike benomyl-tolerant (1,4,9) and DCNA-tolerant (10) strains, those tolerant to both DCNA and benomyl had reduced mycelial growth and sporulation rates. Geeson (5) reported that some UV-induced carbendazin and DCNA-carbendazin tolerant mutants had slower growth rates than the field isolates of *B. cinerea* from which they were selected, but otherwise they could not be distinguished morphologically from the field isolates. Multiple-tolerant strains were pathogenic, but they were less vigorous than isolates from which they were derived when colonizing peach fruit and tomato stems.

Multiple tolerant strains had higher ED_{50}s for growth inhibition by benomyl than the parent benomyl-tolerant isolates, except strain C75T-2T, which was less tolerant to DCNA (Table 1). Furthermore, unlike the other multiple-tolerant strains, strain C75T-2T did not show significant reduction in linear growth in the absence of fungicides, compared with its parent isolate (Table 2). In the presence of benomyl, ED_{50}s for growth inhibition by DCNA for multiple-tolerant strains were lower (Table 1), but germination rates and initial germ tube elongation were similar (Table 3) to those in tests on PDA amended with DCNA alone. This would indicate that multiple tolerance to DCNA and benomyl could develop from benomyl-tolerant conidia on substrates treated with DCNA alone or combined with benomyl, but a reduced rate of colonization would be expected in the presence of benomyl.

Postharvest treatments of stone fruits such as sweet cherries use a combination spray of DCNA and benomyl to reduce decays caused by *Monilinia fructicola* (Wint.) Honey, *M. laxa* (Alderh. & Ruhl.) Honey, *B. cinerea*, and *Rhizopus stolonifer* (Ehrenberg ex Fr.) Lind (7). This treatment has remained effective against *B. cinerea* even with benomyl-tolerant *B. cinerea* present in the orchards (8). It has been assumed that the combination treatment of DCNA and benomyl would provide control of decays caused by *B. cinerea* tolerant to benomyl. Our data indicate that DCNA residues of 12.9 μg/g are sufficient to reduce decays caused by benomyl-tolerant isolates of *B. cinerea*, but the potential exists for selection of strains with tolerance to both DCNA and benomyl. Residues of 17.4 μg of DCNA per gram did not reduce the decay caused by the multiple tolerant strains (Table 4).

Tolerance to DCNA in *B. cinerea* has not been found in the field, where this fungicide has only limited use. In crops such as grapes, however, where combinations of benomyl and DCNA are used to control *B. cinerea* in the vineyard and where benomyl-tolerance in *B. cinerea* occurs, the potential for tolerance to both DCNA and benomyl exists.

These studies indicate that multiple-tolerant strains could be at a competitive disadvantage in the absence of DCNA and/or benomyl because their growth rates and ability to sporulate are reduced. Studies are needed to ascertain the potential importance of this phenomenon under field conditions.

TABLE 3. Growth of germ tubes from DCNA-benomyl multiple-tolerant and parent benomyl-tolerant conidia of *Botrytis cinerea* on potato-dextrose agar (PDA) and PDA amended with DCNA, benomyl, or DCNA plus benomyl

Isolate or strain	Germ tube length (mm) after 12 hr at 20 C[a]			
	PDA	10 μg DCNA/ml PDA	10 μg benomyl/ml PDA	10 μg DCNA and 10 μg benomyl/ml
9B3T[b]	112.2	0.0	128.1	0.0
9B3T-1T[c]	141.5	46.4	104.9	42.7
HR75T[b]	113.5	0.0	104.9	0.0
HR75T-2T[c]	141.5	28.1	117.1	43.9
C75T[b]	79.3	0.0	75.6	0.0
C75T-2T[c]	151.3	42.7	137.9	50.0
9L8T[b]	111.0	0.0	117.1	0.0
9L8T-1T[c]	135.4	24.4	134.2	25.6

[a] Average of four replications of 10 germ tube measurements per isolate or strain.
[b] Benomyl-tolerant parent isolate.
[c] DCNA-benomyl multiple-tolerant daughter strain.

TABLE 4. Effect of postharvest DCNA and/or benomyl treatment on the ability of DCNA and benomyl-sensitive, benomyl-tolerant, and DCNA-benomyl multiple-tolerant strains of *Botrytis cinerea* to decay peach fruit

Postharvest treatment[b]	Average lesion diameter (mm)[a]		
	39 (DCNA and benomyl sensitive)	9B3T (benomyl tolerant)	9B3T-1T (DCNA-benomyl tolerant)
None	37.0	42.5	28.8
Benomyl	8.5	51.7	22.0
DCNA	9.0	10.3	23.9
DCNA and benomyl	8.7	10.8	21.4

[a] Average lesion diameter of four inoculated fruit per treatment after 5-day incubation at 20 ± 2 C.
[b] Peach cultivar Rio Oso Gem fruits were dipped for 15 min in 900 μg of DCNA, 300 μg of benomyl, or 900 μg of DCNA plus 300 μg of benomyl per milliliter of water. Treated fruit were air-dried before inoculation by placing a drop of a spore suspension from each isolate or strain on an injury made with the point of a sterile 2-mm diameter glass rod.

TABLE 5. Pathogenicity of DCNA-benomyl multiple-tolerant strains and parent benomyl-tolerant isolates on tomato cultivar Ace 55 stems

Isolate or strain	Length of stem colonized[a] (mm)
9B3T[b]	40.0
9B3T-1T[c]	20.4
HR75T[b]	38.6
HR75T-2T[c]	12.2

[a] Average of five replications per isolate or strain. Eight-centimeter stem sections placed upright in moist sterile sand in plastic boxes were inoculated with 4-mm diameter mycelial plugs from each isolate or strain. Inoculated stems were incubated at 21 ± 2 C for 9 days. No lesions developed on noninoculated controls.
[b] Benomyl-tolerant parent isolate.
[c] DCNA-benomyl multiple-tolerant strain.

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