Tolerance of Botrytis cinerea to 2,6-Dichloro-4-nitroaniline

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ABSTRACT

Isolates of *Botrytis cinerea* grown on media containing 2,6-dichloro-4-nitroaniline (DCNA) gave rise to DCNA-tolerant variants. The growth of tolerant strains on the chemical was normal and usually equal to that on check plates. Subsequent hyphal-tip and single-spore cultures revealed that tolerant isolates may be heterocaryotic for tolerance and capable of growing on 7,000 ppm DCNA. Most isolates maintained tolerance even after growing for extensive periods in the absence of DCNA. Tolerant and susceptible isolates did not differ significantly in ability to rot peach and cherry fruits except, of course, on DCNA-treated fruits. Some DCNA-tolerant isolates showed tolerance also to other fungicides. Spores did not differ from mycelium in tolerance. Phytopathology 60:1489-1492.

Additional key words: pathogenicity, heterocaryosis, strain.

DCNA (2,6-dichloro-4-nitroaniline) (Botran, Dicloran, Allison) is used widely for controlling diseases on crops caused by a variety of pathogens, including *Rhizopus stolonifer, Botrytis cinerea* Fr., and *Monilinia fructicola* (12). Sharples (11) demonstrated that DCNA is fungistatic to mycelium and spores of *B. cinerea,* delaying spore germination and severely checking hyphal growth. Clark & Hams (1) reported that linear growth of *B. cinerea* was controlled 86% on agar plates containing 5 ppm DCNA, and completely inhibited by 10 ppm.

Tolerance may develop in laboratory cultures (6, 13), however, and Locke (5) recently reported various degrees of Botran tolerance in *Sclerotium cepivorum* from a field treated with Botran to control white rot of onions.

Priest & Wood (10) found that strains of *B. allii* selected for resistance to chlorinated nitrobenzenes were also resistant to Botran. Resistance was retained for at least 18 months under ordinary laboratory conditions. Georgopoulos (2, 3) had similar results with *Hypomyces solani,* although nitrobenzene-tolerant strains were not tolerant to captan.

Our earlier study on tolerance of *Rhizopus stolonifer* to DCNA (13) indicated that such tolerance may be due to a heterocaryotic condition and may be conditioned by the number of nuclei containing tolerant factors. *Botrytis cinerea* is reported tolerant to other fungicides, and has a nuclear behavior that makes it ideal for the study of heterocaryosis as a possible mechanism in variation of tolerance. Since strains of *Botrytis* tolerant to other chemicals (9) may also show tolerance toward DCNA, the reverse was tested. This paper thus reports on initial selection of strains tolerant to DCNA, tolerances to varied concn, a possible mechanism conditioning degree of tolerance, comparisons of spores and mycelium on DCNA-containing media, stability of tolerance, cross-resistance tests with other chemicals, and pathogenicity of tolerant strains to treated and untreated fruits.

MATERIALS AND METHODS.—Most tests were carried out on Dion's medium (DM): 30 g glucose, 10 g yeast extract, 1.0 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $ZnSO_4$, 1.0 g $CaCO_3$, 15 g agar, and distilled H_2O to make 1 liter. The ingredients were routinely combined and autoclaved at 121 C for 30 min. Potato-dextrose agar (PDA) was prepared by boiling 200 g diced peeled potatoes in a cheesecloth bag for 30 min. The broth was combined with 10 g dextrose, 15 g agar, and enough tap water to make 1 liter, and autoclaved at 121 C for 20 min. When particularly large numbers of conidia were required, a grape medium was prepared by placing a layer of presterilized Thompson Seedless grapes in 125-ml flasks and autoclaving for 5 min at 121 C. The original isolates of *Botrytis cinerea* were obtained from tomato (Bc-5), cherry (Bc-3 and Bc-2), and prune (Bc-4). The DCNA used was technical grade, dissolved in 10 ml of acetone/liter of the medium to be used. This solution was added to molten agar and agitated just before the plates were poured. Inoculum was usually small agar plugs (3-5 mm in diam) containing mycelium and spores (DM). All incubations were at 25 ± 2 C in the laboratory.

The pathogenicity tests were carried out on harvested ripe peach fruits surface-sterilized, punctured approximately 1 mm deep with a glass rod, and inoculated with a drop of spore suspension. DCNA treatments of fruits were with a dip of Botran 50% wettable formulation. Fruits were incubated at 25 C in plastic boxes with raised wire-mesh floors. When a high relative humidity was wanted, water was added to each of the boxes.

Cross-resistance tests were carried out on PDA plates. Before incorporation in media, the test chemicals: 1) DCNA (technical), captan (technical), benomyl 50W, Dithianon 75W, dichlone (technical) 90%, and 2-chloro-3-phenyl-1,4-naphthoquinone (WK-1) were dissolved in acetone; while 2) pentachloronitrobenzene (PCNB 75W), griseofulvin (technical), tetrachloroethylmercaptocyclohexenedicarboximide (Difolatan 80W), trichloronitrobenzene (TCNB 70W), *N*-(dichlorofluromethylthio)-*N'*, *N'*-dimethyl-*N*-phenylsulfamide (Bay 47531), 4-tertiarybutyl-7-decylamino-3,4,5,6 tetra-hydro-2H-azepine-chlorohydrate (GS16306) 50W, and ferbam (Fermate 76W) were dissolved in glass-distilled water.

RESULTS AND DISCUSSION.—Selection of DCNA-tolerant strains of *B. cinerea* was first attempted as follows: isolates Bc-2, Bc-3, Bc-4, and Bc-5 were grown on Thompson Seedless grapes to obtain spores. Three plates (a, b, c) at each concn of DCNA were inoculated with conidia of each of the four isolates.

TABLE 1. Growth of four isolates of *Botrytis cinerea* on various concn of 2,6-dichloro-4-nitroaniline (DCNA), and designation of tolerant isolates obtained

	Concn of DCNA (ppm)									
Isolate	50	100	150	200	250	300	350	400	450	500
Bc-2	1a[a]	[b]	3a	4a	5a		7a	8a		10a
	1b		3b	4b	5b	6b	7b	8b	9b	10b
	1c	2c		4c			7c	8c	9c	10c
Bc-5									12a	
										13b
								11c	12c	
Bc-4							23a		25a	
	22a							24b		26b
Bc-3	27a		28a	29a		31a		32a		
	27b			29b	30b	31b		32b	33b	
			28c	29c	30c	31c		32c		

[a] Numerals represent original plates and DCNA concn. Lower case letters represent replications of these concn. As noted in some cases, *B. cinerea* did not grow on all three replications; i.e., with Bc-2 at 100 ppm, only replication c produced growth. Thus 2c becomes code for tolerant isolate selected from 100 ppm throughout the remainder of the study.

[b] Where blanks appear there was no growth in any of the plates for the isolates at the indicated DCNA concn.

Table 1 shows the responses after 7 days of incubation. The isolates differed in ability to grow on DCNA-containing media, with no seeming relation to the chemical concn. Thus, only one of three plates might show growth on a given concn, and isolate Bc-5 grew only at higher concn. Growth was usually asymmetrical and similar to a sector from the point of inoculation.

Any symmetrical colonies produced were generally surrounded by a "clear halo" extending about 2 mm from the margin of the colony. Hyphal-tip isolations were made from the margins of the areas of growth on DCNA and transferred to PDA plates. These were coded as shown in Table 1. After the plates were grown out, stock tube cultures of each were made for use in subsequent studies.

The tolerant isolates derived from the four original isolates did not differ significantly from them in production of conidia and sclerotia.

Tests were made to determine if the isolates established from hyphal tips from the originally tolerant sectors differed in linear growth and tolerance at the same concn of DCNA. Linear growth of 17 of these isolates is shown on Table 2. The majority showed near-equal linear growth after 5 days on both 400 and 500 ppm DCNA, regardless of the DCNA concn in the plates from which they were selected, but they still seemed to differ in degree of tolerance as evidenced by differences in linear growth.

The isolates shown in Table 2 were grown in PDA tubes, followed by three subsequent transfers on PDA in the absence of DCNA. The level of tolerance shown in Table 2 was maintained except in isolates 28-a and 29-c, which showed considerably less tolerance after the successive growth in the absence of DCNA. The only other changes were slight increases in linear growth over growth prior to the successive transfers.

TABLE 2. Comparison of linear growth on 400 and 500 ppm 2,6-dichloro-4-nitroaniline (DCNA) of hyphal-tip cultures of *Botrytis cinerea* selected from colonies growing on plates containing various concn of DCNA

		Linear growth (mm)			
Isolate		Check, DM[a]	DM + Acetone	DM + 400 ppm DCNA	DM + 500 ppm DCNA
Bc-2	6b	80[b]	70	39	40
	7b	70	80	42	42
	8a	60	68	30	33
	9b	60	65	40	33
	10a	70	75	43	38
Bc-5	11c	50	50	40	32
	12a	50	50	40	43
	13b	50	50	42	40
Bc-4	24b	80	75	30	30
	25a	45	45	0	0
	26b	35	37	27	19
	23a	40	55	55	47
Bc-3	28a	80	80	32	32
	29c	40	50	33	33
	31b	50	47	0	20
	32a	70	75	30	25
	33b	70	65	33	35

[a] Dion's medium.

[b] Mean 3 replications/treatment; 5 days' incubation.

Tolerance of spores vs. mycelium.—Since previous workers have suggested that spores of *B. allii* differ from mycelium in ability to serve as inoculum on fungicide-containing media (9), *B. cinerea* was tested for this possibility. Three isolates of differing tolerance were selected: B-5, sensitive parent; 25-a, intermediate tolerance; and 13-b, tolerant (equal growth with and without DCNA). The inocula were loops of spore suspensions and agar plugs from young colonies containing only mycelium. Mycelium alone as inoculum did not differ significantly from conidia alone in tests for tolerance on 100, 300, or 500 ppm DCNA.

Experiments with single-spore isolates.—Although the four original isolates were essentially sensitive to DCNA, tolerant strains were selected quite easily when media containing DCNA were inoculated with either mycelium or spores. No "training" period for obtaining tolerant mutants appeared necessary. Apparently mutation, followed by selection or selection for tolerance existing at a low level in the original isolates, could account for the appearance of tolerant strains. Since tolerance was not observed in all plates containing DCNA and inoculated with mycelium and/or spores from the same isolate, certain cultures may in fact be heterocaryotic or mosaic for tolerance, which places importance on the area of the colony from which inoculum is taken. This is quite possible, since it is well known that both mycelium and conidia of *B. cinerea* are multinucleate (4). Being heterocaryotic for tolerance might also explain the ability of some isolates to grow on low concn of DCNA and not on higher concn. To test this possibility, a series of single-spore isolations were carried out followed by tests for tolerance to DCNA. The term "generation" is used here to designate each successive set of single-spore isolations.

In the first generation from isolate B-5 (sensitive to DCNA), 9 of 50 single-spore isolates grew on media containing 300, 500, or 700 ppm of DCNA. In a second generation from a selected tolerant isolate (B-5-41), 1 of 20 single-spore isolates grew on 300 ppm. In the third generation from the single tolerant isolate (B-5-41-8), 9 of 10 isolates grew on 300 ppm of DCNA. None of 10 single-spore isolates from a sister susceptible isolate (B-5-41-7) grew on 300 ppm in this third generation. Thus, an almost pure tolerant strain can be isolated from a susceptible isolate, and repeated testing can yield an apparently homocaryotic strain for susceptibility (B-5-41-7).

In the first generation from a DCNA-tolerant isolate, 13-b (originating from B-5 in previous tests), an average of 29 of 50 single spore isolates grew on 300, 500, or 700 ppm of DCNA. In the second generation from a tolerant selection (13-b-42), 8 of 19 isolates were tolerant to DCNA. From a tolerant isolate in the second generation (13-b-42-3), 10 of 10 in the third generation and 10 of 10 in the fourth generation were tolerant to DCNA up to 7,000 ppm. A sister isolate of 13-b-42-3 from the second generation, 13-b-42-5, selected for intermediate tolerance showed 4 of 10 isolates that were tolerant in the third generation. Thus, 13-b-42-3 was apparently homocaryotic for tolerance and 13-b-42-5 was still heterocaryotic.

Pathogenicity tests.—Tolerant strains would be of less significance, of course, if the development of tolerance was accompanied by a loss of virulence or inability to rot DCNA-treated fruits. Pathogenicity tests were carried out to compare tolerant (13-b-42-3) and susceptible (B-5-41-7) isolates in ability to rot DCNA-treated and untreated fruits. Three treatment concn were tested, equivalent to 1, 2, and 3 lb. of DCNA/100 gal H_2O. Ripe peaches were surface-sterilized for 1 min in a 1:10 0.525% sodium hypochlorite solution and air-dried. Those to be treated were then dipped in appropriate concn of Botran 50W for 1 min and air-dried. Residue analysis of peaches thus treated was: 1 lb., 11 ppm/fruit; 2 lb., 22.6 ppm/fruit; and 3 lb., 31.0 ppm/fruit. Fruits were punctured 1 mm deep with a glass rod, then inoculated with a drop of a spore suspension containing approximately 50,000 spores/ml. After 6 days of incubation, there was no significant difference between the tolerant (13-b-42-3) and the susceptible strain (B-5-41-7) in amount of rot on untreated fruits. Likewise, there was no difference in amount of rot between Botran-treated and untreated fruits inoculated with 13-b-42-3. In contrast, B-5-41-7 did not rot fruits treated with 2 lb./100 gal or 3 lb./100 gal, and caused only slight rot in a few peaches treated at 1 lb./100 gal. Thus, tolerance does not affect the ability of *B. cinerea* to rot untreated peach fruits, and the dip treatment with Botran was not effective against the tolerant strain. Results were similar in repeated experiments, and also with cherry fruits.

Cross-resistance tests.—Since some strains of *Botrytis allii* selected for tolerance to PCNB have also shown tolerance to DCNA (10), *B. cinerea* isolates tolerant to DCNA were tested for tolerance to other fungicides. Table 3 shows that 13-b-42-3 grew better than B-5-41-7 on 100 ppm of six of the chemicals tested, and poorer or not at all on two of the test chemicals. This indicates the importance of such cross-resistance tests if strains tolerant to a specific chemical became predominant in nature.

Discussion.—The tolerant strains rotted fruits treated with DCNA at rates currently used commercially, while the susceptible strains were unable to rot DCNA-treated fruits. Thus far we have not obtained tolerant strains from nature, but the importance and probability of their occurrence is obvious.

Theoretical claims (7) for the importance of the phenomenon of heterocaryosis are supported by the demonstration that heterocaryosis may serve as the mechanism in maintaining low levels of tolerance to DCNA in this multinucleate fungus, and that tolerant strains emerge when selection is exerted by the chemical.

The possibility should not be overlooked that DCNA may be mutagenic to *B. cinerea,* especially since it is not known whether the original strains used in this study had ever been exposed to DCNA in nature.

Thus, no conclusion can be reached at this time as to the original occurrence of tolerance in the strains used to initiate the study. The rapid selection of

tolerant strains, coupled with the observations that both homo- and heterocaryotic strains for tolerance maintain their tolerance and appear to grow normally, indicates that factors conditioning tolerance are not adverse to normal growth; otherwise one might have expected an alteration of nuclear ratio back toward susceptibility, or possibly complete elimination of the tolerant type during vegetative growth in the absence of DCNA.

The results further emphasize the need for additional study on the mode of action of fungicides. It would be particularly interesting to compare the nature of the reactions to PCNB, TCNB, and DCNA in the tolerant and susceptible strains (Table 3).

Parry & Wood (8, 9) suggested that differences in tolerance may be due in some cases to differences between susceptible and tolerant strains in permeability of the cell wall. They particularly noted a difference in the tolerance expressed when spores or mycelium were used as inoculum, with the spores being less tolerant. The present study showed no such differences in *B. cinerea*. The possibility should not be overlooked, however, that the greater growth of the DCNA-tolerant strain (13-b-42-3) than of the susceptible strain (B-5-41-7) on various chemicals at different concn may be due to differential permeability between these strains.

TABLE 3. Growth of DCNA-susceptible (B-5-41-7) and DCNA-tolerant (13-b-42-3) strains of *Botrytis cinerea* after 7 days on PDA containing 10 and 100 ppm active ingredient of 13 different chemicals

Chemicals tested	B-5-41-7		13-b-42-3	
	10 ppm	100 ppm	10 ppm	100 ppm
Dithianon	++[a]	++	++	++
Dichlone	++	++	++	++
Ferbam	++	++	++	++
Captan	++	+	++	++
WK 1	++	++	+	+
Difolatan	+	+	++	++
GS16306	+	+	++	++
Griseofulvum	+	+	++	+
DCNA	−	−	++	++
PCNB	+	+	++	++
TCNB	−	−	+	−
Bay 47531	+	−	−	−
Benomyl	−	−	−	−

[a] − = No growth; + = growth of colony not more than 20 mm in diam; ++ = growth of colony 40 mm or more in diam. Represents average values obtained in 3 different tests with 3 replications/test. Incubation time (7 days). See Materials and Methods for coined names or chemical names.

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