Shoot and Flower Blight of Leucospermum cordifolium Incited by a Benomyl-Tolerant Strain of Botrytis cinerea

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ABSTRACT

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Botrytis cinerea consistently was isolated from blighted shoots and flowers of *Leucospermum cordifolium* in Kula, Hawaii. Several benomyl-tolerant isolates were isolated at the Maui Agricultural Research Center, Kula, after continuous applications of benomyl. However, benomyl-tolerant isolates were not present in an adjacent field where benomyl had not been applied. A *B. cinerea* isolate (B1) tolerated high concentrations of benomyl in spore germination and mycelial growth tests. At the highest concentration of benomyl (500 μg/ml) spore germination of B1 was 98.6%, whereas a benomyl-sensitive isolate (B4) showed 8.9% germination. Mycelial growth of B1 was inhibited slightly (81.6% and 60.3%) by benomyl at 250 μg/ml and 500 μg/ml. In contrast, two benomyl-sensitive isolates (B3 and B4) were inhibited completely at the lowest benomyl concentration tested (1.0 μg/ml). Isolate B1 also exhibited tolerance to methyl-thiophanate and thiabendazole at 250 μg/ml and 500 μg/ml, respectively, whereas the benomyl-sensitive isolates (B3 and B4) did not. Captan was the least effective in inhibiting the growth of any isolate. At a high concentration (500 μg/ml) mancozeb reduced the growth of all isolates. On the other hand, chlorothalonil required low concentrations (10 μg/ml and 25 μg/ml) to inhibit the growth of all isolates. Both the benomyl-tolerant isolate (B1) and the benomyl-sensitive isolate (B3) induced blight of flowers and young shoots of container-grown *L. cordifolium* plants and produced symptoms similar to those observed in the field.

Additional key words: ornamental protea, *Banksia speciosa,* benzimidazole fungicides.

Blighted young shoots were observed on several *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade, plants in Kula, Hawaii, during the late spring and early summer months in 1974 and 1975. *Botrytis cinerea* Pers. ex Fr. consistently was isolated from these blighted shoots. This disease was especially prevalent at the Maui Agricultural Research Center at Kula where benomyl sprays were used as a general control practice to control *Botrytis* on all Proteaceae plants, including *L. cordifolium*. There are several reports of benomyl tolerance in fungi, including in *Botrytis* (3). Thus, it was suspected that benomyl-resistant strains of *Botrytis* may have developed on the *L. cordifolium* and *B. speciosa* R. Br. plants.

The purpose of this research was to compare benomyl-tolerant and -sensitive isolates for pathogenicity, mycelial growth, and spore germination.

MATERIALS AND METHODS

Fungal isolates.—All test isolates of *B. cinerea* were obtained from infected plant material from Kula. The isolates included: B1 from shoots of *L. cordifolium* at the Maui Agricultural Research Center, B3 from shoots of *L. cordifolium* at a commercial field in Kula, and B4 from shoots of *B. speciosa* at a commercial field in Kula. Monospore cultures of the isolates were maintained on potato-dextrose agar (PDA) slants.

Pathogenicity tests.—Isolates B1 and B3 were subcultured on PDA plates for 2 weeks and then suspensions of spores and hyphal fragments were prepared by adding 20 ml of sterile distilled water plus one drop of Tween-20 to each of five plates. The mycelium was scraped with a rubber policeman and the resulting suspensions were swirled. These suspensions then were filtered through a one-ply tissue paper (Kimwipe) and adjusted with water to 1×10^6 spores/ml by counts made with a hemocytometer. Approximately 10 ml of the spore suspension was atomized onto 10 cut flowers at three different stages of inflorescence development and the leaves of 10 rooted cuttings. The stages of flower development were: (i) the stage at which the perianths and styles have emerged and elongate at the same rates from the receptacle; (ii) the stage at which the perianths have stopped elongating but the styles have continued to grow (at this stage the stigmas are tightly held next to the stamens situated in the spoon-like depressions of the perianth lobes which causes the styles to form loops); and (iii) the stage at which the stigma is released from the perianth lobes allowing the styles to reflex outward. Inoculated plants were placed in a polyethylene bag overnight and examined after 7 and 14 days.

Fungicides.—Benlate 50W (benomyl) was obtained from E. I. duPont de Nemours and Company, Wilmington, Delaware; Mertect 50W (thiabendazole) from Merck Chemical Company, Rahway, New Jersey;

Topsin M 70W (methyl-thiophanate) from Agchem-Decco Division of Pennwalt Corporation, Tacoma, Washington; Bravo 75W (chlorothalonil) from Diamond Shamrock Chemical Company, Cleveland, Ohio; Orthocide 50W (captan from Chevron Chemical Co., Richmond, California; and Dithane M-45 80W (manganese ethylene bisdithiocarbanate plus zinc ion or mancozeb) from Rohm and Haas Chemical Company, Philadelphia, Pennsylvania.

Tolerance of fungal pathogen strains to benzimidazole and other fungicides.—Preliminary screening of *Botrytis* isolates was conducted on water agar containing 100 μg/ml of benomyl. Subsequent tests were made to determine radial growth and spore germination of isolates on media containing various concentrations of the different fungicides.

Mycelial agar disks (4 mm in diameter) were removed from 2- to 3-day-old PDA cultures of the isolates B1 and B4, and placed on PDA plates containing various concentrations of the different fungicides. Inoculated plates were incubated at 24 C and the radial growth was measured daily. Average growth rate was based on an average of five plates. This experiment was repeated two times.

Spore suspensions were made from 1- to 2-week-old cultures of *Botrytis* isolates (B1, B3, and B4) using sterile distilled water and one drop of Tween-20. Approximately 2 ml was placed on the surface of plates containing water agar plus various concentrations of different fungicides. These plates were incubated at room temperature under constant illumination. One day after inoculation, the percent germination was determined by the number of spores germinated per 500 spores on an average of five plates. This experiment was repeated two times.

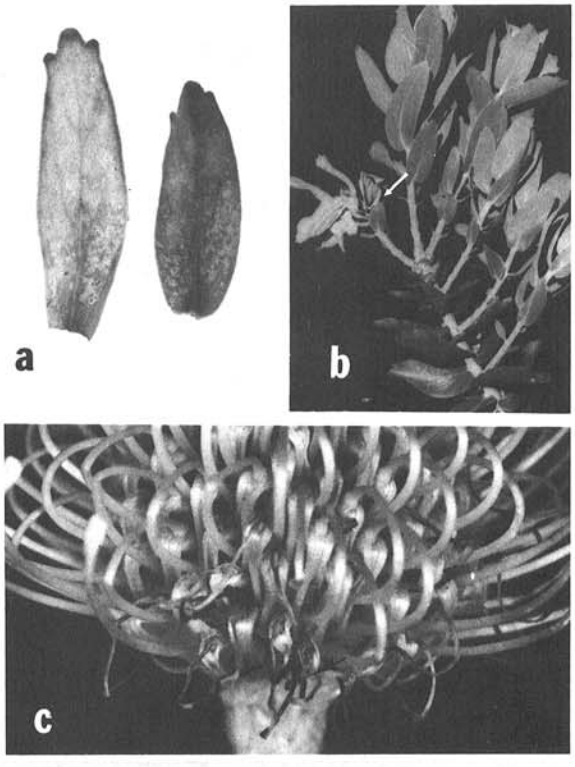

Fig. 1-(a to c). *Botrytis cinerea* symptoms on *Leucospermum cordifolium* plants. **a)** Water-soaked circular lesions on two young inoculated leaves. **b)** Collapsed shoot tip and leaves of inoculated shoot (left), compared with healthy shoots (right), **c)** Collapsed stigmas and styles near the base of inoculated flowers.

RESULTS

Disease symptoms and pathogenicity tests.—*Botrytis cinerea* consistently was associated with dried young shoots of field-grown *L. cordifolium*. The incidence and severity were highest during the winter months. Also *B. cinerea* was recovered from the dead, dried young shoots of rooted cuttings growing in propagating benches.

Initial symptoms on inoculated *L. cordifolium* plants in the greenhouse progressed from water-soaked circular lesions on the young succulent leaves (Fig. 1-a), to a collapse of the entire leaf and a girdling of the young stem resulting in death of the shoot (Fig. 1-b). All 10 plants were affected. The development of symptoms was rapid under these conditions with severe symptoms within 7-10 days after inoculation. These symptoms were similar to those symptoms occurring in the field. No difference could be detected in severity between isolates B1 and B3 under test conditions.

Flowers of *L. cordifolium* inoculated with the fungus indicated that all three stages of inflorescence development were susceptible. All of the inoculated flowers were infected. Symptoms occurred initially on the styles and perianths as small water-soaked lesions 3 days after inoculation. These lesions generally advanced into the flower head (Fig. 1-c).

Sensitivity of Botrytis to various fungicides.—*Isolation of tolerant strains.*—Benomyl resistance was suspected where benomyl was applied on a weekly schedule at the Maui Agricultural Research Center. Several plants had symptoms of shoot dieback even after several benomyl spray applications. All 10 isolates made at the Research Center from young infected shoots grew on water agar plates containing benomyl; however, all 10 isolates from similarly diseased tissues from an adjacent field, where benomyl was not used, grew only on the medium without benomyl.

Radial growth studies.—A presumed benomyl-tolerant isolate, B1, and two benomyl-sensitive isolates, B 3 and B4, were evaluated for susceptibility to various fungicides by measurement of radial growth and determination of spore germination. Isolate B1 was tolerant to high levels of benomyl and methyl-thiophanate (Table 1). The B3 and B4 isolates were inhibited completely by 1 μg/ml of benomyl, methyl-thiophanate, or thiabendazole.

High levels (250 μg/ml and 500 μg/ml) of mancozeb and captan were required to inhibit B1, whereas chlorothalonil was effective at 10 μg/ml. Isolates B3 and B4 were inhibited slightly at low levels of mancozeb and captan and required high concentrations (250 μg/ml and 500 μg/ml) for inhibition of growth. Chlorothalonil suppressed B3 growth at low levels (25 μg/ml) showing inhibition similar to that of the B1 isolate.

Spore germination studies.—The effect of fungicides on spore germination is summarized in Table 2. Captan at

500 μg/ml was the least effective in inhibiting spore germination of B1 (93.6% germinated) and B4 (84.8% germinated). In contrast, chlorothalonil inhibited spore germination of both isolates at 10 μg/ml. Increasing concentrations of benomyl (10 μg/ml, 50 μg/ml, 125 μg/ml, 250 μg/ml, and 500 μg/ml), resulted in an increasing reduction of spore germination (95%, 86.4%, 63.5%, 68.2%, and 8.9%, respectively) of isolate B4. However, benomyl had little inhibitory effect on the germination of isolate B1.

TABLE 1. The effect of various fungicides on the radial growth of *Botrytis cinerea* isolates B1, B3, and B4

Fungicide	Concentration (μg/ml)	Radial growth[a] of isolate: B1 (mm)	B3 (mm)	B4 (mm)
Benomyl	1	...	0	0
	10	77.5	0	0
	25	...	0	0
	50	77.8	0	0
	125	72.5	0	0
	250	62.0	0	0
	500	45.8	0	0
Thiabendazole	1	74.0	...	...
	10	81.0	0	0
	25	...	0	0
	50	78.8	0	0
	125	76.3	0	0
	250	72.1	0	0
	500	52.7	0	0
Chlorothalonil	1	...	...	...
	10	31.4	...	...
	25	...	9.5	...
	50	25.6	10.5	...
	125	22.5	12.5	...
	250	20.5	12.0	...
	500	18.1	8.5	...
Methyl-thiophanate	1	74.0	0	0
	10	81.0	0	0
	25	...	0	0
	50	78.8	0	0
	125	76.3	0	0
	250	72.1	0	0
	500	52.4	0	0
Mancozeb	1	...	...	...
	10	81.0	61.0	74.5
	25	...	68.5	73.0
	50	81.0	63.5	74.5
	125	80.5	61.0	65.0
	250	68.2	43.5	52.5
	500	53.4	32.5	35.5
Captan	10	80.1	67.0	73.5
	25	80.4	68.5	77.5
	50	80.4	61.0	76.0
	125	79.0	62.5	72.0
	250	79.7	52.0	74.5
	500	65.5	48.0	69.0
Control	...	76.0	78.0	75.5

[a]Values represent the average radial growth of five plates after 72 hours of growth repeated twice.

Thiabendazole and methyl-thiophanate were more toxic to isolate B4 based on inhibition of spore germination, as compared with isolate B1. Reduction in the percent germination of the B4 isolate was observed at 10 μg/ml of either fungicide. On the other hand, inhibition of the B1 isolate required concentrations of 50 μg of thiabendazole per milliliter, and 250 μg of methyl-thiophanate per milliliter.

High rates of mancozeb (125 μg/ml for B1 and 250 μg/ml for B4) were needed to inhibit spore germination of isolates B1 and B4, respectively.

DISCUSSION

Several reports on benomyl tolerance have been published (2, 3, 7, 8, 10). Therefore, it was not unusual to find benomyl-tolerant *Botrytis cinerea* strains where benomyl had been used weekly for a period of 2 years. Unlike the reports indicating reduction in other functions

TABLE 2. The effect of various fungicides on spore germination of *Botrytis cinerea* isolates B1 and B4

Fungicide	Concentration (μg/ml)	Germination at 24 hours[a] for isolate: B1 (%)	B4 (%)
Benomyl	10	99.6	95.3
	50	99.1	86.4
	125	99.5	63.5
	250	99.1	68.2
	500	98.6	8.9
Thiabendazole	10	98.9	90.1
	50	89.4	37.2
	125	38.3	12.7
	250	20.1	17.7
	500	22.5	12.6
Chlorothalonil	10	0	0
	50	0	0
	125	0	0
	250	0	0
	500	0	0
Methyl-thiophanate	10	99.3	86.9
	50	98.2	83.0
	125	91.0	65.3
	250	76.7	50.6
	500	58.4	19.3
Mancozeb	10	92.5	91.6
	50	97.7	93.6
	125	51.6	91.5
	250	17.5	56.7
	500	3.0	2.3
Captan	10	99.0	96.2
	50	91.1	93.3
	125	98.4	89.9
	250	95.1	86.3
	500	93.6	84.8
Control	...	99.7	99.3

[a]Values represent the average percent spore germination per 500 spores observed on five water agar plates repeated twice.

such as growth, reproduction, and pathogenicity in benomyl-tolerant fungal strains (7), no difference was detected between the benomyl-tolerant isolate (B1) and the benomyl-sensitive isolates (B3 and B4) in respect to growth, germination, and pathogenicity.

Radial growth and spore germination studies indicated that tolerance to benomyl also resulted in a concomitant tolerance to methyl-thiophanate and thiabendazole. This is similar to other reports (3, 5). Both methyl-thiophanate and benomyl from methyl 2-benzimidazolecarbamate (MBC) in aqueous solution (4, 9). Cross resistance between benomyl- and thiabendazole-resistant fungal strains has also been reported in some fungi (1, 6), suggesting that the fungitoxic mode of action of these fungicides may be similar. The mycelial growth of the benomyl-tolerant isolate (B1) tolerated high levels of benomyl, methyl-thiophanate, and thiabendazole. This resistance suggests that toxicity of these fungicides is similar. However, in spore germination studies, B1 was more tolerant to higher levels of benomyl than methyl-thiophanate and thiabendazole (Table 2). This may indicate that there may be differential permeability to these fungicides or differential conversion to the toxic moiety (7).

The development of benomyl-tolerant *B. cinerea* strains appears to have been induced by the continual use of benomyl on *L. cordifolium*. The benzimidazole fungicides are quite effective in inhibiting the growth of sensitive or wild-type *B. cinerea* strains, and when used in combination or alternated with other fungicides which exhibit another mechanism for inhibition of growth, should reduce the chances of development of tolerant strains. In this respect, both chlorothalonil and mancozeb appear to be effective protectant fungicides.

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