Synergy Between a Benzimidazole-Sensitive Isolate and Benzimidazole-Resistant Isolates of *Penicillium digitatum*

B. L. Wild and J. W. Eckert

Graduate research assistant and professor of plant pathology, respectively, Department of Plant Pathology, University of California, Riverside 92521. Present address of senior author: Gosford Horticultural Postharvest Laboratory, Gosford, NSW, 2250 Australia.
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ABSTRACT

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A benzimidazole-sensitive isolate of *Penicillium digitatum* increased the infectivity of a benzimidazole-resistant isolate when mixtures of the two were inoculated into oranges subsequently treated with 500 mg of benomyl per liter. Benomyl and carbendazim reduced only slightly the percent germination of sensitive isolate spores, but the germ tubes were severely stunted and distorted. Spores of the sensitive isolate germinating in liquid culture containing carbendazim produced 50–70% of the pectolytic enzyme activity that was measured in the cultures without fungicide. The infectivity of four benzimidazole-resistant isolates was increased by the addition of the dialyzed culture filtrate from the carbendazim-inhibited isolate. The synergy between resistant and sensitive isolates of *P. digitatum* during infection of oranges suggests that the effectiveness of the benomyl fruit treatment could be improved by strict sanitation of packinghouses to minimize spore populations of both resistant and sensitive isolates.

Additional key words: citrus fruit, methyl benzimidazolecarbamate, postharvest decay control.

Green mold incited by *Penicillium digitatum* Sacc. is initiated by germination of airborne conidia in injuries in the peel (pericarp) of citrus fruits. This disease is a limiting factor in long-distance marketing of citrus fruits, especially in crops produced in arid production areas. Since the early 1970s the benzimidazole fungicides, benomyl, carbendazim, and thiabendazole, have been used extensively to prevent infection of citrus fruits by *P. digitatum* and other postharvest pathogens (3,7). These fungicides are suspended in water or wax formulations that are applied to the fruits in the packinghouse, preferably within 24 hr after harvest.

Benzimidazole-resistant isolates of *P. digitatum* and *P. italicum* were observed in California packinghouses about 15 mo after thiabendazole was adopted as a prestorage treatment for lemons (11,12). Lemon packinghouses that used thiabendazole intensively had a high frequency of benzimidazole-resistant isolates of *Penicillium* spp. in the spore population of the atmosphere. Benzimidazole-resistant isolates of *P. digitatum* and *P. italicum* have been reported in all citrus production areas of the world (12) and were isolated from a high percentage of decayed fruit in Rotterdam, a major international citrus market (14). All benzimidazole-resistant isolates of *P. digitatum* and *P. italicum* tested were resistant also to the other benzimidazole fungicides, ie, benomyl, carbendazim, thiophanate-methyl, and thiabendazole (3,11,12,18).

Benzimidazole-resistant isolates have exhibited the same range in virulence as benzimidazole-sensitive isolates when inoculated individually into untreated citrus fruits (11,12,18). However, most sensitive isolates predominated in the spore population produced on oranges inoculated with a mixture of conidia of the two types (18). The resistant isolate decreased to a low percentage of the spore population after the initial spore mixture passed several sequential life cycles in oranges. We anticipated that the benomyl treatment would be effective when the benzimidazole-resistant component of the spore population decreased below the inoculum threshold for the resistant spores alone. Contrary to expectation, the benomyl treatment was not effective until the benzimidazole-resistant spores became a small fraction of the total spore population of the inoculum. This observation suggested that the conidia of the sensitive strain in the inoculum mixture somehow increased the infectivity of the conidia of the resistant strain.

We report here on the synergistic interaction between benzimidazole-resistant and benzimidazole-sensitive isolates of *P. digitatum* during infection of benomyl-treated oranges.

MATERIALS AND METHODS

Fruit treatments. *P. digitatum* isolate M6R is sensitive to benzimidazole fungicides (benomyl, carbendazim, thiophanate-methyl, and thiabendazole) and isolate M1 is representative of the resistant isolates found in citrus packinghouses of California. Isolates M16, M13, M1, and M20S belong to carbendazim-resistance categories I, II, III, and IV, respectively (18, and Table 1).

Washington navel oranges were surface sterilized by immersion

TABLE 1. The effect of dialyzed culture filtrate of carbendazim-inhibited germlings of *Penicillium digitatum*[a] on the infection of oranges by benzimidazole-resistant isolates

Benzimidazole-resistant isolate	ED_{50}[b] (μg/ml)	Infection[c] in oranges inoculated with 50 benzimidazole-resistant spores suspended in: Dialyzed culture medium only (control)	Dialyzed filtrate from carbendazim-inhibited germlings
M16	1.30	12.5	72.5
M13	5.50	22.5	57.0
M1	7.20	52.5	92.0
M20S	36.30	7.5	40.0

[a] Benzimidazole-sensitive isolate M6R; $ED_{50} = 0.03$ μg of carbendazim per milliliter.
[b] Carbendazim concentration required to inhibit colony growth on potato dextrose agar by 50%.
[c] Mean percent infection of three replicates, each consisting of 16 fruit each with four inoculation sites; treatment differences are significant by analysis of variance, $P = 0.05$.

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for 30 sec in 70% v/v aqueous ethanol. A microsyringe was used to inject 5 μl of spore suspension (containing 10^5 conidia per milliliter) 3 mm deep into the peel at each of four sites equidistant on the equator of the fruit. The percentage of resistant spores in the inoculum was varied from 0–100%. Each spore mixture was inoculated into three replicate lots of 16 fruit each. The inoculated fruit were incubated for 18 hr at 26 C, each lot was dipped 30 sec in an aqueous suspension of 500 mg benomyl (methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate; Benlate, 50% WP) per liter, and dried. Fruit lots inoculated with the sensitive isolate only were treated with benomyl in the same manner. After 5 days at 26 C, the inoculation sites on the fruit were inspected and the degree of infection was expressed as percent infection (100 × [decaying sites]/[total inoculated sites]).

Cylinders (3 mm diameter) of fruit peel that included an unsuccessful inoculation site (no lesion) were removed from the benomyl-treated fruit after incubation at 26 C for 5 days. The peel tissue was fixed in FAA (formalin-acetic acid-alcohol-water) (6:4:50:40, v/v) for 24 hr and placed for 10 min each in boiling 10% KOH and boiling 0.1 N HCl. The peel tissue was placed in gently boiling 0.05% (w/v) aqueous trypan blue for 5 min and mounted in clear lactic acid-phenol-glycerol-water (20:20:40:20, w/w) and viewed intact.

Assay of pectolytic enzyme activity in cultures of germinating conidia. Conidia of benzimidazole-sensitive isolate M6R were incubated in reconstituted orange juice (one part Calfame brand concentrate and three parts water), pH 4, containing 0, 1, or 2 μg of carbendazim (methyl 2-benzimidazolecarbamate) per milliliter. After 24 hr of incubation at 25 C, the germlings were removed by filtration through a 5-μm Millipore filter and the culture filtrate was dialyzed against running tap water at 22 C for 24 hr. The pectolytic activity in the dialyzed filtrate was determined by measuring the reduction in viscosity of a 0.5% (w/v) aqueous pectin solution buffered at pH 5.0 in an Ostwald viscosimeter at 25 C (4).

RESULTS

Synergy between the benzimidazole-sensitive isolate and the benzimidazole-resistant isolate. The benomyl treatment prevented infection of oranges inoculated with 500 spores of the benzimidazole-sensitive isolate of *P. digitatum*, but did not control decay in fruit inoculated with the same number of spores of the resistant isolate (Fig. 1). The percent infection (62%) decreased as the number of benzimidazole-resistant spores in the inoculum was reduced. Twenty-five spores, the minimum inoculum level tested, resulted in 4% infection. The addition of benzimidazole-sensitive spores to inoculum consisting of 25–250 benzimidazole-resistant spores substantially increased percent infection. The inoculum consisted of 500 spores total in each case and the benzimidazole-sensitive component was varied from 95 to 50% of the population.

Germination and pectolytic enzyme production by germinating benzimidazole-sensitive conidia. The benzimidazole-sensitive conidia germinated 85% in control liquid cultures and about 60% in cultures containing 1–2 μg of carbendazim per milliliter. However, the germ tubes in the carbendazim cultures were severely distorted, and rarely exceeded 20 μm in length after 24 hr of incubation at 25 C (Fig. 2C). Germlings of similar appearance were observed at unsuccessful inoculation sites on benomyl-treated fruit (Fig. 2B).

The benzimidazole-sensitive isolate M6R produced significant pectolytic enzyme activity during the 24-hr germination period in liquid cultures containing 1–2 μg of carbendazim per milliliter, despite the extremely stunted and malformed condition of the germ tubes. In fact, the enzyme activity in the filtrates of the cultures with carbendazim was about 50–70% of that measured in the filtrates of the control cultures (Fig. 3A). Addition of carbendazim to cultures after 12 hr of incubation (to simulate benomyl treatment of inoculated oranges) had no significant effect upon the level of pectolytic enzyme activity found in the filtrate of the 24-hr culture (Fig. 3B).

Effect of dialyzed filtrate of sensitive isolate M6R upon infection of oranges by benzimidazole-resistant isolates. The addition of dialyzed culture filtrate of carbendazim-inhibited germlings of the sensitive isolate M6R to inoculum (10^4 spores per milliliter) of each of four benzimidazole-resistant isolates increased infection of

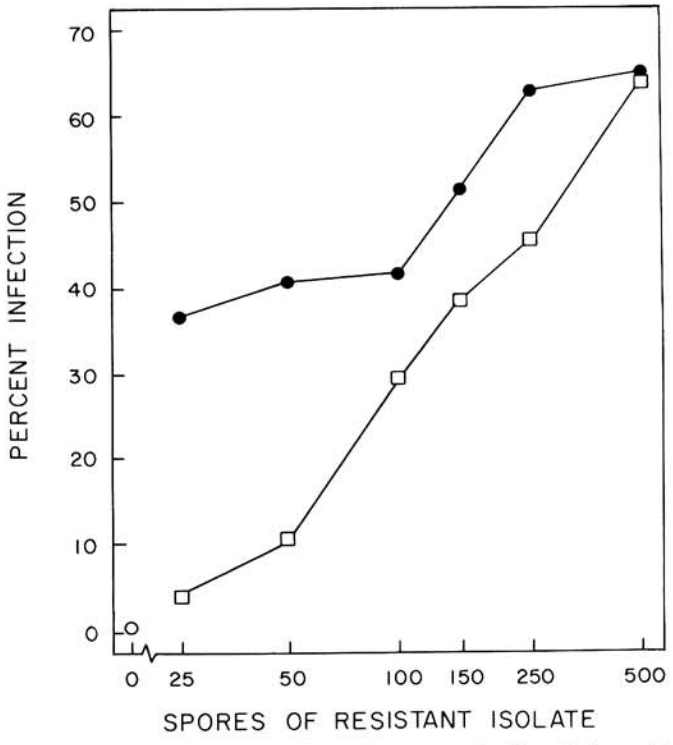

Fig. 1. Infection of benomyl-treated oranges by inoculation with benzimidazole-resistant spores of *Penicillium digitatum* alone (□) or mixed with benzimidazole-sensitive spores (●). Each mixture consisted of 500 spores total. (○) 500 benzimidazole-sensitive spores alone.

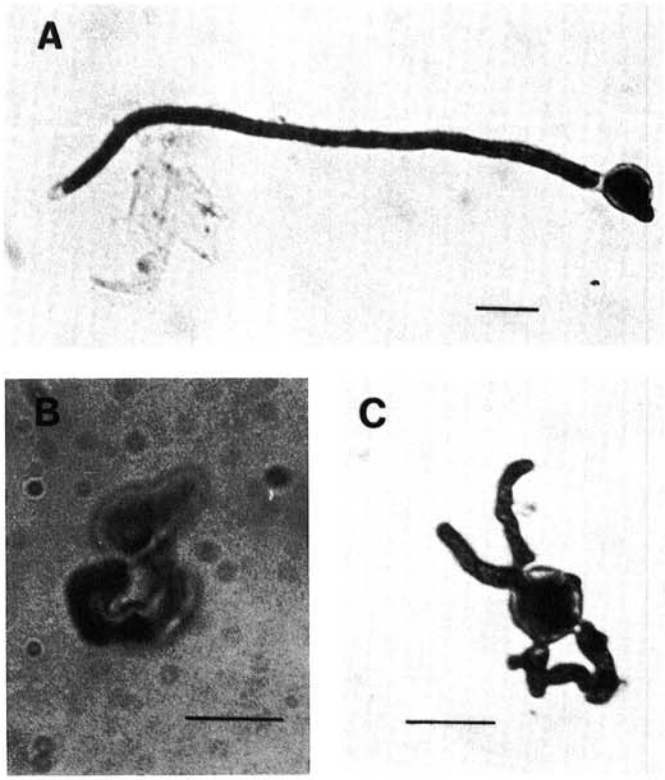

Fig. 2. Germlings of *Penicillium digitatum*: **A,** normal growth in liquid culture medium after 24 hr; **B,** distorted growth at the inoculation site on a benomyl-treated orange after 5 days; and **C,** distorted growth of a germling after 24 hr in liquid culture medium containing 1 μg of carbendazim per milliliter. Horizontal bars represent 10 μm.

benomyl-treated fruit severalfold over comparable fruit inoculated with spores of the resistant strains in water only (Table 1).

DISCUSSION

Bollen (2) discussed the interaction of fungicide-resistant and fungicide-sensitive species under selection pressure and stressed that the fungicide treatment influences not only the sensitive component of the fungus population, but also the resistant component that interacts with it. The most familiar interaction of this type is the predominance of the resistant species when the sensitive species competing for substrate is suppressed by the fungicide treatment. The suppressed sensitive species may also participate actively in the interaction through antibiosis or probiosis (2), as revealed in the present investigation.

Treatment of citrus fruit with benomyl or thiabendazole at 0.5–1.0 g/L prevents infection by benzimidazole-sensitive isolates of *P. digitatum* if the treatment is applied within 24 hr after inoculation (3,16). These treatments are so effective that the presence or absence of sensitive strains of *Penicillium* in the population is rarely considered in discussions of the benzimidazole-resistance problem. The present investigation has revealed, however, that conidia of a benzimidazole-sensitive isolate of *P. digitatum* may germinate in the presence of benomyl or carbendazim and stimulate the infection of treated fruit by resistant isolates in the inoculum population. Sensitive isolates alone cannot infect benomyl-treated fruit. Spores of the sensitive isolate germinating in the presence of benomyl or carbendazim produced stunted and distorted germlings similar to those of *P. atrovenatum* exposed to thiabendazole (10). The benomyl treatment greatly reduced the infectivity of the sensitive isolate, but did not prevent the production of pectolytic enzymes by the germinating spores. Pectinases associated with the pathogenesis of *P. digitatum* (4) apparently are secreted by the benomyl-inhibited germlings and degrade the walls of cells surrounding the inoculation site, releasing nutrients that increase the infectivity of the benzimidazole-resistant spores in the inoculum. Kavanagh and Wood (13) demonstrated that several constituents of orange peel significantly increased the infectivity of conidia of *P. digitatum*.

Several synergistic interactions in pathogenesis by fungi have been described (6,8) and this phenomenon may be more common than implied by the paucity of the literature on the subject. Garrett (9) suggested that synergism during the infection process is probable when the inoculum consists of spore aggregates (eg, as in the inoculation of oranges with a suspension of spores of *P. digitatum*). Synergism is most apparent when one of the interacting microorganisms is inhibited by some environmental factor, yet retains the ability to stimulate the pathogenic activities of the associated microorganism. Savastano and Fawcett (17) demonstrated a strong synergistic interaction between *P. digitatum* and *Geotrichum candidum* when oranges inoculated with a mixture of the two species were incubated at a temperature that was optimum for *G. candidum*, but inhibitory to *P. digitatum*. Morris et al (15) reported a synergistic interaction between the same two pathogens in oranges treated with benomyl, thereby suppressing selectively the development of *P. digitatum*.

Wild (18) found that the percentage of resistant spores in the population of *P. digitatum* decreased on untreated oranges that were inoculated with a mixture of benzimidazole-resistant and benzimidazole-sensitive spores. This observation suggests that abstaining from the use of benomyl might be helpful in managing the practical problem of benzimidazole-resistance in *P. digitatum* (12). However, the present investigation revealed that the incidence of decay in benomyl-treated oranges depends not only on the number of resistant spores in the inoculum, but also upon the presence of sensitive spores. The synergistic interaction between resistant and sensitive spores dictates that sanitation to decimate both types of spores must play a major role in any strategy aimed at improving the effectiveness of benzimidazole fungicides on citrus fruits. The participation of pectolytic enzymes in the synergistic interaction suggests further that inhibitors of these enzymes (1,5) might improve the performance of benzimidazole fungicides against Penicillium decay when resistant isolates are present at a low level in the spore population.

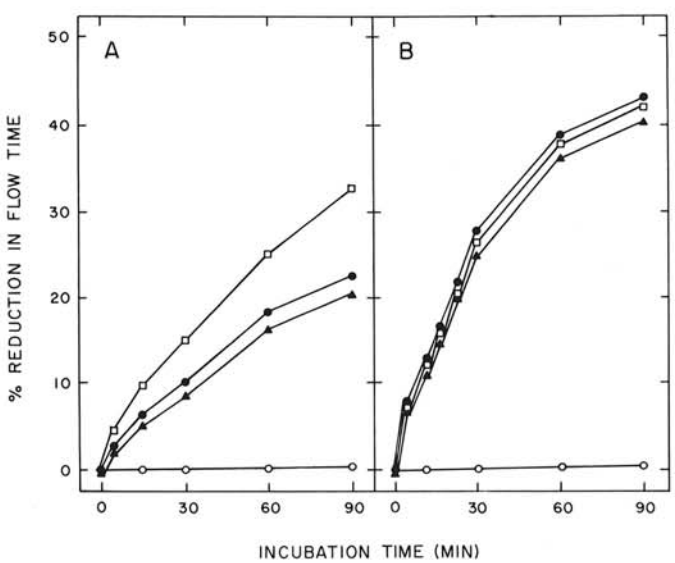

Fig. 3. Reduction in viscosity of pectin solutions by extracellular enzymes produced during a 24-hr incubation period by germlings of *Penicillium digitatum* (benzimidazole-sensitive isolate M6R). Dialysate of filtrate of: (□) culture without carbendazim, (●) culture with 1 μg of carbendazim per milliliter, (▲) culture with 2 μg/ml carbendazim, (○) nutrient medium only. **A,** Carbendazim added to spore suspension at the beginning of the incubation period; **B,** carbendazim added to germlings after 12 hr of incubation.

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