Growth, Sporulation, and Virulence of Isolates of *Penicillium digitatum* Resistant to the Fungicide *sec*-Butylamine

J. L. Smilanick 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 first author: Horticultural Crops Research Laboratory, USDA-ARS, 2021 South Peach Avenue, Fresno, CA 93727.

This research was supported in part by the California Citrus Research Board and the U.S.–Israel Binational Research and Development Fund (BARD).

We thank J. F. Petri for advice on monitoring spore populations in citrus packinghouses and M. J. Kolbezen and B. Bretschneider for advice and assistance in certain phases of the work.

Portion of a Ph.D. dissertation submitted by the senior author to the University of California, Riverside.

Accepted for publication 6 March 1986.

ABSTRACT

Smilanick, J. L., and Eckert, J. W. 1986. Growth, sporulation, and virulence of isolates of *Penicillium digitatum* resistant to the fungicide *sec*-butylamine. Phytopathology 76:805-808.

Isolates of *Penicillium digitatum* resistant to the fungicide *sec*-butylamine (SBA) were obtained by ultraviolet irradiation or collection from natural resistant populations in citrus packinghouses. Resistant isolates were not controlled on inoculated lemons by practical SBA fruit treatments. The EC_{50} in liquid culture was 1–2 μmol/ml and 10–40 μmol/ml SBA for sensitive and resistant isolates, respectively. The growth rates, sporulation, and virulence of resistant and sensitive isolates were similar both in culture and in untreated lemons. The relative fitness of the isolates was tested by inoculating fungicide-free lemons with a 1:1 mixture of spores of two isolates and measuring the frequency of SBA-resistance in the spores collected from the diseased fruit. In 27 pairs of resistant and sensitive isolates, followed for four spore generations, the proportion of the resistant spores increased in 19, decreased in five, and did not change in three pairs. These results suggest that the acquisition of SBA resistance is not usually accompanied by a decrease in the vigor or parasitic fitness of *P. digitatum*.

The buildup of variants of *Penicillium digitatum* Sacc. that are resistant to postharvest fungicides has become a serious problem in marketing citrus fruits worldwide (3,5,9,10). An effective strategy to suppress the level of resistance in the pathogen population must consider the pathogenic fitness, reproduction rate, and dispersal of the sensitive and resistant isolates of the pathogen. Wild (19) showed that benzimidazole-resistant isolates of *P. digitatum* were less fit than sensitive isolates and did not persist in a mixed population with the latter throughout several disease cycles in oranges that were not treated with a benzimidazole fungicide. *sec*-Butylamine (SBA)-resistant isolates of *P. digitatum*, however, appear to behave differently. Observations in commercial packinghouses on the dynamics of populations of *P. digitatum* indicated that SBA-resistant isolates could persist for at least 5 yr after the SBA treatment was discontinued (9). Our investigation compared growth, sporulation, and virulence of SBA-sensitive and SBA-resistant isolates that could influence their persistence in the pathogen population in the absence of fungicide selection pressure.

MATERIALS AND METHODS

Cultures. SBA-resistant and SBA-sensitive isolates of *P. digitatum* were obtained by exposure of petri plates containing a selective medium (16) for 2–5 min to the atmosphere of citrus packinghouses or adjacent groves in Southern California or Arizona. Isolates were classified as SBA-resistant if the spores germinated >90% on potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 500 μg/ml SBA and grew freely on PDA with 5,000 μg/ml SBA. SBA-sensitive isolates neither germinated nor grew on PDA amended with more than 300 μg/ml SBA. In addition to isolates collected from the environment, resistant mutants were produced by irradiation of our sensitive isolate M6R. Spores suspended in 0.01% Triton X-100 were exposed to a dosage of ultraviolet (UV) light that killed approximately 90% of the spores. The irradiated spores were streaked onto PDA amended with 1,000 μg/ml SBA and the cultures incubated at 25 C for 4 days in darkness. Of 40 colonies examined, six had luxuriant growth and sporulation, normal pigmentation, and maintained SBA-resistance after several transfers. These isolates were designated UV4, UV13, UV19, UV23, UV27, and UV34. All isolates used in these experiments were single-spored and stored on silica gel (17) at 5 C. They were recultured on PDA for 7–10 days at 25 C when needed. To produce large amounts of spores, lemons were surface-disinfested in 70% ethanol for 1 min and aseptically inoculated with an isolate of *P. digitatum*. Five inoculated fruit were incubated at 25 C for 10–14 days in sterile fiber cartons, when spores were removed with a brush and stored over silica gel at 1 C.

Chemicals. SBA sulfate was prepared by alkaline distillation from a commercial formulation (Deccotane, 25% a.i., Pennwalt Corp., Monrovia, CA) and neutralized with H_2SO_4. The distilled amine solution was used to determine EC_{50} values in liquid cultures, and the Deccotane formulation was used for fruit dip treatments. The concentration of SBA in these solutions was determined by distillation and titration of the amine with HCl.

Growth rate of *P. digitatum*. Dry spores of each isolate were suspended (1 mg/ml) in an orange juice-asparagine-salts medium (2), pH 4.5, and the cultures incubated at 27 C for 12 hr on a rotary shaker (2.5-cm displacement; 180 rpm). The germlings were filtered from the medium and washed twice with distilled water and once with fresh orange juice medium. The germlings were then resuspended at 5 mg/ml (fresh weight) in the same medium and the cultures equilibrated for 30 min at 25 C on a reciprocating shaker (3.8-cm displacement; 120 strokes per minute) before the addition of SBA. Four replicate samples of the cultures were removed immediately and 5 hr after the SBA was added. They were filtered through glass-fiber filters, dried for 24 hr at 90 C, and weighed. The EC_{50} for SBA (concentration that decreased the growth rate 50%) was determined from the dry weight of hyphae produced over the 5-hr period in cultures containing a range of SBA concentrations (0.25–0.40 mM). The EC_{50} was estimated by the regression analysis

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

procedure of Goldstein (6) for nonquantal bioassays. The EC_{50} for one sensitive and two resistant isolates was measured on three occasions to estimate the 95% confidence interval. Growth rates of the isolates without SBA were determined from the dry weights of the control treatments after 5 hr from the above experiment. These were compared by a one-way analysis of variance procedure followed by Duncan's multiple range test.

Disease control. Twenty surface-disinfested lemons were inoculated with an isolate of *P. digitatum* by means of a steel puncture tool (1 mm diameter × 2.5 mm deep puncture), dipped into a suspension of 10^6 spores per milliliter in 0.01% Triton X-100. The lemons were incubated at 23 C for 12 hr and immersed 1 min in 2% (w/v) SBA in 0.01% Triton X-100 adjusted to pH 8, or in the Triton X-100 solution without SBA. Fiber boxes containing treated fruit were randomly stacked and stored at 25 C for 10 days and the decayed fruit counted.

Development of *P. digitatum* in infected lemons. Lemons, selected from commercial harvest bins for similar size (9 cm length × 6 cm diameter) and smooth surface texture, were surface-disinfested and aseptically injected 2.5 mm deep into the peel with 5 μl of a suspension of 10^6 spores per milliliter in 0.01% Triton X-100. The fruit were incubated at 25 C in stainless steel trays. The diameters of 12 decay lesions for each of the 15 isolates were measured 72 and 80 hr after inoculation. Sporulation of the isolates on each of five diseased fruit was measured after 7 days. Two 7-mm-diameter plugs of the decayed peel, removed 20 mm from the inoculation site, were placed in 25 ml of a polyglycol ether surfactant (0.1% Tergitol XD). The solution was agitated to free the spores, and the optical density of the suspension was measured at 425 nm. Spore concentration was estimated by reference to a standard curve relating optical density to *P. digitatum* spore concentration measured with a hemacytometer. A suspension of 1.5×10^6 spores per milliliter had an optical density of 0.1. The lesion expansion rates and spores produced per square millimeter were independently analyzed using a one-way analysis of variance procedure followed by Duncan's multiple range test.

Inoculations with mixtures of isolates of *P. digitatum*. Six sensitive isolates and nine resistant isolates were matched in 27 unique resistant-sensitive isolate pairs to evaluate the competitive behavior of each isolate in decaying fruit that was not treated with SBA. SBA-sensitive and natural SBA-resistant isolates used in the experiment are characterized in Figures 1–3. Two additional sensitive isolates were included in this experiment to increase the size of the pathogen population characterized. Isolates from silica gel stocks were cultured simultaneously on PDA for 1 wk at 25 C. The spores of each isolate were suspended in 0.01% Triton X-100, the suspensions adjusted to 10^6 spores per milliliter, and equal volumes of two isolates were combined so that the inoculum consisted of exactly 0.5×10^6 spores per milliliter of a sensitive and a resistant isolate, respectively. Five μl of this inoculum was injected 2.5 mm into the peel of four surface-disinfested lemons and the fruit were incubated in sterilized steel trays at 25 C 1 wk. Spores were collected from the surface of the decayed fruit, the suspension adjusted to 10^6 spores per milliliter, and 5 μl was injected into another lot of lemons. The number of viable spores of the resistant and sensitive isolates in the initial inoculum and in each successive crop of spores was determined by plating spore samples collected on a sterile cotton swab 20 mm from the inoculation point onto both PDA and PDA amended with SBA at 500 μg per milliliter. The media contained 3 μg of dicloran per milliliter of medium to suppress colony expansion and merging. Colonies were counted after 3 days incubation at 25 C. The proportion of resistant spores was determined in this manner in samples collected in four successive disease cycles.

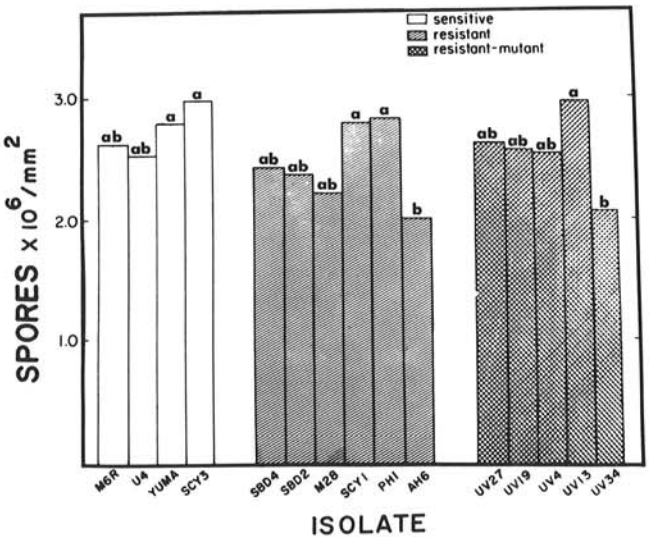

Fig. 1. Effect of a commercial *sec*-butylamine (SBA) treatment (2% w/v) on decay of lemons inoculated with SBA-sensitive, naturally SBA-resistant, and SBA-resistant UV-mutants of *Penicillium digitatum*. Decayed fruit were counted after incubation of the fruit at 25 C for 10 days.

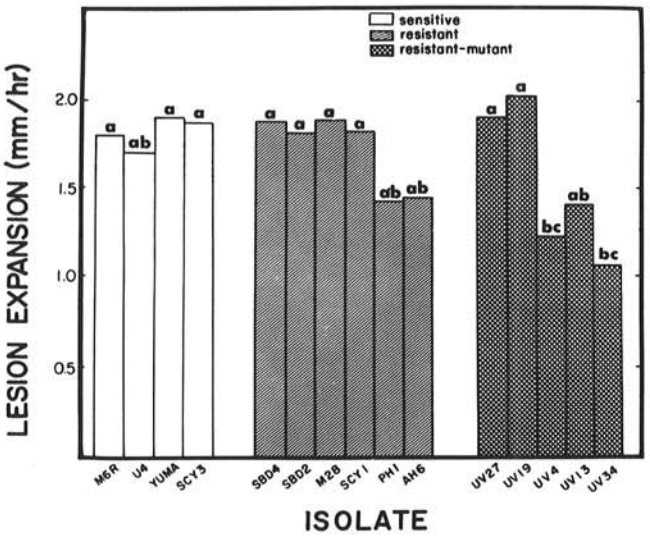

Fig. 2. Expansion of decay lesions at 25 C in lemons inoculated with *sec*-butylamine (SBA)-sensitive, naturally SBA-resistant, and SBA-resistant UV-mutants of *Penicillium digitatum*. Bars having the same letter are not significantly different at $P \leq 0.05$.

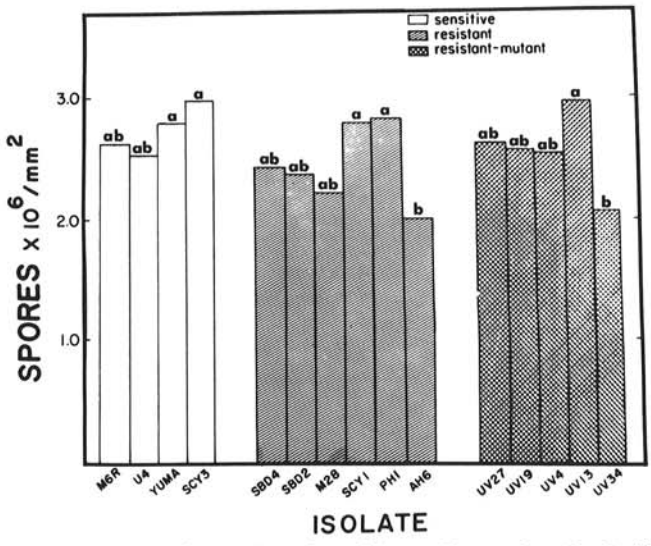

Fig. 3. Spore production on the surface of decayed lemons inoculated with *sec*-butylamine (SBA)-sensitive, naturally SBA-resistant, and SBA-resistant UV-mutants of *Penicillium digitatum* and incubated 1 wk at 25 C. Bars having the same letter are not significantly different at $P \leq 0.05$.

TABLE 1. EC_{50} values for *sec*-butylamine (SBA)-sensitive, naturally SBA-resistant, and SBA-resistant, UV-mutant isolates of *Penicillium digitatum*[y]

Isolate	SBA-resistance class	EC_{50} (μmol/ml SBA)
M6R	Sensitive	0.99 ± 0.28[z]
U4	Sensitive	1.95
YUMA	Sensitive	0.90
SBD2	Resistant	40.08 ± 0.94[z]
M28	Resistant	18.49
PH1	Resistant	17.98
UV19	Resistant UV-mutant	21.97 ± 1.41[z]
UV13	Resistant UV-mutant	12.16
UV27	Resistant UV-mutant	9.83

[y] Growth in an orange juice-asparagine-salts medium at 25 C determined by dry weight.
[z] Mean of three experiments, ± 95% confidence interval.

RESULTS

Growth and SBA-sensitivity of isolates. The means EC_{50} of the SBA-sensitive, naturally SBA-resistant, and UV-induced SBA-resistant mutants were 1.28 mM, 25.52 mM, and 14.65 mM SBA, respectively (Table 1). Mean growth rates in liquid culture of these isolates were 8.9, 8.6, and 8.8 mg dry weight per milliliter per hour, respectively. These values are not significantly different (Duncan's multiple range test, $P \leq 0.05$). Infection of lemons by the SBA-sensitive isolates was controlled by treatment of inoculated fruit with 2% SBA; the SBA-resistant isolates were not controlled by this treatment (Fig. 1).

Virulence and sporulation of isolates on lemons. Lesion expansion rates of the SBA-sensitive, naturally-resistant, and UV-induced, resistant mutants ranged from 1.7 to 1.9, 1.4 to 1.9, and 1.1 to 2.0 mm per hour, respectively, but each resistance class contained isolates with lesion expansion rates similar to those of sensitive isolates (Fig. 2). The lesion expansion rates of the most SBA-resistant naturally-resistant isolate (SBD2) and UV-induced resistant mutant (UV19) were not significantly different from the SBA-sensitive isolates. Only two isolates, both UV-induced, resistant mutants (UV4 and UV34), produced lesions that expanded at a significantly lower rate ($P \leq 0.05$) than many other isolates tested. Spore production varied among isolates (Fig. 3), but was not related to SBA-sensitivity. Only one naturally resistant isolate (AH6) and one UV-induced, resistant mutant (UV34) produced significantly ($P \leq 0.05$) fewer spores than the isolates with the highest production. One isolate (UV34) was significantly ($P \leq 0.05$) below the most vigorous isolates in both lesion expansion rate and spore production.

Development of isolates in mixed infections. In 27 unique pairs of SBA-sensitive and SBA-resistant isolates inoculated together into lemons, the proportion of spores of the resistant isolate increased over four disease cycles in 19 pairs of isolates, decreased in five pairs, and remained unchanged in three pairs (Fig. 4). One pair, SBA-sensitive isolate M6R combined with UV-induced, resistant mutant isolate UV4 (derived from M6R), did not change in composition from the initial 1:1 ratio of resistant to sensitive spores over four disease cycles.

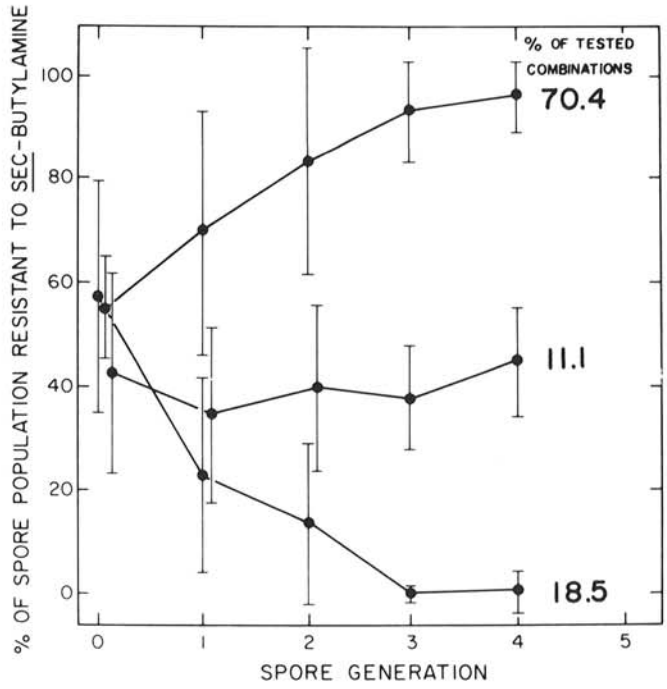

Fig. 4. Competition between 27 pairs of *sec*-butylamine (SBA)-sensitive and SBA-resistant isolates coinoculated on untreated decaying lemons. Each lot of fruit was inoculated with spores from the preceding spore generation. The mean value of each category ± one standard deviation is shown.

DISCUSSION

The persistence of resistant biotypes in the absence of fungicide selection pressure appears to depend on the nature of the resistance and the properties of the individual isolates. After inoculation of untreated plants or fruits with a mixture of spores of fungicide-resistant and fungicide-sensitive isolates, the frequency of benzimidazole-resistant biotypes of *Sclerotinia homoeocarpa* Bennett (18), *P. expansum* Link ex. Thom (12), and *P. digitatum* (19) decreased in successive cycles. Similar experiments showed that isolates of *P. italicum* Wehmer (4) and *P. expansum* (7) that are resistant to ergosterol biosynthesis inhibitors and of *P. expansum* resistant to dicarboximides (7) also decreased in the population when these fungicides were discontinued. Conversely, no significant change was noted in the frequency of cadmium-resistant isolates of *S. homoeocarpa* (18) or benzimidazole-resistant isolates of *P. italicum* (8), *Venturia inaequalis* (Cooke) Wint. (11), *V. pirinia* Aderh. (15), *Cercospora beticola* Sacc. (13), or *Sphaerotheca fuliginiea* (Schlecht.) Pollacci (14) after these fungicides were deleted from the control program. Unfortunately, some conclusions regarding the relative fitness and persistence of fungicide-resistant biotypes in the pathogen population have been based upon experiments involving only one or few resistant isolates. The behavior of individual biotypes may not be representative of the entire subpopulation.

Our investigation showed that isolates of *P. digitatum* can exhibit a 10- to 40-fold greater tolerance to SBA than representative wild types, without a correlated decrease in growth rate, lesion expansion in fruit, in vivo sporulation, or frequency in the spore population throughout four disease cycles on lemon fruit that were not treated with SBA. These resistant isolates cannot be controlled on inoculated fruit treated with the highest concentrations of SBA used in commercial packinghouses. Therefore, SBA-resistant biotypes proliferate rapidly in the pathogen population when citrus fruits are treated after harvest with SBA. The persistence for several years of resistant biotypes in packinghouses after SBA was discontinued can be explained by our observation that in 81.5% of the pairs of resistant/sensitive isolates evaluated, the resistant biotype persisted at the initial frequency or increased in the population over several disease cycles. In contrast to the behavior of many benzimidazole-resistant pathogens, the frequency of SBA-resistant isolates in the *P. digitatum* population cannot be selectively reduced by eliminating the SBA treatment. An effective strategy for reduction of SBA-resistant biotypes in the packinghouse must be based on other methods that reduce the entire *Penicillium* spore population, i.e., equipment sanitation procedures (1) and nonselective fruit treatments such as sodium carbonate solution (43 C) or imazalil, which are effective against both fungicide-resistant and -sensitive strains of *P. digitatum*.

LITERATURE CITED

1. Bancroft, M. N., Gardner, P. D., Eckert, J. W., and Baritelle, J. L.

1984. Comparison of decay control strategies in California lemon packinghouses. Plant Dis. 68:24-28.
2. Bartz, J. A., and Eckert, J. W. 1972. Studies on the mechanism of action of 2-aminobutane. Phytopathology 62:239-246.
3. Dawson, A. J., and Eckert, J. W. 1977. Problems of decay control in marketing citrus fruits: Strategy and solutions, California and Arizona. Proc. Int. Soc. Citric. 1:255-259.
4. DeWaard, M. A., Groeneweg, H., and Van Nistelrooy, J. G. M. 1982. Laboratory resistance to fungicides which inhibit ergosterol biosysnthesis in *Penicillium italicum*. Neth. J. Plant Pathol. 88:99-112.
5. Eckert, J. W., and Wild, B. L. 1983. Problems of fungicide resistance in Penicillium rot of citrus fruits. Pages 525-555 in: Pest Resistance to Pesticides. G. P. Georghiou and T. Saito, eds. Plenum Press, New York. 809 pp.
6. Goldstein, A. 1964. Biostatistics: An Introductory Text. MacMillan Co., New York. 272 pp.
7. Gullino, M. L., and De Waard, M. A. 1984. Laboratory resistance to dicarboximide and ergosterol biosynthesis inhibitors in *Penicillium expansum*. Neth. J. Plant Pathol. 90:177-179.
8. Gutter, Y., Shachnai, A., Schiffmann-Nadel, M., and Dinoor, A. 1981. Biological aspects of citrus molds tolerant to benzimidazole fungicides. Phytopathology 71:482-487.
9. Kaplan, H. J., Dave, B. A., and Petri, J. F. 1981. Tolerance of citrus pathogens to current packinghouse treatment. Proc. Int. Soc. Citric. 2:788-791.
10. McDonald, R. E., Risse, L. A., and Hillebrand, B. M. 1979. Resistance to thiabendazole and benomyl of *Penicillium digitatum* and *P. italicum* isolated from citrus fruit from several countries. J. Am. Soc. Hortic. Sci. 104:333-335.
11. McGee, D. C., and Zuck, M. G. 1981. Competition between benomyl-resistant and sensitive strains of *Venturia inaequalis* on apple seedlings. Phytopathology 71:529-532.
12. Prusky, D., Bazak, M., and Ben-Aire, R. 1985. Development, persistence, survival, and strategies for control of thiabendazole-resistant strains of *Penicillium expansum* on pome fruits. Phytopathology 75:877-882.
13. Ruppel, E. G., Jenkins, A. D., and Burtch, L. M. 1980. Persistence of benomyl-tolerant strains of *Cercospora beticola* in the absence of benomyl. Phytopathology 70:25-26.
14. Schepers, H. T. A. M. 1984. Persistence of resistance to fungicides in *Sphaerotheca fuliginea*. Neth. J. Plant Pathol. 90:165-171.
15. Shabi, E., and Katan, T. 1980. Fitness of *Venturia pirina* isolates resistant to benzimidazole fungicides. Phytopathology 70:1172-1174.
16. Smilanick, J. L., and Eckert, J. W. 1982. A selective medium for *Penicillium digitatum* (Sacc.), citrus green mold. (Abstr.) Phytopathology 72:1008.
17. Trollope, D. R. 1975. The preservation of bacteria and fungi on anhydrous silica gel: An assessment of survival over four years. J. Appl. Bacteriol. 38:115-120.
18. Warren, C. G., Sanders, P. L., Cole, H., Jr., and Duich, J. M. 1977. Relative fitness of benzimidazole- and cadmium-tolerant populations of *Sclerotinia homoeocarpa* in the absence and presence of fungicides. Phytopathology 67:704-708.
19. Wild, B. L. 1980. Resistance to citrus green mold *Penicillium digitatum* Sacc. to benzimidazole fungicides. Ph.D. dissertation. University of California, Riverside. 89 pp.