Tolerance of Verticillium malthousei to Benomyl

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ABSTRACT

Three isolates of *Verticillium malthousei,* ML2, ML4, BC69, were evaluated for benomyl sensitivity. Isolate response in the presence of benomyl measured by linear growth, sporulation, and germinability of spores harvested from benomyl-amended plates of potato-dextrose agar was consistent. Isolate ML2 was benomyl-tolerant, whereas ML4 and BC69 were benomyl-sensitive. When *Agaricus bisporus* sporocarps were dipped into benomyl and inoculated with the isolates, results coincided with the three in vitro tests. However, ML2 was neither an aggressive nor virulent pathogen which suggests that this benomyl-tolerant isolate has little epidemiological significance. The 1958 collection date of ML2 is significant since it antecedes the introduction and use of benomyl. Since one isolate from the natural *V. malthousei* population was benomyl-tolerant, other benomyl tolerant isolates may be a threat to mushroom farms where *Verticillium* is controlled with benomyl.

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Fungitoxicity of benzimidazole compounds (3) to Deuteromycetes suggested that Verticillium disease (17, 18) of mushrooms might be controlled by benomyl. The effectiveness of benomyl for control of this disease has been confirmed (4, 8, 16, 19, 20, 21). Initial screening of fourteen *Verticillium malthousei* Ware isolates suggested that variations in benomyl sensitivity existed in naturally occurring strains of the pathogen (20) prior to commercial use of benomyl.

Fungicide tolerance may be expressed various ways. MacKenzie (11) and MacKenzie et al. (12) reported that single gene tolerance of *Cochliobolus carbonum* Nelson to cadmium compounds and the cycloheximide antibiotic Actidione was expressed as a 2- to 3-fold increase in growth rate and a reduction in spore germination on fungicide-amended agar. When corn was inoculated with tolerant and sensitive isolates, and plot areas were sprayed with cadmium or Actidione fungicides, the sensitive isolates predominated.This result contradicts the theoretical advantage provided by fungicide tolerance.

Goldberg and Cole (6) reported a complete failure with benomyl applied to control *Sclerotinia homoeocarpa* on a commercial golf course. Fungal isolates from the problem area grew normally on benomyl-amended agar containing 100× greater concn than that which completely suppressed a sensitive isolate. In this instance, the population of *S. homoeocarpa* on the golf course was benomyl-tolerant and the sensitive isolates were absent.

Bollen and Scholten (2) reported in 1971 that cyclamen soft rot caused by *Botrytis cinerea* was not controlled by benomyl. In this instance, tolerant isolates were able to grow on benomyl-amended agar at 2,000× the concn required for complete inhibition of sensitive isolates.

Our report concerns the influence of benomyl on linear growth and sporulation. In addition, germination of spores harvested from cultures grown on benomyl-amended agar and signs of the pathogen which developed following inoculation of benomyl-treated suscept tissue were investigated with selected *V. malthousei* isolates. All isolates antecede the introduction and use of benomyl. As such, these isolates allowed the occurrence of fungicide tolerance to be evaluated prior to selection pressure on the natural population of *V. malthousei* by benomyl.

MATERIALS AND METHODS.—The isolates of *V. malthousei* Ware used in this study were obtained from diseased sporocarps of *Agaricus bisporus* (Lange)

Imbach and represent the spectrum of response reported for fourteen isolates (20). Isolate ML2 was from a white Pennsylvania mushroom collected in 1958; isolate BC69 originated from a golden-white Pennsylvania mushroom collected at the Butler County Mushroom Farm, Inc. in 1969; and ML4 was from a cream-colored California mushroom collected in 1965. Cultures were maintained by mass transfer onto potato-dextrose, yeast-extract agar (PDYA) every 60 days, and each isolate was single-spored prior to its use in these experiments.

Isolates ML4 and BC69 were typical of *Verticillium malthousei* Ware (18). Isolate ML2 was atypical and possessed a number of asymmetric phialospores. In comparative studies using an isolate of *V. psalliotae* Treschow from the Commonwealth Mycological Institute, Kew, England, ML2 seemed to be intermediate between *V. malthousei* and *V. psalliotae*. Both species reportedly cause a disease of *A. bisporus* (17, 18). As a result of our observations, and those of Fassatiova (5), we considered all three isolates to be *V. malthousei*.

Growth of isolates was measured on potato-dextrose agar (PDA) after 14 days incubation at ca. 22 C under continuous fluorescent light. The PDA was amended with benomyl at 0, 1, and 10 μg/ml of medium prior to plate pouring. The plates were centrally seeded with 4.5-cm diam disks cut from the periphery of 14-day-old PDA cultures. Each treatment consisted of three replicate plates for each isolate at each fungicide concn; the experiment was repeated three times, and the data from one representative experiment are presented.

Sporulation of ML2 and BC69 was determined by spreading 0.1 ml of an aqueous spore suspension (2×10^5 spores/ml) from 7-day-old PDA cultures onto PDA plates infused with 0, 1, and 10 μg/ml benomyl. Test plates were incubated for 8 days at room temp after which they were flooded with 10 ml of water, and the spore concn ascertained with the aid of a haemocytometer. Three individual counts were made for each isolate and treatment; this experiment was conducted twice.

To determine the germination of ML2 and ML4 spores harvested from PDA plates amended with 0, 1, and 10 μg/ml benomyl, four drops of an aqueous spore suspension containing 2.0×10^5 spores/ml were spread over the surface of 2% water agar contained within a 50-mm diam plastic, sealable petri plate. Germination was ascertained after 18 h at 12 C in the dark. Spores in 12 microscope fields at ×100 magnification were counted and percentage germination calculated. Each treatment was replicated three times and the entire experiment was repeated once. Data from one experiment are presented in the results section for this and the sporulation study.

Data from the preceding experiments were analyzed by analysis of variance and means separated at the 0.05 level using Duncan's Multiple Range Test.

Mature (veil tightly closed) sporocarps of PSU 310, *A. bisporus,* were harvested. Within 4 h of harvest eight sporocarps were immersed for 5 min into an agitated aqueous system containing 0, 100, or 1,000 μg/ml benomyl. Treated mushrooms were air-dried and an aqueous spore suspension of either ML2, ML4, or BC69 containing 1.5×10^5 spores/ml was spread over the surface with a curved stirring rod. The mushrooms were divided randomly into two groups of four and were incubated in moist chambers at 18 C in the dark for 3 days. Superficial mycelial development by the pathogen was indexed by a system which accounted for both presence of mycelium and/or sporulation and their intensity of development. These were valued as follows: no aerial mycelium = 1; aerial mycelium present = 2; aerial mycelium with branching = 3; aerial mycelium, branching, plus visible sporulation = 4. The intensity of mycelial development and/or sporulation was rated according to the following system: none = 1; very few = 2; sparse = 3; moderate = 4; abundant = 5. The index was obtained by the formula: (no. of mushrooms) × (sum of pathogen signs) × (sum of sign intensity) = (index of signs).

This experiment was repeated five times during the period between August 1971 and March 1972. Data presented are from one experiment and are typical of results obtained in the other experiments.

RESULTS.—Mean colony diam of the three isolates following 14 days of incubation varied between 47 and 70 mm on nonamended PDA plates. BC69 and ML4 had significantly more radial growth than ML2 (Fig. 1). In plates amended with 1 and 10 μg/ml benomyl, however, growth of ML2 was greater than that of the other two isolates.

Sporulation of ML2 and BC69 differed by a magnitude of 160× (Fig. 2) with ca. 3,700 spores from BC69 and 23 from ML2 both reduced by 1×10^5 after 8 days from nonamended PDA plates. During incubation in the presence of 1 and 10 μg/ml of benomyl, the difference in magnitude of sporulation was reduced to 64× and 12×, respectively. Thus, the effect of benomyl on sporulation of ML2 was proportionately less than on BC69.

Germination of ML2 spores harvested from cultures grown on PDA alone was almost 50% less than that of ML4 on similar nonamended control plates (Fig. 3). In the presence of benomyl, the reduction in germination expressed as percent of germination on nonamended check demonstrated that ML4 was affected to a greater extent than ML2; 71 vs. 26% at 1 μg/ml and 68 vs. 56% at 10 μg/ml.

ML2 possessed the lowest index of signs (Fig. 4). ML4 and BC69 had higher and similar indices on untreated mushrooms. Mushrooms treated with benomyl supported a higher index rating with ML4 and BC69 than ML2. When the data are considered as percent reduction compared to control, there was no reduction for ML2 at 100 μg/ml and 25% reduction at 1,000 μg/ml. The index for ML4 and BC69 was reduced by 54 and 37% of their respective controls at 100 μg/ml. At the higher concn, 1,000 μg/ml, the reduction for ML4 and BC69 was 66 and 41%, respectively.

DISCUSSION.—Results of the four different experimental procedures were similar; in all tests, ML2 exhibited greater benomyl tolerance than the other isolates. This type of benomyl tolerance was probably present in the natural population since the isolates were collected before benomyl was developed or used at commercial mushroom farms. The significance of such tolerance in a commercial situation is open to question, since ML2 is an anomalous isolate.

Information allowing insight to the mechanism of action of benomyl appears in Fig. 3. Spores harvested

from cultures grown on benomyl-amended agar did not germinate as well as spores from nonamended plates. Benomyl may be systemic within the fungus as has been demonstrated in green plants (13). If benomyl is systemic, it may affect the reproductive physiology of *V. malthousei* by impairing either spore formation, the germination process, or both. Further investigations are needed to explain the observed results. One can assume with cautious certainty that little, if any, benomyl was present in the supernatant; the low water-solubility of benomyl coupled with the amount of water added to adjust spore concns lends credence to this assumption.

Fungicide tolerance is well documented in laboratory experiments (1, 7, 9, 10, 15), although its origin is unknown. Use of "acquired tolerance" (2) to describe this phenomenon is presumptuous. Few reports exist

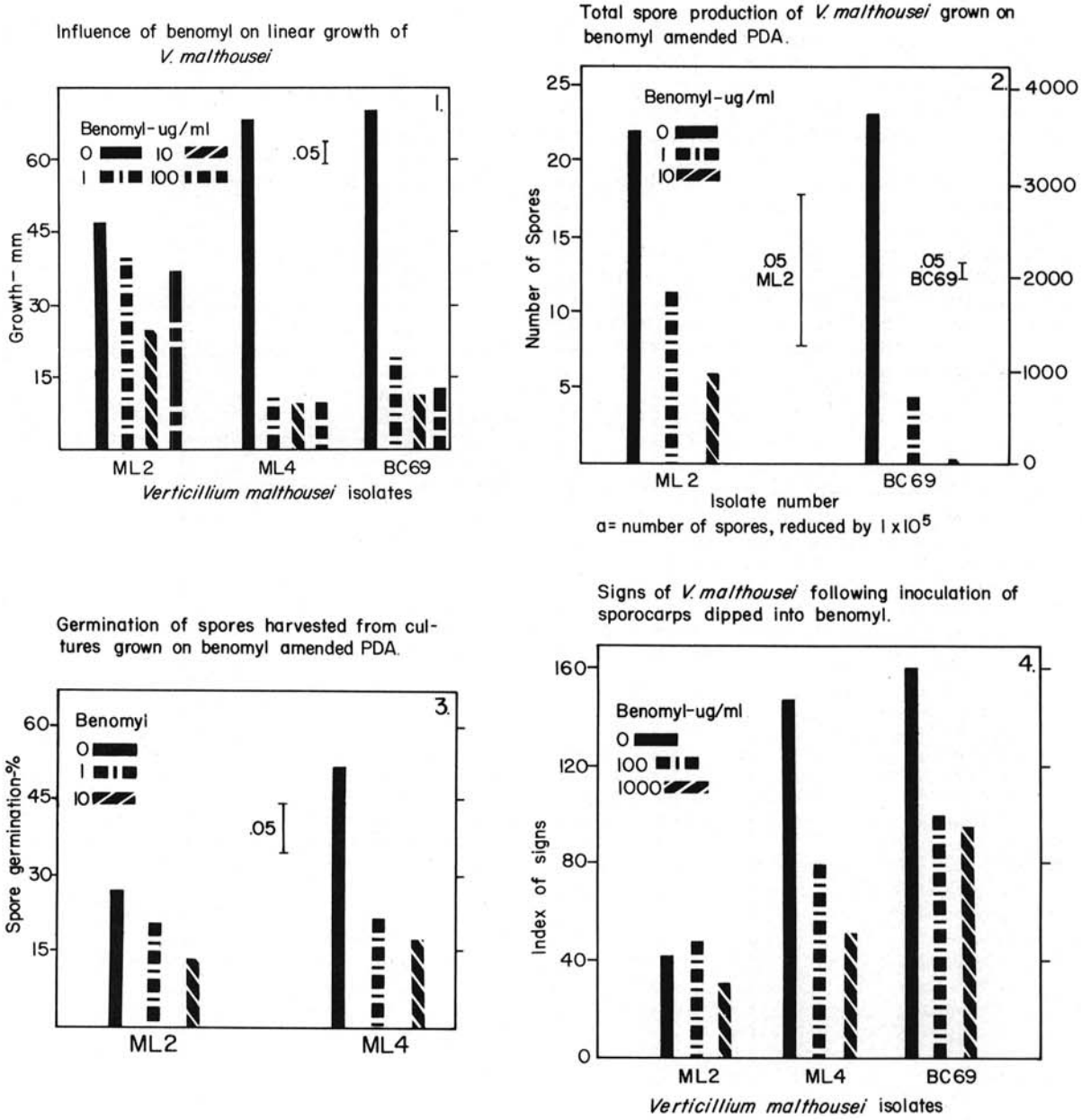

Fig. 1-4. 1) Linear growth of two *Verticillium malthousei* isolates on potato-dextrose agar amended with benomyl. **2)** Spore production per plate of two *V. malthousei* isolates grown on benomyl-amended, potato-dextrose agar. Spore production reduced by 1×10^5; note that the left axis refers to isolate ML2 and right axis to isolate BC69. **3)** Germination of *V. malthousei* spores harvested from two isolates grown on benomyl-amended, potato-dextrose agar. **4)** Index of signs of three *V. malthousei* isolates following inoculation of *Agaricus bisporus* sporocarps dipped into benomyl. The index is based on the presence and intensity of mycelium and/or sporulation.

regarding the survival potential (fitness) and pathogenicity of fungicide-tolerant isolates in field experiments (12, 14). This facet of fungicide tolerance is of great significance.

Fungicide tolerance of *V. malthousei* and *Cochliobolus carbonum* (11) were similar in the laboratory. The fungicide-tolerant isolates of *C. carbonum* neither proliferated nor remained a part of the pathogen population under field conditions irrespective of fungicide applications or lack thereof. This suggests that introducing *V. malthousei* isolates ML2 and ML4 or BC69 into a commercial mushroom production facility might result in the disappearance of ML2 from the population whether benomyl was used for disease control or not. Realizing that ML2 demonstrated less reproductive and pathogenic capacity than either ML4 or BC69 supports this position.

Other reports and my research results indicate that for fungicide tolerance to be of field significance, an isolate must demonstrate a measure of tolerance in culture combined with fitness characteristics necessary for the pathogen to survive. Such factors as aggressiveness and virulence, along with benomyl tolerance will determine whether Verticillium disease of mushrooms will be controlled under widespread commercial conditions as effectively in years hence as has been reported (4, 8) in the past.

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