Acid Production, a Possible Basis for Benomyl Tolerance in Verticillium malthousei

D. H. Lambert and P. J. Wuest

Graduate Assistant and Professor, respectively, Department of Plant Pathology, 211 Buckhout Laboratory, The Pennsylvania State University, University Park 16802.

Contribution No. 832 from the Department of Plant Pathology, The Pennsylvania Agricultural Experiment Station. Authorized for publication 8 January 1975 as Journal Series Paper No. 4794.

Accepted for publication 23 January 1976.

ABSTRACT

LAMBERT, D. H., and P. J. WUEST. 1976. Acid production, a possible basis for benomyl tolerance in Verticillium malthousei. Phytopathology 66: 1144-1147.

Benomyl tolerance for 12 strains of *Verticillium malthousei* was associated closely with ability to produce acid; e.g., for supplemented Czapek Dox broths, the pH's of culture filtrates of tolerant strains ranged from 3.5 to 5.4 vs. 6.2 to 7.4 for culture filtrates of sensitive strains. Benomyl effectiveness also was altered by buffering of media to differing pH. These results suggest that for this species, benomyl tolerance is not related directly to the fungicide's mode of action, but may result partially or entirely from an alteration in fungal metabolism that conditions decreased sensitivity to benomyl, possibly by a pH-regulated reduction in uptake or binding.

Additional key words: mushroom disease.

Benomyl, methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate breaks down in water to its primary active form methyl-2-benzimidazolecarbamate (MBC) (2, 12). Benomyl appears to inhibit nucleic acid replication, possibly by interfering with spindle-fiber function during cell division (4, 6). Benomyl tolerance, which results in loss of disease control by that fungicide, is now common in many fungi.

Verticillium malthousei Ware, the pathogen considered in this paper, infects the commercial mushroom *Agaricus bisporus* (Lange) Imbach causing "Verticillium disease." Tolerance of this pathogen to benomyl often has occurred at commercial mushroom farms in as little as 9 months or less of continued benomyl usage. The variability in the response of *V. malthousei* to benomyl has been investigated since 1970 (1, 8, 9, 10, 17, 19, 20), although strains with a high level of tolerance were not common in the Pennsylvania mushroom industry until 1973.

Preliminary investigations have indicated that benomyl-tolerant strains have greater sensitivity to 8-azaguanine and lower sensitivity to Zn^{+2}, Mn^{+2}, Cd^{+2}, and Na_2-EDTA (7), with a variable response to Fe, which is dependent upon ion concentration and initial valence. Inasmuch as most of these responses may be explained by possible differences in medium acidity, the relationship of pH to benomyl tolerance was investigated and is reported here.

MATERIALS AND METHODS

Twelve strains of *V. malthousei* were selected, including four isolated in 1973 which were highly tolerant of benomyl–O1, P5, T1, and T5, the latter from California and the others from different localities in Pennsylvania; and eight strains either highly sensitive or with a slight amount of tolerance–B0, C7, G1, 90, 87, E1, S1, and K2, isolated prior to 1973 in Pennsylvania, or in the latter three cases, England, Switzerland, and Korea, respectively. These isolates are available from the American Type Culture Collection (ATCC) as *V. fungicola* Gams (syn. *malthousei* Ware) with accession numbers 32837 to 32849 excluding 32841 in the following order: B0, C7, E1, G1, K2, O1, P5, S1, T1, T5, 87, and 90.

Three solid media were used, all with Czapek Dox sucrose base (CDA). The first medium was amended with 5 μM benomyl, 10 dishes per treatment per strain. These were used in experiments to establish levels of tolerance for each strain. A medium used for dose-response curves contained 0, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0, 50, or 100 μM benomyl per liter of medium. Three dishes per concentration for the three representative strains (viz., benomyl-sensitive G1, intermediate 87, and tolerant T5) were used. The third medium, used for determining the effect of medium pH on tolerance, was amended with 0 or 5 μM benomyl, and the pH was adjusted to 4.5, 5.5, 6.5, or 7.5 with 0.5 M NaH_2PO_4 + Na_2HPO_4 with two petri dishes per treatment per strain. All amendments were added after autoclaving, and the media were inoculated with 5-mm diameter plugs from the periphery of 2-week-old cultures. Tolerance in each case was computed by dividing the diameters of the colonies on benomyl-containing media by the colony diameters on corresponding media without benomyl. Five millimeters was subtracted from the actual diameters to compensate for the inoculum plugs. Data were collected after 13 days incubation at 20 C.

To assess acid production, two liquid media were used. Medium A contained 15 g mannitol (the predominant mushroom sugar), 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeCl_3$, and 5 g each of Difco yeast extract and nutrient broth per liter. Medium B contained 30 g sucrose, 2 g $NaNO_3$, the other minerals used in Medium

Copyright © 1976 The American Phytopathological Society, 3340 Pilot Knob Road, St. Paul, MN 55121. All rights reserved.

A, plus 0.5 g each of yeast extract and nutrient broth base. Five flasks containing 100 ml of Medium A and four replicates of Medium B received 5-mm diameter agar plugs from sporulating cultures and were held for 10 days at 20 C without shaking. Equivalent flasks of each medium that did not receive fungus plugs served as controls. After incubation, the mycelium was removed and the culture filtrate pH was recorded.

Subsequently, four tolerant and four sensitive strains of *Sclerotinia homeocarpa* were screened in Medium A, and four tolerant strains (obtained from A. H. McCain) and four sensitive strains of *Botrytis cineria* were screened on Medium B.

In a final experiment, liquid Medium B was buffered to pH's 3.5, 5.0, 6.5, and 8.0 with 0.25 M phosphates containing equal concentrations of Na^+ and K^+ ions. Five-millimeter disks of tolerant strains P5, O1, T1, and T5 and sensitive strains B0, G1, C7, and E1 were added to flasks with and without 5 μM benomyl for each pH. The broth pH and mycelium dry weight from each flask were determined following 14 days incubation. After these procedures, the broths remaining from the pH 5.0 and 6.5 treatments were saved and adjusted to pH 6.5 with Na/K hydroxide or phosphoric acid, diluted to half-strength with distilled water and supplemented with 23 g/liter of Difco nutrient agar base. These media were autoclaved, poured into three petri dishes per treatment, and received 5-mm diameter plugs from the margin of benomyl-sensitive strain B0. Linear growth of this strain on the various media was measured after 15 days and percent tolerance was calculated for each of the original pH-strain combinations. The degree of inhibition due to residual benomyl was compared to the mycelial dry weights and percent tolerances from the original broth cultures by covariance analysis.

Data from the series of phosphate buffer dishes and broths were analyzed with a paired *t*-test with different pH levels compared and like strains paired. Other tolerances and pH measurements were analyzed by Duncan's (Bayesian) least significant difference test (DMLSD).

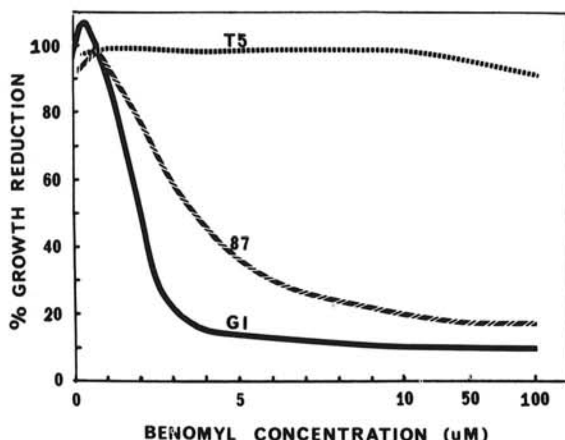

Fig. 1. Dosage-response curves of three *Verticillium malthousei* strains sensitive (G1), tolerant (T5), or intermediate (87) in their response to benomyl.

RESULTS

For the three representative strains, ED_{50}'s were 2 μM for the sensitive strain, 4 μM for the strain with slight tolerance, and more than 100 μM for the strain with high tolerance (Fig. 1). Growth rate of the sensitive and slightly tolerant strains was not further reduced at concentrations of benomyl higher than saturation, which continued to increase at 50 and 100 μM. Strain G1 and possibly 87 have a region of growth stimulation at low benomyl concentrations, also reported for other *V. malthousei* strains (W. D. McIlveen, *personal communication,* 1973).

Benomyl tolerance on agar media was closely related to acid production in the absence of benomyl in the two different broths (Table 1). In general, strains with slight tolerance; e.g., 87 and S1, were intermediate in acid production.

Response to benomyl in liquid and agar culture was pH-dependent (Table 2). On buffered agar, sensitive strains were less inhibited by benomyl as pH decreased. In liquid media, this effect was significant only at the lowest

TABLE 1. Benomyl tolerance and acid production for 12 strains of *Verticillium malthousei*

Strain	Tolerance[1] (%)	Broth pH[2]	
		Medium A	Medium B
Noninoculated	. . .	6.84 d	7.22 b
B0	11.7 a[3]	7.22 ab	6.23 e
G1	12.9 ab	7.10 bc	7.35 b
K2	13.6 bc	7.16 b	6.85 d
C7	14.8 cd	7.34 a	7.28 a
90	16.3 de	7.07 bc	6.98 d
E1	19.0 e	6.92 c	7.05 abcd
S1	22.6 f	5.54 ef	7.06 c
87	30.0 g	6.35 e	4.03 fg
P5	91.3 h	5.35 fg	4.10 f
O1	97.8 h	4.74 g	3.80 g
T5	99.0 h	3.96 h	3.49 h
T1	99.1 h	4.86 fg	3.82 g

[1]Linear growth with 5 μM benomyl as percentage of control – Czapek Dox agar, 13 days incubation at 20 C.
[2]Supplemented Czapek Dox broths without benomyl, incubated 10 days at 20 C.
[3]Values followed by the same letter do not differ significantly, $P = 0.05$.

pH, where responses of the sensitive strains were not significantly different from those of the tolerant ones. The influence of pH on the tolerant strains was more complex. In both media, benomyl had little effect on growth rate at the lowest pH. At pH 5.0 (liquid) and 5.5 (agar), a stimulation was observed which resembled that of sensitive strains grown with low concentrations of benomyl. Without buffering, growth stimulation of strain T5 did not occur (Fig. 1), nor for P5 in a separate trial. However, growth of other tolerant *V. malthousei* strains appeared to be stimulated by 9 μM benomyl (1). At higher pH, benomyl inhibited the growth of tolerant strains, but its effectiveness was not substantially increased.

The pH of broth after growth of benomyl-tolerant and -sensitive *Sclerotinia* and *Botrytis* cultures did not differ significantly. Tolerant strains of *S. homeocarpa* produced a final average pH of 6.89 vs. 6.83 for the sensitive strains. Broth from flasks in which *B. cinerea* was grown had a final average pH of 4.51 for tolerant strains vs. 4.20 for sensitive strains.

Bioassays for residual activity of benomyl in liquid media with initial pH of 5.0 and 6.5 showed less activity in broth from tolerant strains than in broth from sensitive strains. There was a highly significant negative linear correlation between residual activity and mycelial dry weight, r = −0.83; i.e., benomyl activity decreased in proportion to the amount of mycelial growth. There was an equally strong positive correlation between residual activity and tolerance as determined in broth cultures, r = +0.82. The initial pH of the media had little effect on residual benomyl activity as determined using the original broth control flasks.

DISCUSSION

Benomyl-tolerant strains of *V. malthousei* produce an unknown acid in culture, and media acidification reduces benomyl's activity. Effectiveness of benomyl in reducing growth of sensitive and tolerant strains becomes proximate at pH 4.2. For tolerant strains, buffering over a range of pH simulates a dose-response pattern normally seen for sensitive strains at benomyl concentrations from 0 to 1 μM. However, tolerance is not substantially reduced in media at high pH. This may reflect either an ineffectiveness of the buffer at the appropriate sites within the tolerant fungi, or that increased acid production is a minor consequence of tolerance rather than its principal cause. In either case, increasing the pH of a benomyl suspension will not affect growth of tolerant strains sufficiently to make this an alternative procedure in a disease control program.

Differential acid production is not associated with benomyl tolerance for the two other fungal species investigated nor *Fusarium roseum* (16). Growth of tolerant and sensitive strains of *S. homeocarpa* resulted in broths with a pH approximating those of benomyl-sensitive *Verticillium* strains, whereas the broths of all *B. cinerea* strains had a pH equivalent to those of tolerant strains. This implies that more than one mechanism for tolerance exists, and that factors conditioning tolerance to benomyl differ among fungal species.

If the primary cause of fungicide tolerance in this species is acid production, several processes might be involved. Since benomyl as MBC is more soluble and is not detoxified at low pH (6, 12), these factors do not contribute to the tolerance mechanism. Neither the work presented here nor preliminary work supports the possibility that tolerant *V. malthousei* strains have a special ability to detoxify benomyl extracellularly. Likewise, we have found no heat-stabile components of the medium or mycelium from tolerant cultures which subsequently reduce the activity of benomyl (D. H. Lambert, *unpublished*).

Effects of pH on the uptake of organic compounds whose charges are pH-dependent have been reported for numerous systems (2, 11, 13, 15, 18). In the above cases, uptake was maximal in the pH range where the molecules were uncharged, decreasing as pH approached either a higher or lower pKa. This phenomenon usually relates to the solubility of neutral organic compounds in the lipid layer of membranes. Since the pKa values for MBC are 3.95 and 10.55 (Charles Delp, DuPont de Nemours Co.,

TABLE 2. Effect of medium pH on benomyl tolerance of *Verticillium malthousei* strains sensitive or tolerant to benomyl

Initial buffer pH	Linear growth on Czapek Dox agar — Average tolerance[1] (%): Tolerant strains (4)	Sensitive strains (8)
4.5	100 b[2]	34 d
5.5	106 a	27 e
6.5	97 bc	19 f
7.5	94 c	16 g

	Mycelial dry weight from broth culture			
	Tolerant strains (4)		Sensitive strains (4)	
Initial buffer pH	Final pH	Tolerance (%)	Final pH	Tolerance (%)
3.5	3.9	99 ab	4.2	70 b
5.0	5.0	120 a	5.2	12 c
6.5	6.4	83 b	6.4	9 c
8.0	7.6	89 b	7.9	15 c

[1]Growth with 5 μM benomyl as percentage of untreated control growth.
[2]Values followed by the same letter do not differ significantly, $P = 0.05$.

personal communication), the behavior of the benomyl–*V. malthousei* system is consistent with the foregoing models. A decrease in pH may alter the charge distribution at fungal sites as well as on MBC, affecting binding at the fungicide's site of action or elsewhere. Actual pH within the cytoplasm of the *Verticillium* strains tested was not determined, although the pH range undoubtedly was more restricted than that of the media in which they were grown (14). Even so, a cell wall pH < 4 has been reported (5).

Although commercial mushroom growers report that *Verticillium* disease is either controlled or not controlled by benomyl, a range of responses to the fungicide exist among tolerant (1) and sensitive (8) isolates of the pathogen. This indicates that during the evolution towards tolerance, physiological changes occur which are expressed quantitatively, or are conditioned by other factors among different strains such that disease control is observed in a quantitative rather than a qualitative mode.

The functional alteration (mutation) causing benomyl tolerance in *V. malthousei* may occur as a change in metabolism or permeability rather than a change at benomyl's proposed primary site of action (4, 7). However, tolerance might be expressed at the site of action due to impaired binding, or elsewhere by a reduction in the activity or concentration of the fungicide. As a result of this change, tolerant strains of *V. malthousei* excrete greater amounts of acid, are less sensitive to Na_2EDTA and certain toxic metal ions, and are more sensitive to 8-azaguanine and zineb (9). This latter characteristic may be pH-related or due to a greater vulnerability of the metabolic function which has been altered by the development of tolerance. Though a reduction in pH can substantially decrease the activity of benomyl in this species, the nature of the tolerance mechanism is not yet certain.

LITERATURE CITED

1. BOLLEN, G. J., and A. VAN ZAAYEN. 1975. Resistance to benzimidazole fungicides in pathogenic strains of Verticillium fungicola. Neth. J. Plant Pathol. 81:157-167.
2. BOSUND, I. 1960. The bacteriostatic action of benzoic and salicylic acids. V. Influence of pH on the total uptake of benzoic acid by Proteus vulgaris and baker's yeast. Physiol. Plant. 13:793-799.
3. CLEMONS, G. P., and H. D. SISLER. 1969. Formation of a fungitoxic derivative from benlate. Phytopathology 59:705.
4. CLEMONS, G. P., and H. D. SISLER. 1971. Localization of the site of action of a fungitoxic benomyl derivative. Pestic. Biochem. Physiol. 1:32-43.
5. CONWAY, E. J., and M. DOWNEY. 1950. pH values of the yeast cell. Biochem. J. 47:355-360.
6. FUCHS, A., G. A. VAN DEN BERG, and L. C. DAVIDSE. 1972. A comparison of benomyl and thiophanates with respect to some chemical and systemic fungitoxic characteristics. Pestic. Biochem. Physiol. 2:191-205.
7. HAMMERSCHLAG, R. S., and H. D. SISLER. 1973. Benomyl and methyl-2-benzimidazolecarbamate (MBC): biochemical, cytological and chemical aspects of toxicity to Ustilago maydis and Saccharomyces cervisiae. Pestic. Biochem. Physiol. 3:42-54.
8. LAMBERT, D. H. 1973. Population studies of Verticillium species associated with Agaricus bisporus (Lange) Imbach. M. S. Thesis, The Pennsylvania State University, University Park, Pa. 81 p.
9. LAMBERT, D. H. 1975. Increased sensitivity to zineb for Verticillium malthousei strains tolerant to benomyl. Phytopathology 65:637-638.
10. LAMBERT, D. H., and P. J. WUEST. 1974. A possible basis for benomyl tolerance in Verticillium malthousei. Proc. Am. Phytopathol. Soc. 1:138.
11. MILLS, D. 1972. Effect of pH on adenine and amino acid uptake during sporulation in Saccharomyces cervisiae. J. Bact. 112:519-526.
12. PITBLADO, R. E., and L. V. EDGINGTON. 1972. Movement of benomyl in field soils as influenced by surfactants. Phytopathology 62:513-515.
13. RUBBERY, P. H., and A. R. SHELDRAKE. 1973. Effect of pH and surface charge on cell uptake of auxin. Nature New Biol. 244:285-288.
14. RYAN, J. P., and H. RYAN. 1972. The role of intracellular pH on the regulation of cation exchanges in yeast. Biochem. J. 128:139-146.
15. SIMON, E. W., and H. BEEVERS. 1952. Effect of pH on the biological activities of weak acids and bases. New Phytol. 51:163-190.
16. SMILEY, R. W., and R. J. HOWARD. 1976. Tolerance to benzimidazole-derivative fungicides by Fusarium roseum on Kentucky bluegrass turf. Plant Dis. Rep. 60:91-94.
17. SNEL, M., and J. T. FLETCHER. 1971. Benomyl and thiabendazole for the control of mushroom diseases. Plant Dis. Rep. 55:120-121.
18. TISDALE, J. H., and A. G. DEBUSK. 1970. Developmental regulation of amino acid transport in Neurospora crassa. J. Bact. 104:689-697.
19. WUEST, P. J., and H. COLE, JR. 1970. Effect of three fungicides on vegetative growth of Verticillium malthousei and Agaricus bisporus isolates. Phytopathology 60:1320 (Abstr.).
20. WUEST, P. J., H. COLE, JR., and P. L. SANDERS. 1974. Tolerance of Verticillium malthousei to benomyl. Phytopathology 64:331-334.