In Vitro Activity of Etaconazole, Bitertanol, and Fenarimol on Fungi Causing Summer Diseases of Apples

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ABSTRACT

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The in vitro sensitivity, as measured by radial growth inhibition, of *Botryosphaeria dothidea, Physalospora obtusa, Glomerella cingulata, Colletotrichum gloeosporioides, Helminthosporium papulosum, Mycosphaerella pomi, Gloeodes pomigena,* and *Zygophiala jamaicensis* to etaconazole, bitertanol, and fenarimol was determined. Except for *G. pomigena*, EC_{50} values were less than 10 μg a.i./ml for each fungus-fungicide combination. Fenarimol was generally the least active of the compounds tested. Isolates of each fungus varied in their sensitivity to the compounds, indicating the potential for the selection of less sensitive strains under orchard conditions. Poor control of the summer diseases in the orchard by sterol-inhibiting fungicides may be due to their lack of persistence or to poor uptake of the compounds by fruit.

Sterol-inhibiting fungicides have been widely tested during the past 10 yr for apple disease control. This group of compounds has exceptional activity against apple scab (*Venturia inaequalis* (Cke.) Wint.), powdery mildew (*Podosphaera leucotricha* (Ell. & Everh.) Salm.), and cedar apple rust (*Gymnosporangium juniperi-virginianae* Schw.). In studies with *V. inaequalis*, the sterol-inhibiting compounds were excellent eradicants but relatively poor protectants (10,13,18,19).

Performance of sterol-inhibiting fungicides against the summer diseases of apples under orchard conditions has been inconsistent and generally less effective than that of the standard commercial fungicides (2,3,7,8,12,15,16,22). Summer diseases include Brooks spot (*Mycosphaerella pomi* (Pass.) Lindau), white rot (*Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & de Not.), black rot (*Physalospora obtusa* (Schw.) Cke. = *B. obtusa* (Schw.) Shoemaker), bitter rot (*Glomerella cingulata* (Stonem.) Spauld. & Schrenk, perfect stage of *Colletotrichum gloeosporioides* Penz.), black pox (*Helminthosporium papulosum* Berg.), flyspeck (*Zygophiala jamaicensis* Mason), and sooty blotch (*Gloeodes pomigena* (Schw.) Colby. Bitertanol gave better control of Brooks spot, sooty blotch, flyspeck, and bitter rot than other sterol-inhibiting fungicides tested in Virginia, Ohio, Pennsylvania, and North Carolina (4,16,20,21). Yoder (20) suggests that the residual activity of bitertanol may be better than that of other sterol-inhibiting fungicides. However, bitertanol performed no better than other sterol-inhibiting fungicides as a protectant when apple trees were inoculated with *V. inaequalis* after fungicide application and simulated rainfall (18,19). It is possible that some of the fungi causing summer diseases are more sensitive to bitertanol than to other sterol-inhibiting compounds; however, there is little data to support this.

The potential development of resistance to the sterol-inhibiting compounds is a major concern of Jones (9). He has suggested that limits of variation in sensitivity should be established for natural populations of *V. inaequalis* before the sterol-inhibiting compounds are used commercially. Limits of sensitivity also should be established for other major pathogens of apple.

The purpose of this study was to determine and compare the in vitro sensitivity of isolates of *P. obtusa, B. dothidea, C. gloeosporioides, Glomerella cingulata, H. papulosum, Gloeodes pomigena, Z. jamaicensis,* and *M. pomi* to bitertanol, etaconazole, and fenarimol, to possibly explain the inconsistent performance of these fungicides under field conditions, and to begin to determine the ranges of sensitivity of the pathogens to these fungicides.

MATERIALS AND METHODS

Fungicides. Technical-grade and formulated etaconazole (Vangard 10W), fenarimol (Rubigan 2E), and bitertanol (Baycor 50W) were used in this study. Concentrated stock solutions of each fungicide were made in distilled water, and appropriate amounts were added to potato-dextrose agar (PDA) after autoclaving to achieve the desired concentration. One to 2 ml of ethanol, propanol-2, or acetone were used as solvents for technical etaconazole, bitertanol, and fenarimol, respectively, when preparing the stock solution. These solvents were also used when formulated etaconazole or bitertanol were used. Controls with comparable amounts of solvents were used in initial tests. The solvents had no effect on the growth of the seven fungi tested and were not added to the controls in subsequent tests.

All tests with *P. obtusa, B. dothidea, Glomerella cingulata,* and *C. gloeosporioides* were conducted with technical material only. Tests with *H. papulosum, Gloeodes pomigena, M. pomi,* and *Z. jamaicensis* were conducted with technical and formulated materials. In several tests with each fungus, technical and formulated materials inhibited mycelial growth similarly when compared at the same level of active ingredient. Tests using either technical or formulated materials were combined for data analysis.

Media were prepared 2–3 days before use. About 15 ml was added to each 9-cm-diameter petri dish. Concentrations of fungicides used in most tests were 0.01, 0.1, 1.0, 2.5, 5.0, 7.5, and 10.0 μg a.i./ml.

Isolates. Isolates of *C. gloeosporioides* (conidial strains), *Glomerella cingulata* (perithecial strains), *B. dothidea, P. obtusa,* and *M. pomi* were obtained from infected fruit 2–6 mo before testing and stored in slant tubes on PDA at 2 C. Isolates of *Gloeodes pomigena* and *Z. jamaicensis* were isolated from apple fruit and maintained in a culture collection for about 2 yr. All isolates were obtained from unsprayed fruit or from fruit that had not been sprayed with a sterol-inhibiting compound. Fruit were collected from the Mountain Horticultural Crops Research Station, Fletcher, NC, or Central Crops Research Station, Raleigh, NC. Isolates were grown on PDA in petri dishes for appropriate times before

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testing.

Measurements of radial growth. A 5-mm-diameter plug of mycelium was removed from the margin of an actively growing culture of each fungus and placed in the center of each petri plate. Three or four replicates per concentration for each fungicide were used in individual tests. Most tests were conducted three times. *M. pomi* was incubated at 20 C for 3 or 4 wk, *H. papulosum* at 20 C for 2 or 3 wk, *Gloeodes pomigena* and *Z. jamaicensis* at 20 C for 2 wk, *B. dothidea* and *P. obtusa* at 28 C for 2 or 3 days, *C. gloeosporioides* at 28 C for 6 or 7 days, and *Glomerella cingulata* at 28 C for 5–6 days. Radial growth was determined at the conclusion of each test from two perpendicular colony diameter measurements.

Calculation of EC_{50} values. EC_{50} values for radial growth inhibition were obtained from regression equations that related percentage inhibition on a probit scale to the log of the fungicide concentration. EC_{50} values greater than 10 μg a.i./ml (the highest concentration used in the study) are reported as 10 μg a.i./ml.

Spore germination and germ tube growth of *M. pomi*. Ascospores of *M. pomi* were discharged from moistened, overwintered apple leaves in a spore tower (6) onto four plates each of PDA and PDA amended with 0.01, 0.1, or 1.0 μg/ml of etaconazole, fenarimol, or bitertanol. Plates remained in the spore tower for about 2 min at ambient temperature (20–22 C). Two groups of spores were deposited within a 0.5-mm-diameter circle on each plate. After removal from the tower, plates were incubated at 20 C, and after 4 hr, a drop of cotton blue in lactophenol was placed on one group of spores in each of four plates of PDA and PDA amended with the fungicides. The remaining group of spores in each plate was fixed and stained with cotton blue in lactophenol after 12 hr. Spore germination was determined by counting the germinating spores out of 50 in each group. A spore was considered germinated if its germ tube length was half the length of the spore. Germ tube lengths (10 spores per plate) were measured for only the group incubated for 12 hr.

Autoradiography. To investigate movement of sterol-inhibiting fungicides into fruit, ^{14}C-etaconazole was applied to Golden Delicious and Rome Beauty fruit. The ^{14}C-etaconozaole (specific activity = 26.4 μCi/mg) was dissolved in 0.4 ml of ethanol, then diluted with distilled water to give a stock solution containing 420 μg/ml. A 1.2-cm-diameter circle was drawn on the surface of each fruit, and a 10-μl drop of the stock solution (0.11 μCi/10 μl) was placed in the center of each circle. At 6, 12, 24, 48, 72, and 96 hr after the drops were applied, the application site was washed with three 10-μl drops of ethanol, and the washings from each fruit were placed in 15 ml of scintillation fluid (Oxifluor-CO_2, New England Nuclear, Boston, MA), shaken thoroughly, and counted for 5 min in a Beckman LS7500 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA). After the fruit surface was rinsed, a cork borer was used to remove a cylinder of fruit tissue, including the skin (1.2-cm in diameter and 2-cm long), from below the fungicide application site. Two fruits of each cultivar were evaluated at each time interval. Each cylinder of fruit tissue was cut into four tangential sections (3 mm wide), and the sections were placed on Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY), pressed between two pieces of Plexiglas, and stored at −75 C for 21 days. The films were developed in Kodak GBX Developer and Replinisher.

RESULTS

Bitertanol was significantly more active against *Glomerella cingulata* and *C. gloeosporioides* than etaconazole or fenarimol (Table 1). *G. cingulata* perithecial isolates were generally less sensitive to the sterol-inhibiting fungicides than the *C. gloeosporioides* conidial isolates. Fenarimol was significantly less active on *P. obtusa* and *B. dothidea* than bitertanol and etaconazole. The three materials were more active against *Z. jamaicensis* (flyspeck) than *Gloeodes pomigena* (sooty blotch). EC_{50} values of *G. pomigena* for bitertanol and fenarimol were greater than 10 μg a.i./ml. Etaconazole was the most active compound against *H. papulosum*. One of the four isolates of *H. papulosum* was noticeably less sensitive than the others tested against all the compounds. EC_{50} values for bitertanol and fenarimol were greater than 10 μg a.i./ml for this isolate compared with 2.5–3.5 μg/ml for the other *H. papulosum* isolates tested.

Growth of *M. pomi* was inhibited to a greater extent by etaconazole than by bitertanol and fenarimol (Table 1). Concentrations of bitertanol and fenarimol greater than 5 μg a.i./ml had little additional inhibitory effect on mycelial growth. Growth of five of seven isolates of *M. pomi* on PDA amended with 0.01 μg a.i./ml of etaconazole or 0.01 and 0.1 μg a.i./ml of bitertanol or fenarimol was greater than on unamended PDA. Growth of some isolates on PDA amended with 0.01 or 0.1 μg/ml of

Table 1. Relative sensitivity of isolates of fungi causing summer diseases of apple to etaconazole, bitertanol, and fenarimol

Pathogen	No. of isolates tested	Fungicide	Mean EC_{50}[a] (μg a.i./ml)	Range in EC_{50} (μg a.i./ml)	Coefficient of variation[b] (%)
Glomerella cingulata	5	Etaconazole	1.46[c]	0.57–2.43	51
		Bitertanol	0.40	0.34–0.42	14
		Fenarimol	1.77	0.85–2.70	35
Colletotrichum gloeosporioides	5	Etaconazole	0.46	0.35–0.60	18
		Bitertanol	0.24	0.21–0.30	12
		Fenarimol	0.70	0.50–0.94	21
Physalospora obtusa	5	Etaconazole	0.28	0.11–0.36	32
		Bitertanol	0.08	0.01–0.15	55
		Fenarimol	1.94	0.54–2.70	40
Botryosphaeria dothidea	6	Etaconazole	0.55	0.41–1.02	39
		Bitertanol	0.12	0.07–0.20	45
		Fenarimol	4.93	1.14–7.09	40
Mycosphaerella pomi	7	Etaconazole	0.56	0.14–1.12	64
		Bitertanol	>6.56	1.32–>10.0	···
		Fenarimol	>4.83	0.74–>10.0	···
Helminthosporium papulosum	4	Etaconazole	1.35	0.64–2.92	···
		Bitertanol	>4.57	3.33–>10.0	···
		Fenarimol	>6.46	2.61–>10.0	···
Gloeodes pomigena	3	Etaconazole	5.77	0.77–>10.0	···
		Bitertanol	>10.00	2.64–>10.0	···
		Fenarimol	>10.00	2.16–>10.0	···
Zygophiala jamaicensis	2	Etaconazole	0.37	0.35–0.39	···
		Bitertanol	0.87	0.78–1.13	···
		Fenarimol	2.13	1.11–3.29	···

[a] EC_{50} values of isolates >10 μg a.i./ml entered into mean calculation as 10 μg a.i./ml; thus mean EC_{50} values may be greater than reported.

[b] Coefficient of variation not computed if EC_{50} value was >10 μg a.i./ml or if fewer than four isolates were tested.

[c] $LSD_{0.05}$ for EC_{50} values of *G. cingulata, C. glorosporioides, P. obtusa,* and *B. dothidea* are 0.87, 0.15, 0.70, and 1.56, respectively.

fenarimol was 40% greater than on unamended medium. This increase in growth was not explained by an increase in the rate of ascospore germination or an increase in germ tube length. After 4 hr, germination on unamended medium was 72.5%; the percent germination was not greater on medium amended with any of the fungicides tested at 0.01, 0.1, or 1.0 μg/ml and was significantly less than the unamended control at 1.0 μg/ml of etaconazole. There was no significant increase in germ tube length after 12 hr with any fungicide treatment over the unamended control; germ tube length was significantly less in all fungicide treatments except 0.01 μg/ml of fenarimol.

Isolates of the seven fungi varied in their sensitivity to the compounds. The coefficients of variation of the EC_{50} values for *Glomerella cingulata*, *C. gloeosporioides*, *P. obtusa*, and *B. dothidea* generally ranged from 10 to 50% (Table 1). The coefficients of variation of the EC_{50} values were not computed for *H. papulosum* or *Gloeodes pomigena* because predicted EC_{50} values for one or more isolates of each fungus exceeded 10 μg a.i./ml.

Autoradiography. The level of ^{14}C-label on the fruit following the application of ^{14}C-etaconazole declined with time over the duration of the experiment. Fifty percent of the ^{14}C-label was not recovered from the surface of the Golden Delicious fruit after 12 hr and from the Rome Beauty fruit after 24 hr by the washing procedure. The autoradiographs indicated that after 48 hr, the material either remained on or in the cuticle or was retained in the epidermal and subepidermal fruit tissues (Fig. 1). In some fruit sections after 48 hr for Rome Beauty fruit and 96 hr for Golden Delicious fruit, some of the ^{14}C-label appeared to have moved as far as 1 mm into the flesh of the fruit just beneath the application site. No lateral movement was observed.

DISCUSSION

The summer disease fungi varied in their sensitivity to the sterol-inhibiting compounds tested. With the exception of *Gloeodes pomigena*, the mean EC_{50} values for the isolates of the pathogens tested were less than 10 μg a.i./ml. In comparison to other fungi tested for sensitivity to sterol-inhibiting compounds (1), the summer disease fungi would be classed from very sensitive (EC_{50} values <2 μg a.i./ml) to sensitive (2–10 μg a.i./ml). Generally, fenarimol was less active at the same level of active ingredient than bitertanol and etaconazole. This difference was most apparent with *B. dothidea* and *P. obtusa*.

Although only a small number of isolates of each fungus was tested, it is apparent that some variation in sensitivity to the sterol-inhibiting compounds is present in the natural population. Coefficients of variation of EC_{50} values were lowest for *C. gloeosporioides* and greatest for *Gloeodes pomigena*, *H. papulosum*, and *M. pomi*. The range in sensitivities observed with each of the fungi suggests the potential for selection of less sensitive isolates.

C. gloeosporioides conidial isolates were more sensitive to the sterol-inhibiting compounds than *Glomerella cingulata* perithecial isolates. This is interesting since *G. cingulata* is considered the sexual stage of *C. gloeosporioides*. Fruit rot symptoms and the epidemiology of *C. gloeosporioides* and *G. cingualata* differ (14,17).

In our tests, there was an increase in growth of *M. pomi* on medium amended with low concentrations of the sterol-inhibiting fungicides. However, low rates of the sterol-inhibiting fungicides did not stimulate ascospore germination or germ tube growth. Based on these results, it seems unlikely that poor control in the orchard can be related to an increase in the infection efficiency of *M. pomi* ascospores because of the presence of a sterol-inhibiting fungicide.

Although etaconazole, bitertanol, and fenarimol are active against the summer rot pathogens in vitro, they have not consistently provided satisfactory control in the orchard. Bitertanol has provided better control in the orchard than etaconazole or fenarimol (4,16,21); fenarimol has generally provided poor control of the summer diseases (5,16,21). Buchenaur (1) found little correlation between the in vitro and in vivo effectiveness of the sterol-inhibiting compounds. This also appears to be the case with the summer rot pathogens.

The poor performance of the sterol-inhibiting compounds under orchard conditions may be related to their residual activity on the fruit. After 24 hr and simulated rainfall, Szkolnik (19) found that conventional fungicides such as captan, mancozeb, and dodine provided much greater protection against infection by *V. inaequalis* than the sterol-inhibiting compounds. Kelley and Jones (11) have shown that etaconazole residues on the leaf surface rapidly decline over 24 hr. In our experiment with ^{14}C-etaconazole on fruit, 50% of the etaconazole was not recovered from the fruit surface by washing after 12–24 hr.

It is also possible that the poor control in the orchard may be related to uptake of the materials by the fruit. Our results with the ^{14}C-etaconazole showed that most of the fungicide remained in the epidermal and subepidermal tissue and there was little penetration into the flesh of the fruit. Thus, the sterol-inhibiting fungicides are probably not effective eradicants of pathogens such as *Glomerella cingulata* and *B. dothidea*, which rapidly colonize fruit tissue. However, etaconazole has demonstrated eradicant activity against *V. inaequalis*, which develops just beneath the cuticle. Kelley and Jones (11) have shown that etaconazole moves into leaves rapidly, and numerous tests (10,13,19) have demonstrated excellent postinfection and presymptom apple scab control. Kelley and Jones (10) also have reported "healed" apple scab lesions on fruit that were treated with bitertanol or etaconazole. Based on these results with *V. inaequalis*, the sterol-inhibiting fungicides should also control *Gloeodes pomigena* and *Z. jamaicensis*, both of which grow on the fruit cuticle. However, control is poor under orchard conditions. This may result from a lack of sensitivity to the sterol-inhibiting fungicides. Both fungi are considerably less sensitive to etaconazole than *V. inaequalis*. EC_{50} values for isolates of *V. inaequalis* to etaconazole are typically <0.1 μg/ml.

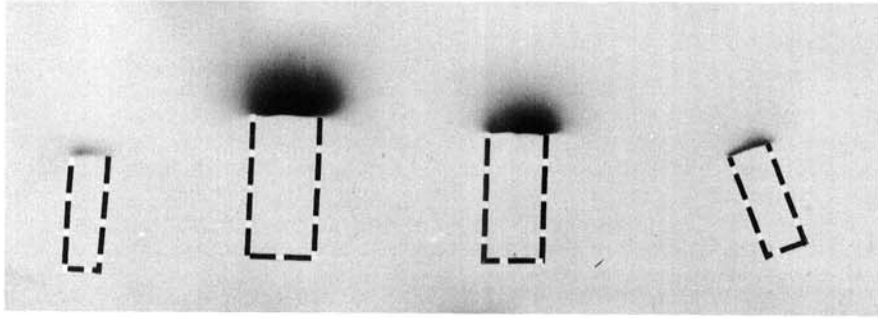

Fig. 1. Autoradiograph showing ^{14}C-etaconazole associated with the cuticle, epidermis, and subepidermal cells of sections of Rome Beauty fruit 48 hr after application.

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LITERATURE CITED

1. Buchenaur, H. 1979. Comparative studies on the antifungal activity of triadimefon, triadimenol, fenarimol, nuarimol, imazalil, and fluotrimazole in vitro. Z. Pflanzenkrankh. Pflanzenschutz 86:341-354.
2. Drake, C. R. 1980. Apple pests and their control at Blacksburg, Va, 1979. Fungic. Nematic. Tests 35:3-5.
3. Drake, C. R. 1982. Evaluation of various fungicides for control of apple diseases, 1981. Fungic. Nematic. Tests 37:2-3.
4. Ellis, M. A., and Hall, F. R. 1981. Evaluation of fungicides for control of apple diseases and European red mite, 1980. Fungic. Nematic. Tests 36:4.
5. Ellis, M. A., Wilson, L. L., and Hall F. R. 1983.

Evaluation of fungicides and fungicide combinations for control of apple scab, fly speck and moldy core, 1982. Fungic. Nematic. Tests 38:137-138.
6. Gilpatrick, J. P., Smith, C. A., and Blowers, D. R. 1972. A method of collecting ascospores of *Venturia inaequalis* for spore germination studies. Plant Dis. Rep. 56:39-42.
7. Hendrix, F. F., Jr., and Britton, K. O. 1983. Evaluation of apple fungicides, 1981. Fungic. Nematic. Tests 38:139140.
8. Hickey, K. D., May, J., and Garretson, M. 1983. Evaluation of sterol-inhibiting fungicides for apple disease control when applied with a conventional airblast sprayer, 1982. Fungic. Nematic. Tests 38:142.
9. Jones, A. L. 1981. Fungicide resistance: Past experience with benomyl and dodine and future concerns with sterol inhibitors. Plant Dis. 65:990-992.
10. Kelley, R. D., and Jones A. L. 1981. Evaluation of two triazole fungicides for postinfection control of apple scab. Phytopathology 71:737-743.
11. Kelley, R. D., and Jones, A. L. 1982. Volatility and systemic properties of etaconazole and fenapanil in apple. Can. J. Plant Pathol. 4:243-246.
12. Nardacci, J. F., and Sutton, T. B. 1982. Apple disease control in western North Carolina, 1981. Fungic. Nematic. Tests 37:12.
13. Schwabe, W. F. S., Jones, A. L., and Jonker, J. P. 1984. Greenhouse evaluation of the curative and protective action of sterol-inhibiting fungicides against apple scab. Phytopathology 74:249-252.
14. Shane, W. W., and Sutton, T. B. 1981. Germination, appressoria formation, and infection of immature apple fruit by *Glomerella cingulata*. Phytopathology 71:454-457.
15. Sutton, T. B. 1980. Summer disease control, 1979. Fungic. Nematic. Tests 35:19.
16. Sutton, T. B. 1982. Summer disease control, 1981. Fungic. Nematic. Tests. 37:16.
17. Sutton, T. B., and Shane, W. W. 1983. Epidemiology of the perfect stage of *Glomerella cingulata* on apples. Phytopathology 73:1179-1183.
18. Szkolnik, M. 1980. Artificial rainfall in the determination of apple scab fungicide retentiveness, 1979. Fungic. Nematic. Tests 35:20.
19. Szkolnik, M. 1981. Physical modes of action of sterol-inhibiting fungicides against apple diseases. Plant Dis. 65:981-985.
20. Yoder, K. S. 1982. Broad spectrum apple disease control with bitertanol. Plant Dis. 66:580-583.
21. Yoder, K. S., Cochran, A. E., II, Warren, J. R., and Nicholls, S. A. 1983. Evaluation of morning and evening reduced volume applications of sterol-inhibiting fungicides of apple disease control and fruit effects, 1982. Fungic. Nematic. Tests 38:159.
22. Yoder, K. S., and Hickey, K. D. 1981. Sterol-inhibiting fungicides for control of certain diseases of apple in the Cumberland-Shenandoah region. Plant Dis. 65:998-1001.