FUNGICIDE SCREENING FOR CONTROL OF VERTICILLIUM WILT OF PEPPERMINT

Fred Crowe and Robin Parks

Abstract

The ability of selected modern fungicides and other products to reduce *Verticillium dahliae* growth and disease development was tested. In the laboratory, the fungicides Quadris (active ingredient: azoxystrobin), Benlate (a.i.: benomyl), Vanguard (a.i.: cyprodinil), and Folicur (a.i.: tebuconazole) were added to an agar medium in different concentrations and the growth of *V. dahliae* was measured. All products inhibited mycelial growth in vitro although a linear relationship was not always detected. All products inhibited growth at relatively low concentrations. The effective dosage to reduce fungal growth by 50 percent ranged from 0.001 to 0.261 g a.i./1 water. In the greenhouse, the same fungicides and several additional materials were drenched onto potted peppermint planted in *V.* dahliae-infested soil. The additional materials included Actigard (a product that elicits plant defense responses), a very low rate of Vapam fumigant, and Acid Replacement Solution (ARS), a product that is purported to adversely influence soil pathogen growth, but not plant growth, by altering the pH of the water. No wilt symptoms developed in the plants, including infested pots left untreated, so this trial was inconclusive. However, for some products, there was a treatment effect on dry biomass weight at harvest that may have been caused by phytotoxicity at the tested application rates. Folicur, Vanguard, and ARS were applied in the field in May 2000, in established mint, which had experienced moderate wilt in previous years. Folicur and ARS likely move into soil with irrigation water, but there was no prior information available on the mobility of Vanguard. Regardless, there was no evidence that any of the products affected mint growth or reduced wilt severity in the field.

Introduction

Verticillium dahliae, the causal agent of Verticillium wilt of many crops, survives in soil as dormant, persistent microsclerotia. Microsclerotia germinate and form actively growing mycelium (strand-like mold) and conidial spores that infect root surface cells of many (perhaps most) plant species, or directly invade root wounds. *V dahliae* infections may be limited to root surface cells, in which case relatively few microsclerotia and no pathogenic symptoms are produced. Infection of the root tips may lead to invasion of vascular tissues. Systemic infections may develop as the fungus proliferates and as conidia are passively carried with the upward transpiration flow. If the strain of *V. dahliae* is pathogenic to mint, the mint plant may develop wilt symptoms and die prematurely. The fungus invades other tissues and may form abundant microsclerotia, especially in stem tissue.

For mint species specifically, Verticillium wilt results from (a) infection of roots by microsclerotia in soil, or (b) carryover infections in rhizomes from season to season and/or from field to field. Rootstock dug from infected fields undoubtedly carries the fungus along in soil and plants.

The objectives of this research were to address the possibility of using modern fungicides to manage wilt. It is worth noting first, however, that there are several forms of systemic movement of chemicals in plants: (1) _*Active movement from cell to cell, across membranes and involving metabolic processes (symplastic),* Many or most chemicals must negotiate active cell regulation of such movement. When most agricultural products are described as "locally systemic," systemic activity is limited to such cell-to-cell movement and may not develop throughout the plant. A "non-systemic" product is not actively passed from cell to cell. Long-distance movement of chemicals in plants usually occurs via the sugar-transport system, which also requires active cell metabolism to occur. If a product moves downward to any extent, it is fully systemic in this manner (2) *Passive movement through cell walls, intercellular spaces and via the water-conducting system of xylem tissues (apoplastic).* Because the xylem in roots is separated from soil by living cells, water and nutrients enter the xylem by diffusion through cell walls and between cells. This movement is not actively regulated. *Verticillium* may grow into the xylem near to the root tip, but is excluded by a protective layer (casparian strip) in more mature roots. Once in the xylem, chemicals and *Verticillium* spores move passively upward in the plant, although the flow rate is regulated by openings in the foliage that allow inflow and outflow of gases in leaf spaces. Conidia of *Verticillium* may lodge at periodic cellular constrictions, and the plant may respond to the presence of *Verticillium* by forming anti-fungal compounds and plugs. This interaction leads to wilt symptoms.

The possibility of using fungicides to control Verticillium wilt was considered seriously when the early era systemic products were introduced. Such products included benomyl, triadimephon, triforine, and iprodione, which appeared in the late 1960's and 1970's. A good overview of this work was published by Erwin (1973); other overviews include those by Edgington (1981) and Edgington et al. (1980). The following discussion borrows heavily from these citations, especially Erwin (1973).

Systemic fungicides applied to soil, seed, and roots typically gain access to xylem and passively are translocated upward. A fungicide caught up in the transpirational stream may only briefly disrupt the activity of *V. dahliae,* but this is not clear. For products applied to foliage, only a fraction crosses the waxy surface layer into cells, and movement thereafter is primarily symplastic, from cell to cell via phloem. Some fungicides seem able to move gradually down the plant from leaves, periodically re-entering the transpirational stream along stems and being shunted upward again Such downward translocation of fungicides in the phloem (symplastic) is poorly documented. Fungicides might similarly move inward from stem surfaces into the xylem. Metabolizing cells next to the xylem (the xylem parenchyma) can pull water and chemicals from the transpirational stream and insert them also into the xylem. These tissues react to the presence of *V. dahliae* by producing anti-fungal compounds and xylem plugging materials.

Some modern products being developed for use against fungi are not true fungicides at all, but instead induce one or more plant defense systems to fight off fungal and/or other pest ingress. These defense mechanisms are collectively termed Systemic Acquired Resistance, or SAR (Kessman et al. 1994). For at least one SAR product (Actigard, Novartis Corp.), and perhaps all SAR products, such defense responses seem to be downwardly translocated in plants, although probably not in the xylem as discussed above (A. Talley, Novartis Corp., personal communication). Chemically induced SAR might provide some measure of protection against root infection, or (less likely) protection in the xylem itself. However, if SAR products access the xylem, they may aggravate wilt by elevating the plant's formation of anti-fungal chemicals and plugging materials. Not every SAR product elicits sufficient responses against every disease. In most cases SAR activity diverts energy away from other plant metabolic processes toward defense responses, so the products cannot be applied too frequently without disrupting normal plant growth and development. Also, such products possibly could aggravate wilt symptoms.

There are at least four ways a chemical could prevent or reduce *V. dahliae* infection or symptom development. For an applied chemical product to be effective it must (1) prevent root infection near the root tip, (2) enter the xylem via roots, stems, or leaves and be present long enough in the stem xylem to stop or retard activity *of V. dahliae,* (3) induce plant defense systems to prevent root infection; or (4) block plant defense systems involved in wilt symptom development, either from access within the xylem or from cells surrounding the xylem, essentially forcing the plant to not respond to infection.

While other products were similarly tested and yielded similar results, the most extensive examples of early testing of systemic fungicides against Verticillium wilt were with the benzimidazoles (benomyl, TBZ, MBC, thiophanate products, etc.) on cotton, potato, tomato, strawberry, and other crops. The following comments came from a review by Erwin (1973). Verticillium wilt could be controlled by benzimidazoles when applied to soil, depending on rate of application and distribution in soil. Products were found in leaf tissue following translocation through the xylem, and some products lasted 5-6 months in soil. For deep-rooted crops such as cotton, it was difficult to provide product to most roots. Shallow-rooted crops imposed less of a problem. Unfortunately, rates of application were cost-prohibitive. Benzimidazole prevented root infection and impacted systemic activity of the *Verticillium* fungus. Combinations of seed, soil, and foliage treatments provided control, but again were not economically feasible. Adjuvants to increase mobility in soil and across foliage cuticles, and chemical modification of the fungicides themselves sometimes increased activity of the benzimidazoles. As products moved through plants, most ended up in leaves, near to veins, although some evidence for symplastic recycling was found. For products applied to foliage, stem applications were more effective against vascular wilts than were applications to leaves, although with benzimidazoles control of wilt was difficult to achieve with either treatment.

There are few if any data since the early 1970s, that re-visit these issues; thus modern products remain untested to our knowledge. *V. dahliae,* while causing widespread and severe plant losses, is not one of the diseases against which product manufacturers themselves test any new fungicides. However, several modern fungicides are highly effective against the group of fungi that includes *V. dahliae.* Some last relatively long in plants or soil, some are relatively mobile in soil, and some are relatively more mobile symplastically in plants. For example, the triazole group of fungicides (e.g., Folicur, a.i. tebuconazole, Bayer Corp.) can move in soil and might be worth considering for soil incorporation or soil drench or injected into drip irrigation lines (K. Noegle, Bayer Corp., personal communication) to control root and rhizome infection by *V. dahliae.* The new strobilurin materials (e.g. Quadris, a.i. azoxystrobin, Zeneca) are highly effective and systemic in plants, although it is unclear whether these materials enter the xylem. They persist in plants probably only a few weeks after application. They do not move readily in soil. It would be worth determining whether spraying foliage and stems might provide some activity against systemic *V. dahliae* infections or symptoms. There are new fungicides worth considering in addition to the triazoles and strobilurins.

Materials and Methods

Laboratory: A mint isolate of *V. dahliae* was used in this study. Materials tested were azoxystrobin supplied as Quadris F (Zeneca), benomyl supplied as Benlate SP (DuPont), cyprodinil supplied as Vanguard WG (Novartis), and tebuconazole supplied as Folicur 3.6F (Bayer Corp.). The concentrations evaluated were 0.0, **0.1,** 1.0, 10.0, and 100.0 ppm of active ingredient in an agar medium. Stock suspensions of fungicides were prepared with sterile deionized water. To obtain the needed concentrations, specific aliquots of each suspension were added to potato dextrose agar (PDA) after it was autoclaved and cooled to 50° C. The amended media was poured into 10-cm-diameter petri dishes. Inoculum consisted of 5-mm plugs of agar and mycelium taken from actively growing cultures of *V dahliae* on PDA. Inverted plugs were placed onto the test medium in the center of the dishes. Each concentration was replicated five times for each chemical. The dishes were stacked in blocks and incubated at room temperature in the dark. Radial growth was measured after 7 days. Microsclerotia development was observed after 11 days. The experiment was performed twice.

Mycelial growth was compared with growth on unamended PDA. The percent inhibition in growth was calculated and plotted as a function of the logarithm of fungicide concentration. Simple linear regression using PROC REG of SAS version 7.0 (SAS Institute 1988) was performed to fit a line to the points and determine the effective dosage of active ingredient that causes a 50 percent reduction in radial growth (ED5o)•

Greenhouse: Rooted cuttings of peppermint var. Black Mitcham were planted in potting soil infested with a mint isolate of *V. dahliae.* The inoculum was produced in the laboratory by growing *V. dahliae* on a modified, minimal agar (Puhalla 1979) overlain with sterile, uncoated cellophane. After a 3-wk incubation, microsclerotia were harvested by blending the cellophane in sterile water and washed on a 38 1.tm sieve. The dried concentrated inoculum was ground with sterile sand with a mortar and pestle. The concentration of microsclerotia/g inoculum was estimated and a specific quantity was added to potting soil and mixed in a cement mixer to achieve 10 microsclerotia/g potting soil. Two cuttings were planted in each 10-in-diameter pot. A control treatment was inoculated but not treated with any product. The materials tested included the fungicides Benlate SP (DuPont), Folicur 3.6F (Bayer Corp.), Quadris F (Zeneca), and Vanguard WG (Novartis Corp.); the SAR material Actigard; an Acid Replacement Solution (ARS) (HPT Research, Inc.); and the fumigant Vapam. After pre-drenching the pots, the fungicides were applied at rates equivalent to registered field rates (Table 1). Benlate was mixed with potting soil 1 day prior to planting and the other materials were sprayed onto the foliage 1 wk after planting. Vapam was applied at 3 gal/acre, a rate found to be subphytotoxic on some crops (W. Strange, AMNAC, personal communication).

ARS was applied as per the manufacturer's suggested rate: the equivalent of 10 oz/200 ft^2 was initially drenched onto pots. Immediately following treatment, the pots were liberally watered to simulate irrigation. The treatments were replicated five times for a total of 40 pots. The pots were arranged on a greenhouse bench in a randomized complete block design.

Within 1 wk of treatment, the plants were assessed for foliar damage. Beginning 8 wk after planting, wilt symptoms were assessed weekly for 5 wk. Plants were cut at the soil line to measure dry biomass 13 wk after planting. The plants were allowed to regrow for 5 wk and wilt symptoms were monitored.

Response variables underwent an analysis of variance using the general linear model, PROC GLM, of SAS, version 7.0 (SAS Institute 1988). Means were separated by Fisher's protected least significant difference (LSD) test.

Table 1. Products tested for inhibition of Verticillium wilt of peppermint var. Black Mitcham in a greenhouse trial, Oregon State University — Central Oregon Agricultural Research Center, Madras, Oregon, 2000.

Product	Type of product	Registered field rate	Applied greenhouse rate (product per 50gal/acre)
Benlate SP	Fungicide	10 mg ai/800 g soil	0.04 g
Folicur 3.6F	Fungicide	1 L/ha	0.003 ml
Quadris F	Fungicide	8 oz/3,000 ft^2	0.27 ml
Vanguard WG	Fungicide	1000 g/ha	0.03 g
Actigard	SAR	1 oz✓acre	0.0002 g
Acid Replacement Solution	Alters pH	10 oz/200 ft^2	5.12 ml
Vapam	Fumigant	3 gal/acre	0.9 ml

Field: A preliminary field trial was established in a field of peppermint variety M83-7 exhibiting moderate Verticillium wilt symptoms near Madras, OR. The materials tested included Folicur 3.6F, Vanguard WG, and ARS (Table 2). Plots were treated in early May 2000. An untreated control was also included. Plots were 10 x 20 ft. Each treatment was replicated five times in a randomized complete block design.

Lodging and the mean height of 10 randomly selected plants per plot were measured in early July. Wilt severity was estimated based on the number of wilt strikes in each plot counted in early July. Due to a miscommunication with the grower, harvest was not taken. Response variables underwent an analysis of variance using the general linear model, PROC GLM, of SAS, version 7.0 (SAS Institute 1988). Means were separated by Fisher's protected least significant difference (LSD) test.

Table 2. Products tested for inhibition of Verticillium wilt of peppermint in a grower's field near Madras, Oregon, 2000.

Product	Applied field rate (amount product)[a]
Folicur 3.6F	14.2 oz/acre
Vanguard WG	0.89 lb/acre
Acid Replacement Solution	10 oz/acre[b]

[a]All products applied in 28.6 gal/acre or 258 L/ha, immediately prior to irrigation with 1.65 inches of water.
[b]As per manufacturer's suggestion we used this amount instead of 2 oz/gal H_2O sprayed. We attempted to achieve a pH in water of 1.5-2.0 or lower, including irrigation water.

Results and Discussion

Similar results were obtained in both laboratory experiments. For simplicity, only the results from the first experiment are shown. Benomyl was included simply as a practical control because it had shown high activity against *V. dahliae* in studies performed in the 1960's and 1970's, even though it was found not to move in soil. All products tested inhibited mycelial growth of *V. dahliae;* however the ED50 values for azoxystrobin, benomyl, and tebuconazole were 98 to 100 times higher than cyprodinil (Table 3) Similarly, more growth was observed at the highest concentration of cyprodinil compared with the other products (Fig. 1 C). Mycelial growth was reduced as concentration of active ingredient increased for all products. Linear regression equations for each fungicide are given in Table 2. Because mycelial growth was inhibited dramatically at a low concentration of benomyl (Fig. 1B), there was no evidence (r^2= 0.5990) of a linear relationship between growth and this active ingredient at these concentrations. Microsclerotia development was observed at all concentrations that allowed mycelial growth. From these preliminary tests, there is evidence that all products tested reduce the growth of *V. dahliae.*

Table 3. In vitro inhibition of *Verticillium dahliae* by azoxystrobin, benomyl, cyprodinil, and tebuconazole (percent inhibition [Y] as a function of $\log_o$ of concentration [X]). Experiment 1.

Active ingredient	Brand name	Linear regression	Correlation coefficient	ED50a (g ai/L H_20)
Azoxystrobin	Quadris F	Y = 0.46 — 0.22X	0.9049	0.001
Benomyl	Benlate SP	Y = 0.43 — 0.33X	0.5990	0.001
Cyprodinil	Vanguard WG	Y = 0.78 — 0.12X	0.7126	0.261
Tebuconazole	Folicur 3.6 F	Y = 0.72 — 0.36X	0.9569	0.004

[a]The effective dosage that causes a 50 percent reduction in radial hyphae growth.

In the greenhouse, no wilt symptoms were observed on any of the plants even after 18 wk. Stems from the plots of one block were plated on a semi-selective agar but *V. dahliae* was not recovered even from the untreated control. This suggests that the microsclerotia inoculation did not result in enough of an infection to induce symptom development.

Although microsclerotia inoculum produces a more natural infection than dipping roots in a suspension of *V. dahliae* conidia, it is more difficult to establish systemic infection. In our experience in past greenhouse experiments, wilt symptoms are evident in microsclerotia-infected mint 10-12 weeks post-planting. However, data on foliar damage following product application and growth were obtained. The Vapam treatment caused significant (P = 0.0052) foliar discoloration compared to the other products tested (Table 4). Dry weights at 13 wk post planting differed significantly (P = 0.0002) among the treatments (Table 4). Plants treated with Actigard, ARS, Vanguard, Quadris, and Vapam weighed the most, respectively; plants treated with Benlate and Folicur weighed the least. This effect on plant growth was surprising considering little or no foliar damage was visible for most treatments following application. If these differences in weight are due to phytotoxicity, it may be that the rate applied was higher than is optimal for mint Because no wilt symptoms were observed, there is no evidence whether these products may affect Verticillium wilt in mint

Table 4. Foliar damage rating and dry hay weight from Verticillium wilt fungicide trial in a greenhouse trial, Oregon State University, Central Oregon Agricultural Research Center, Madras, Oregon 2000.

Treatment	Foliar damage'	Dry hay (g)
Untreated	0.0 B[b]	84.3 A
Actigard	0.0 B	74.4 AB
Acid Replacement Solution	0.0 B	72.9 AB
Benlate	0.0 B	35.2 D
Folicur	0.2 B	46.3 CD
Quadris	0.2 B	71.6 AB
Vanguard	0.2 B	71.8 AB
Vapam	1.4 A	65.2 BC
P-value`	0.0139	0.0006
Treatment	0.0052	0.0002
Block	0.4004	0.1919

'Foliar damage ratings: 1 = slight discoloration of a few leaves, 2 = discoloration of many leaves, 3 = discoloration and/or scorching of at least one-half of plant, 4 = discoloration of most leaves, 5 = dead.

[b]Means followed by the same letter are not significantly different at P 5 0.05 according to Fisher's protected least significant difference (LSD) test.

`Probability of obtaining F 0 os.

Neither peppermint growth nor wilt severity was different among the treatments in the trial located in a grower's field. The percent lodging and stem height did not differ among the treatments (Table 5). Verticillium wilt severity was similar among the treatments as well (Table 5).

Table 5. Peppermint growth and Verticillium wilt severity in a fungicide trial located in a grower's field near Madras, Oregon, 2000.

Product	7 Jul % Lodging	7 Jul Stem height (cm)	7 Jul Wilt severity (no. strikes/200 ft^2)
Untreated	50.0	67.9	2.20
Folicur	52.0	68.6	2.40
Vanguard	52.0	67.4	2.50
Acid Replacement Solution	39.0	65.9	2.60
P-value[s]	0.4420	0.0370	0.7089
Treatment	0.6851	0.3297	0.6372
Block	0.2697	0.0168	0.6067

'Probability of obtaining F0 05.

Conclusions

Clearly, some or all of the fungicides tested were effective directly against *V. dahliae* in laboratory tests on agar. In the greenhouse and field tests, no products proved effective, but test results were inconclusive. The greenhouse test was inconclusive because no wilt occurred in potted peppermint. Interestingly, several products negatively influenced mint growth in the greenhouse test. Originally, the greenhouse test was intended to provide the most conclusive data on product potentials. In the field, none of the products decreased wilt levels or enhanced mint growth. But the field test was intended to provide only supplementary data, at best, because it is not clear that a single application is sufficient or when is the best timing for one or more applications. Probably most disappointing was the inconclusive data for the product Folicur, which has been shown to influence other soil diseases when drenched onto soil (F. Crowe, personal observations). Yet, that effect may have been achieved rather shallow in the soil profile, perhaps more shallow than might be required to influence root infection by *V. dahliae.*

Literature Cited

Edgington, L.V. 1981. Structural requirements of systemic fungicides. Annual Review Phytopathology 19:107-124.

Edgington, L.V., R.A. Martin, G.C. Bruin, and R.M. Parsons. 1980. Systemic fungicides: perspective after 10 years. Plant Disease 64:19-23.

Erwin, D.C. 1973. Systemic fungicides: disease control, translocation, and mode of action. Annual Review of Phytopathology 14:389-422.

Kessmann, H., T. Staub, C. Hofmann, T. Maetzke, J. Herzog, E. Ward, S. Uknes, and J. Ryals. 1994. Induction of systemic acquired disease resistance in plants by chemicals. Annual Review Phytopathology 32:439-459.

Puhalla, J. E. 1979. Classification of isolates of *Verticillium dahliae* based on heterokaryon incompatibility. Phytopathology 69:1186-1189.

SAS Institute 1988. SAS guide for personal computers: Statistics Version 6.0 SAS Institute, Cary, N.C.

Acknowledgements

This research was supported by the Mint Industry Research Council and the Oregon Mint Commission.

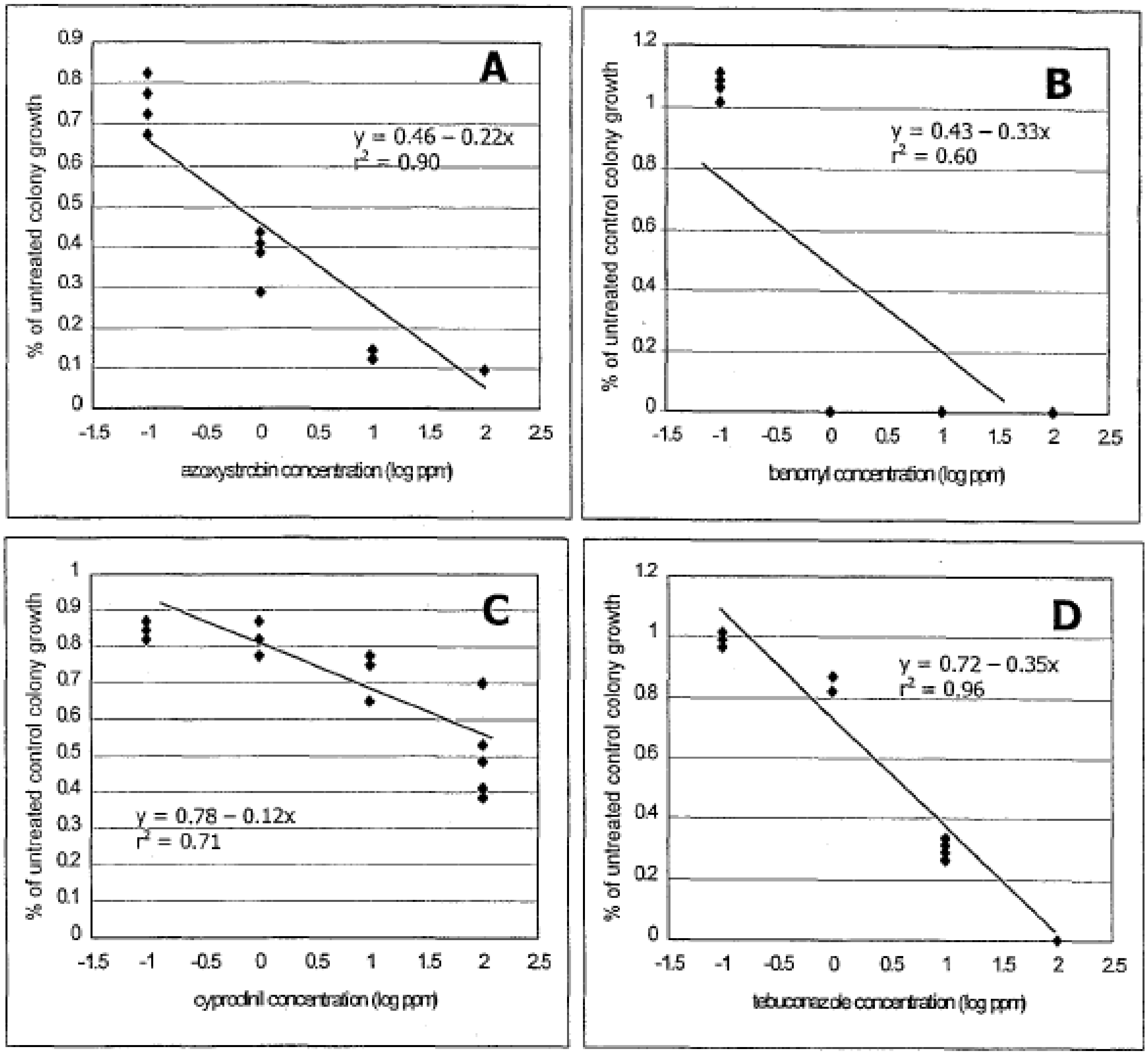

Figure 1. Linear regressions relating the proportion of in vitro *Verticillium dahliae* radial colony growth to the amount of azoxystrobin (A), benomyl (B), cyprodinil (C), or tebuconazole (D) in potato dextrose agar, compared to an untreated control. Experiment 1.