Project Title	Screening new chemicals for V*erticillium* control
Project Number	M 33a
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The results and conclusions in this report are based on an investigation conducted over a period of one year. The conditions under which the experiment was carried out, and the results obtained, have been reported with detail and accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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PRACTICAL SECTION FOR GROWERS

Commercial benefits of the project

The British mushroom industry relies heavily on the fungicide Sporgon 50WP to control *Verticillium*. Partial resistance of some *Verticillium* isolates to Sporgon 50WP has already been reported and if control if this disease was to fail completely, the cost to the industry would be in the region of £10 million compared to an estimated loss of £2-3 million at present.

Good disease control management advocates alternating active ingredients to prevent the build up of resistance among the pathogen population. This is currently not an option for *Verticillium* control strategies. An alternative product therefore needs to be sought in order to prevent any further shifts in the resistance status of *Verticillium* isolates to Sporgon 50WP.

Background and objectives

Verticillium continues to be the major mushroom pathogen affecting the British mushroom industry. Project M 14c demonstrated that control of *Verticillium* by Sporgon 50WP (prochloraz manganese), whilst considerable, was only partial. In addition, a significant proportion of *Verticillium* isolates collected from around Britain (M 14b) have been shown to have higher ED50 (50% of isolates killed at the recommended rate) values for prochloraz manganese, indicating that the sensitivity of the pathogen is shifting towards being more tolerant of this chemical. Control of *Vertcillium* in the industry continues to be costly and troublesome. No immediately promising material resulted from screening a number of chemicals in 1998 (M 33) however the need for an effective alternative fungicide remains acute.

The objectives of this project are to search for, and screen, *in vitro*, new chemicals for the control of *Verticillium.*

Summary of results and conclusions

As a result of the diminished efficacy of Sporgon 50WP, the sole fungicide used to control *Verticillium*, the main objective of this project has been to screen potential fungicides for their effectiveness against this pathogen.

Seven fungicides (Teldor, Zenon, Elvaron Multi, Twist, Trilogy, KIF 3535, KIF 230) were tested *in vitro* for their effect on spore germination and mycelial growth of two *Verticillium* isolates. Their effects on *Agaricus* mycelium were also tested as a measure of likely phytotoxicity. Bavistin DF and Sporgon 50WP were also included in the tests as known materials against which the novel fungicides might be compared.

None of the fungicides tested appeared outstanding when compared to Sporgon 50WP. One chemical, tolylfluanid (Elvaron Multi), might be worthy of further testing as it was

moderately inhibitory to mycelial growth and spore germination but the laboratory results suggest that it may not be as effective as Sporgon 50WP.

The search for an alternative chemical to Sporgon 50WP should continue.

Action points for growers

This project has not identified a viable alternative to Sporgon 50WP so growers should continue to use Sporgon 50WP for *Verticillium* control if appropriate. If *Verticillium* control is not being achieved despite the use of Sporgon 50WP, a number of factors should be considered.

- Under high inoculation pressure control will be difficult to achieve. Every effort should therefore be made to ensure that hygiene measures are effective. Badly infected third flushes should be cooked out or terminated without further picking.

- With the knowledge that Sporgon 50WP levels in casing drop steadily during the cropping period (see HDC report M 14c), it is vitally important to ensure that the levels of fungicide in the casing after treatment are adequate. In the event of poor control, casing samples should be tested for the amount of active ingredient present.

- In the event of unsatisfactory control being achieved, the resistance status of *Verticillium* isolates should be established as it appears that two populations of *Verticillium* are present on British mushroom farms. About 64% of isolates are weakly resistant to Sporgon 50WP while 30% of isolates are more resistant, however, the more resistant isolates appear to be more aggressive pathogens (see HDC report M 14c). Nonetheless, it is likely that the more resistant isolates will be difficult to control thus placing greater emphasis on both good hygiene and efficient pesticide application.

Anticipated practical and financial benefits

Losses due to *Verticillium* are estimated at about £2-3m per annum. The British mushroom industry is heavily dependent on Sporgon 50WP, which has been demonstrated to give good but not total control of *Verticillium*. However, should further resistance to this product emerge then disease control will be severely jeopardised and the cost, in terms of lost production, would be much higher. The industry needs to identify potentially useful chemicals long before the effectiveness of existing chemicals is completely undermined. New chemicals with different modes of action need to be continually screened for their potential to control *Verticillium* and other mushroom pathogens so that growers will have effective fungicides at their disposal to control serious outbreaks of disease.

SCIENCE SECTION

Screening fungicides for potential control of *Verticillium*

1. Introduction

In the late 1960's and early 1970's, the benzimidazole fungicide Benlate, (active ingredient (a.i.) benomyl), was widely used to control *Verticillium*, however, within a few years of its introduction, widespread resistance to this chemical had developed throughout the British *Verticillium* population (Fletcher & Yarham, 1976). In the 1980's, a new chemical, Sporgon 50WP (a.i. prochloraz manganese) was launched to control *Verticillium*, with good results, and this chemical has been relied upon heavily since that time to control *Verticillium* outbreaks in Britain (Fletcher *et al*., 1983).

In recent years, the effectiveness of Sporgon 50WP has been called into question as growers claim that, despite its use, *Verticillium* outbreaks occur where previously they had been controlled. Recently completed HDC-funded research (Grogan *et al.,* 1998 - HDC report M 14b) indicates that a significant proportion (64%) of British *Verticillium* isolates tested were moderately resistant to Sporgon 50WP, and capable of restricted growth at concentrations of prochloraz manganese which would be present in casing. Inoculation studies indicate that Sporgon 50WP will significantly reduce disease losses caused by *Verticillium* (Grogan *et al.,* 1999 - HDC report M 14c) but, under high inoculation loads, it will not give total control.

Mushroom growers can no longer afford the financial losses associated with major disease epidemics yet there is also a strong drive towards reducing the amount of chemicals used in crop production worldwide. This is fuelled by a trend towards pesticide free foods so that future research needs to focus on biological control strategies rather than seeking out new chemicals. In the meantime however growers need to be able to use chemicals to contain and control a disease situation.

The British mushroom industry is heavily dependent on Sporgon 50WP which has been demonstrated to give good but not total control of *Verticillium* and, should further resistance to this product emerge, then disease control will be severely jeopardised. The industry needs to identify potentially useful chemicals long before the effectiveness of existing chemicals is completely undermined. New chemicals with different modes of action are continuously being sought for the major food crops and it is from this area that potentially interesting new fungicides may emerge.

If the effectiveness of Sporgon 50WP is compromised any further, the financial impact on the British mushroom industry will be severe. This research report was commissioned to continue the search for a new fungicide effective against this mushroom pathogen, in order to reduce the heavy reliance and pressure on a single product which carries serious repercussions for the industry in the event of that product failing for whatever reason.

2. Materials and Methods

2.1. Chemicals

Five fungicidal products (including neem oil), which are currently being used in agriculture or horticulture, were provided for study by various chemical companies (Table 1). Two new active ingredients being developed for use were also supplied for testing by Hortichem Ltd. In addition, the fungicides Sporgon 50WP and Bavistin DF were included for comparison giving a total of nine products tested in all.

All major chemical companies were approached on a number of occasions to provide chemicals for testing but with the amalgamation of companies, there are fewer new products coming on the market.

Details of the products tested are given in Table 1 and a brief description of each product is given below.

Table 1. Details of chemicals used for *in vitro* screening against *Verticillium fungicola.*

Active ingredient (a.i.)	**Product and formulation***		**Manufacturer**	**a.i. content**
Prochloraz-Mn	Sporgon 50WP	WP	AgrEvo	46% w/w
Carbendazim	Bavistin DF	WG	BASF	50% w/w
Existing products:				
Fenhexamid	Teldor	WG	Bayer	51% w/w
Spiroxamine	Zenon	EC	Bayer	800 g/l
Tolylfluanid	Elvaron Multi	WG	Bayer	50.5% w/w
Trifloxystrobin	Twist	EC	Novartis	125 g/l
Neem Oil	Trilogy	EC	AgriSense	70% w/v
New chemistry				
(Mepanipyrim)	KIF 3535	WP	Hortichem Ltd.	50% w/w
	KIF 230	WDG	Hortichem Ltd.	15% w/w

* See appendix for explanation of formulation types.

Existing commercial products.

Prochloraz manganese (Sporgon 50WP) is a broad spectrum protectant and eradicant imidazole fungicide. Imidazole fungicides are demethylation inhibitors, which means that they inhibit a demethylation step in sterol biosynthesis. Sterols are an important component of fungal cell membranes.

Carbendazim (Bavistin DF) is a benzimidazole fungicide comprising of methyl benzimidazole carbamate (MBC). Carbendazim inhibits fungal mitosis by binding to the fungal tubulin and preventing cell division.

Fenhexamid (Teldor) is a protectant fungicide of the new hydroxyanilide chemical group. The hydroxyanilide group has come from the carboxamide and oxathiin groups. These types of fungicides are site specific, protectant systemic fungicides.

Spiroxamine (Zenon) is a systemic fungicide from the spiroketalamine class of substances. Spiroketalamines are protective, curative and eradicative fungicides that inhibit the biosynthesis of fungal sterols.

Tolylfluanid (Elvaron Multi) is a wide spectrum, contact protectant fungicide. The mode of action is unclear but it seems to be a multiple-site inhibitor.

Trifloxystrobin (Twist) is a broad spectrum (foliar) fungicide that acts by interfering with the respiration of plant pathogenic fungi. Strobilurin fungicides act in the mitochondrial respiration pathway, which means they are potential inhibitors of spore germination.

Neem oil (Trilogy) is an oil extracted from the neem tree. It is a broad spectrum fungicide and miticide.

New chemistry.

KIF 3535 is an anilinopyrimidine. Anilinopyrimidines inhibit the secretion of hydrolysing enzymes such as cutinase, pectinase and cellulase from fungal cells. It has been discovered that a second mode of action may exist, which inhibits the fungal biosynthesis of methionine.

KIF 230 No information currently available

2.2 Media preparation

All *in vitro* testing was done using commercially available potato dextrose agar (PDA) (Oxoid). Bottles of media were prepared, sterilised by autoclaving at 121^{O}C for 15 minutes, and allowed to cool to 50^{O}C. A fresh stock of fungicide was prepared at a concentration of active ingredient of 1000 mg/litre, equivalent to 1000 ppm. Each fungicide to be screened was incorporated into the medium to give concentrations of 0, 0.2, 2 and 20 ppm using the concentrated stock solution. For the neem oil product, the concentration range was 0, 2, 20 and 200 ppm. Bottles of media with fungicide added were gently shaken to evenly distribute the fungicide within the media, and then poured into sterile 90 mm diameter Petri dishes. Dishes were allowed to cool and dry for two to three days before being used for an experiment.

2.3 Culture preparation

Two different cultures of the mushroom pathogen *Verticillium fungicola* (isolates 182 and 620) were used throughout the screening experiments. Isolate 182 is known to be slightly more resistant to prochloraz manganese than isolate 620 (Grogan *et al*. 1998; HDC report M 14b). In addition, *Agaricus bisporus*, strain Sylvan A15, was also included to determine its sensitivity to the selected chemicals. Stock cultures of *Verticillium* isolates

were grown on PDA (Oxoid) while stock cultures of *Agaricus bisporus* were grown on Malt Extract Agar (MEA) (Oxoid). Stock cultures were prepared two weeks in advance of the *in vitro* fungicide screening tests so that they had a good growing margin from which plugs could be taken to place on fungicide-amended media.

2.4 *In vitro* tests

Mycelial growth.
Culture plugs of each *Verticillium* isolate and *A. bisporus* were taken from the growing edge of a fresh culture and placed on a series of Petri dishes containing PDA medium amended with 0, 0.2, 2 and 20 ppm of a given chemical. Three replicate plates were prepared for each organism at each concentration of active ingredient. Radial growth was measured weekly over a period of up to 3 weeks. When growth measurements were completed, the growth of organisms on fungicide amended media was expressed as a percentage of the growth on media containing no fungicide. Results were then compared with those for Sporgon 50WP and Bavistin DF to determine if the test chemicals were more or less inhibitory than these two fungicides, and whether or not they appeared to have potential for further testing.

Spore germination
Spore suspensions of the two *Verticillium* isolates were prepared to give in the region of 1 million spores per ml (1×10^6 spores/ml). A dilution series was then prepared to give 1×10^2, 1×10^4 and 1×10^6 spores/ml. A 0.1 ml aliquot of each spore dilution was then plated out on to PDA medium amended with the test chemicals, to give 10, 1000 and 100 000 spores per plate. Two replicate plates were prepared for each spore concentration at each concentration of the test chemical. Germination levels were expressed as a percentage based on the number of spores germinating at each fungicide concentration compared with the controls.

Results from both the mycelial growth and spore germination tests were then compared with those for Sporgon 50WP and Bavistin DF to determine if the test chemicals were more or less inhibitory than these two fungicides, and whether or not they appeared to have potential for further testing.

3. Results and Discussion

None of the chemicals tested gave very encouraging results in terms of a potential new chemical for use in the battle against *Verticillium.* There was little difference in the responses of the two different *Verticillium* isolates to any chemical.

One active ingredient, tolylfluanid, gave good inhibition of spore germination, and some inhibition of mycelial growth, at 20 ppm only but the level of inhibition was no better than that obtained with prochloraz manganese (Sporgon 50WP), which was more inhibitive at lower concentrations. Trifloxystrobin gave moderate inhibition of mycelial growth, but no inhibition of spore germination although the mode of action of the strobilurin fungicides would have suggested the opposite result. There is an unconfirmed report that this product may not perform well under laboratory conditions so perhaps it should be tested under cropping conditions.

In view of the poor results obtained from *in vitro* fungicide screening, the question needs to be asked whether or not chemicals should also be routinely screened under cropping conditions so that a more realistic evaluation of their potential can be obtained. This would be unnecessary if there were a large number of potentially useful fungicides to choose from. However, in view of the small number of chemicals available for screening it might be prudent to be more certain that they are not potentially useful, rather than to dismiss them based on laboratory tests only.

The results for each chemical are discussed individually below.

3.1 Prochloraz manganese (Sporgon 50WP)

Mycelial growth. Figure 1 shows the growth responses of the two *Verticillium* isolates and *A. bisporus* to prochloraz manganese. *Agaricus* is largely unaffected at concentrations of 0.2 and 2 ppm but is inhibited at 20 ppm whereas both *Verticillium* isolates show significant inhibition from 0.2 upwards. Isolate 182 was less inhibited than isolate 620.

Spore germination. Spore germination of both *Verticillium* isolates was completely inhibited by prochloraz manganese at 20 ppm (Table 2). Spore germination was reduced, but not completely inhibited, at 0.2 and 2 ppm. Subsequent growth of those spores, which germinated, however was significantly reduced.

This chemical is still relatively effective against *Verticillium* while being not very toxic to *Agaricus*. **Thus any potentially interesting new chemical for further tests must inhibit *Verticillium* growth and spore germination at concentrations of 0.2 or 2 ppm.**

3.2 Carbendazim (Bavistin DF)

Mycelial growth. Figure 2 shows the growth responses of two *Verticillium* isolates and *A. bisporus* to carbendazim. *Agaricus* is increasingly inhibited with increasing concentrations of carbendazim with total inhibition at 20 ppm. Both *Verticillium* isolates

grew well at 0.2 and 2 ppm but with some inhibition detected at 20 ppm. This indicates that the chemical is unlikely to effective.

Spore germination. Carbendazim slightly reduced the germination of *Verticillium* spores at 2 and 20 ppm only, to approximately 75% indicating that the chemical is unlikely to be effective.

3.3 Fenhexamid (Teldor)

Mycelial growth. Fenhexamid had only a slight inhibitory effect on *Verticillium* growth and no inhibitory effect on *Agaricus* growth (Figure 3) over the range of concentrations tested.

Spore germination. Some inhibition of germination was recorded for Verticllium spores at 2 and 20 ppm (Table 2) but at levels not considered to be very effective.

Fenhexamid is not considered to be worth including in any future tests concerning *Verticillium*.

3.4 Spiroxamine (Zenon)

Mycelial growth. Spiroxamine significantly inhibited the growth of both *Verticillium* isolates (Figure 4) but not as severely as prochloraz manganese (Sporgon 50WP)(Figure 1). *Agaricus* was also significantly more inhibited by Spiroxamine than prochloraz manganese.

Spore germination. Some inhibition of germination was recorded for *Verticillium* spores at 0.2, 2 and 20 ppm (Table 2) but at levels not considered to be very effective.

Spiroxamine is not considered to be worth including in any future tests concerning *Verticillium*.

3.5 Tolylfluanid (Elvaron Multi)

Mycelial growth. Tolylfluanid only inhibited the growth of *Verticillium* isolates at 20 ppm (Figure 5). *Agaricus* was also inhibited at 20 ppm only.

Spore germination. Tolylfluanid significantly reduced *Verticillium* spore germination at 20 ppm by 95% but not at 0.2 or 2 ppm (Table 2). This level of inhibition is approaching that of prochloraz manganese (Sporgon 50WP) so that this product may have some potential.

Tolylfluanid may be worth considering in any future tests in view of its good inhibition of spore germination but it is unlikely to be better than prochloraz manganese (Sporgon 50WP).

3.6 Trifloxystrobin (Twist)

Mycelial growth. Trifloxystrobin inhibited the growth of both *Verticillium* isolates over the range 0.2 to 20 ppm (Figure 6) but inhibition levels were slightly less than for prochloraz manganese (Sporgon 50WP)(Figure 1). *Agaricus bisporus* growth was also significantly inhibited over this concentration range.

Spore germination. Trifloxystrobin had no inhibitory effect on *Verticillium* spore germination (Table 2). This result was not as expected, given the inhibitory effect on mycelial growth (recorded above), as the strobilurin fungicides are believed to be more effective against germination and growth from spores rather than against established colonies. There are unconfirmed reports that this product may not work well under laboratory conditions and this product should perhaps be tested using a crop based test.

Trifloxystrobin may be worth considering in further tests in view of its moderate inhibition of mycelial growth and the unexpected ambiguous results obtained for spore germination.

3.7 Neem oil (Trilogy)

Mycelial growth. Neem oil had no inhibitory effect on either *Verticillium* or *Agaricus bisporus* (Figure 7) over the range of concentrations tested.

Spore germination. Neem oil had no inhibitory effect on *Verticillium* spore germination (Table 2).

Neem oil is not considered to be worth including in any future tests.

3.8 KIF 3535

Mycelial growth. KIF 3535 had a slight inhibitory effect on the growth of both *Verticillium* and *Agaricus* at 20 ppm (Figure 8).

Spore germination. KIF 3535 had no inhibitory effect on *Verticillium* spore germination (Table 2).

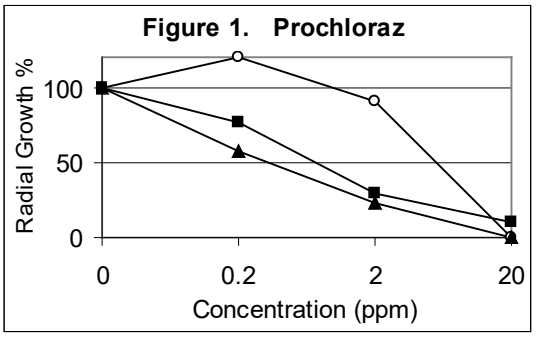
KIF 3535 is not considered to be worth including in any future tests concerning *Verticillium*.

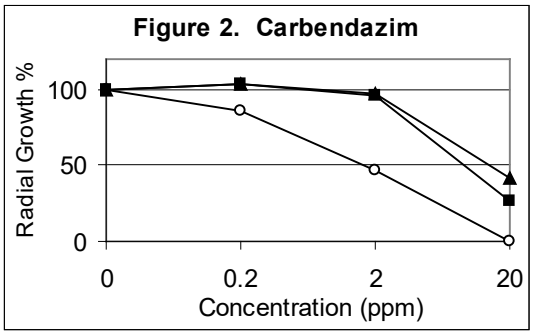
3.9 KIF 230

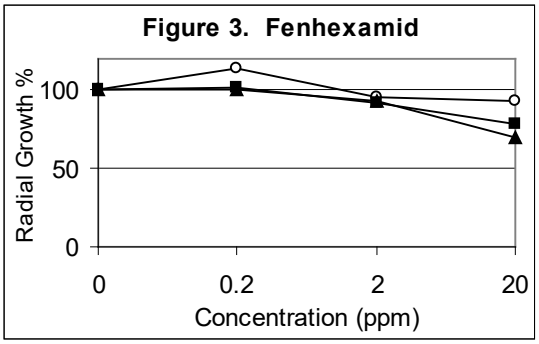
Mycelial growth. KIF 230 had only a very slight inhibitory effect on the growth of either *Verticillium* or *Agaricus* (Figure 9).

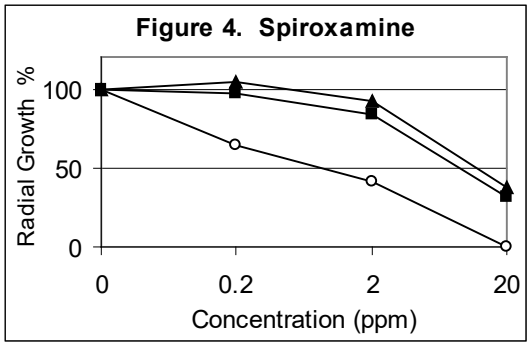
Spore germination. KIF 230 had no inhibitory effect on *Verticillium* spore germination (Table 2).

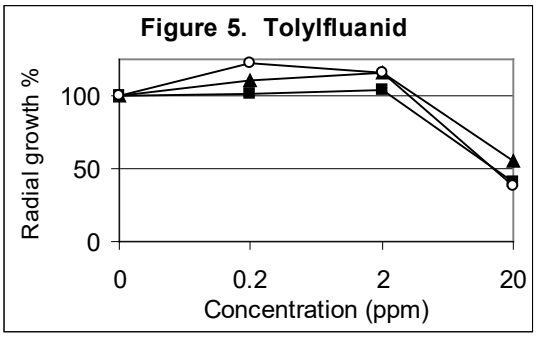
KIF 230 is not considered to be worth including in any future tests concerning *Verticillium*.

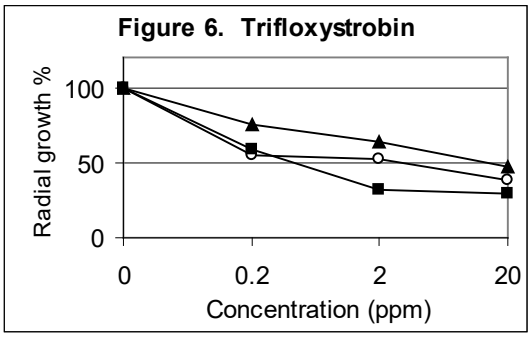


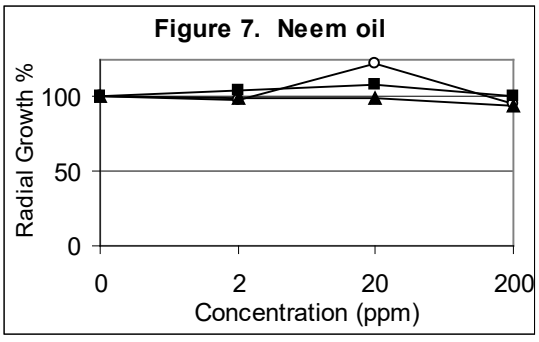


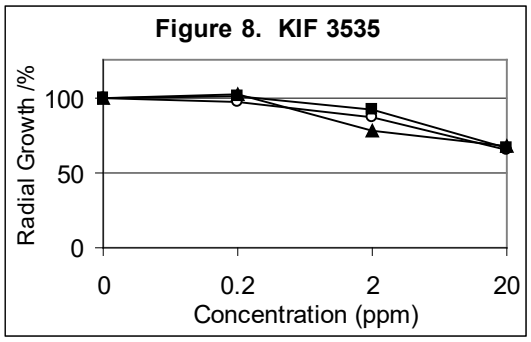












Figures 1-9. Mycelial growth rate of two *Verticillium* isolates and *Agaricus bisporus* in the presence of various fungicides.

Table 2. Percentage germination of *Verticillium* spores after seven days in the presence of various chemicals at concentrations from 0 to 20 ppm.

		Concentration of a.i. (ppm)			
Chemical (a.i.)	**Product**	***0***	***0.2***	***2***	***20***
Prochloraz manganese	(Sporgon 50WP)	100%	52%	90%	0%
Carbendazim	(Bavistin DF)	100	100	74	78
Existing products:					
Fenhexamid	Teldor	100	100	79	85
Spiroxamine	Zenon	100	89	71	96
Tolylfluanid	Elvaron Multi	100	100	100	5
Trifloxystrobin	Twist	100	100	100	100
Neem Oil	Trilogy 70EC	100	#	93	100
New chemistry					
(Mepanipyrim)	KIF 3535	100	76	100	100
	KIF 230	100	96	100	100

n.t. = not tested

4. Conclusions

- Any chemical which is to give better control of *Verticillium* than Sporgon 50WP, would be expected to inhibit mycelial growth by at least 50% at 2 ppm, and by at least 75% at 20 ppm. Spore germination should also be significantly inhibited at 2 and 20 ppm. In addition, *Agaricus* growth must not be totally inhibited over this range of concentrations, although total inhibition at 20 ppm should not be a deterrent as both prochloraz manganese (Sporgon 50WP) and carbendazim (Bavistin DF) inhibit *Agaricus in vitro* at this concentration.

- None of the chemicals tested completely inhibited the growth of *Verticillium* at 20 ppm with three chemicals having only moderate inhibitory effects at this concentration, namely tolylfluanid, trifloxystrobin and spiroxamine, with only tolylfluanid having any significant effect on both spore germination and mycelial growth.

- Tolylfluanid might be worthwhile examining further but the laboratory tests suggest it may be no better than Sporgon 50WP.

- Trifloxystrobin might be worth further investigation under cropping conditions as there are unconfirmed reports that this product may not work well under laboratory conditions.

- The search for an alternative chemical to Sporgon 50WP must continue.

5. References

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6. Appendices.

Appendix 1.

Explanation of formulation abbreviations

WP	Wettable Powder
WG	Wettable Granule
EC	Emulsifiable Concentrate
WDG	Wettable Dry Granule