Project Title	In vivo response of prochloraz resistant *Verticillium* to Sporgon 50WP
Project Number	M 14c
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Report	Final Report (June 1999)
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Location of Project	Horticulture Research International Wellesbourne Warwick CV35 9EF
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Date Project Commenced	1 July 1998
Date Project completed	31st May 1999
Key words	prochloraz manganese, dry bubble, control, efficacy, *Verticillium*, Sporgon 50WP

Practical Section for Growers.

Background

Growers have for some time reported increasing difficulty in *Verticillium* control despite the use of Sporgon 50WP. A survey of *Verticillium* isolates carried out in 1997 (HDC project

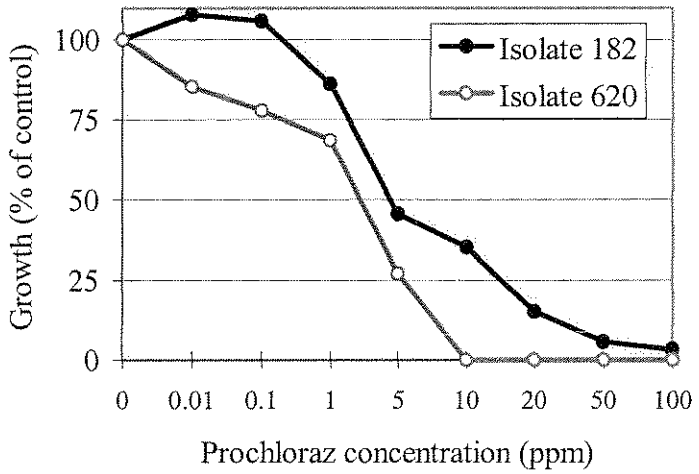

Figure 1. *In vitro* response to prochloraz (a.i. in Sporgon) of two *Verticillium* isolates.

M 14b) indicated that two distinct populations of *Verticillium* were present in Britain. Roughly two-thirds were weakly resistant (represented by isolate 182) whilst one-third were moderately sensitive (represented by isolate 620) (Figure 1). This project set out to examine the degree of control by Sporgon 50WP that could be expected in a cropping situation, when used against representatives of the two types of *Verticillium*.

Summary of Results

Despite high inoculation treatments levels of dry bubble were significantly reduced for both isolates (Figure 2).

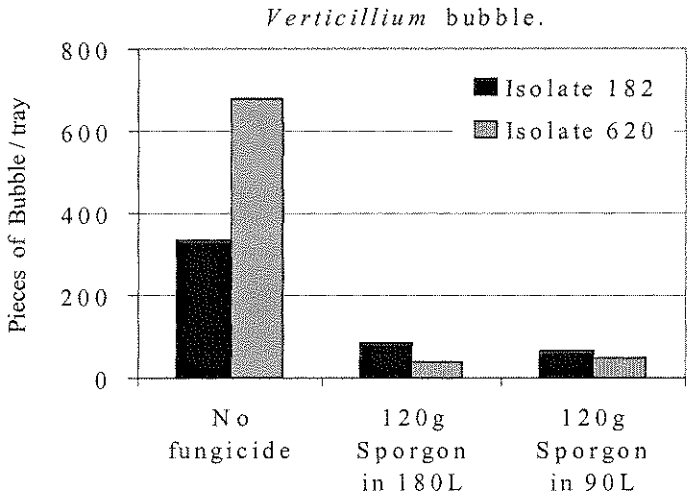

Figure 2. Number of bubble pieces harvested over two flushes.

Although the levels of disease were similar for both isolates, comparison with untreated inoculated plots showed that the degree of control for the sensitive strain (620) was much greater. Ironically, this strain was a more virulent pathogen and, if untreated, produced about twice as much bubble as isolate 182.

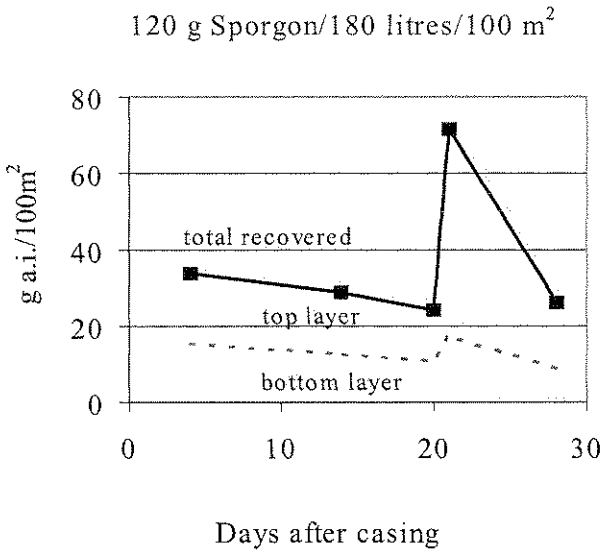

Figure 3. Levels of prochloraz (a.i. in Sporgon) recovered from top and bottom layers of casing.

The project also demonstrated that levels of Sporgon 50WP in the casing dropped quite rapidly throughout the crop (Figure 3).

Action Points for Growers

If *Verticillium* control is not being achieved despite the use of Sporgon 50WP a number of factors should be considered.

- Under high inoculation pressure neither isolate was totally controlled. Every effort should therefore be made to ensure that hygiene measures are effective.

- With the knowledge that Sporgon 50WP levels drop, it is vitally important to check that the levels of active ingredient in the casing, after treatment, are adequate. In the event of poor control casing samples should be tested.

- In the event of unsatisfactory control being achieved, the resistance of the isolate concerned should be established. Despite the compensation effects of sensitivity and virulence found in this project, it is likely that in many instances the more resistant isolates will be more difficult to effectively control thus placing greater emphasis on both good hygiene and efficient pesticide application.

Science Section

1. Introduction

A survey of *Verticillium* isolates from British mushroom farms, carried out in 1997, indicated that two distinct populations of this pathogen existed which differed in their *in vitro* sensitivity to the fungicide Sporgon 50WP (HDC Report M 14b, Grogan *et al.*1998). Sixty-four percent (64%) of the isolates tested were weakly resistant to prochloraz manganese, the active ingredient (a.i.) in Sporgon 50WP, and they had ED50 values (the concentration of a.i. which reduces growth by 50%) in the region of 5 to 8 ppm. Thirty percent (30%) of isolates were more sensitive to prochloraz manganese and they had ED50 values in the region of 1 to 4 ppm. Recent studies in the Netherlands and France have also identified *Verticillium* isolates which are less sensitive to prochloraz manganese *in vitro* (Geels, 1996; Desrumeaux *et al.*, 1998).

In Britain, Sporgon 50WP is relied upon heavily to control *Verticillium fungicola* - a pathogen which can cause severe losses due to spotting and dry bubble formation. It produces masses of small sticky spores which are easily dispersed in water droplets and by flies. *Verticillium* is a true parasite of A. *bisporus*, deriving its nutrition from the host following penetration of mushroom mycelium (Draght *et al.*, 1995). If infection occurs at an early stage in mushroom development, then undifferentiated masses of mushroom tissue will develop to give the characteristic "dry bubble" symptom. If maturing mushrooms are infected then spotting symptoms will develop making the mushrooms unmarketable. At present, the losses to the British industry due to *Verticillium* are estimated to be in the region of £2-3 million but if Sporgon 50WP was to fail completely (as Benlate did in the 1980's) this loss would increase to an estimated £10 million.

The detection, in project M 14b, of an apparent shift in the sensitivity of *Verticillium* isolates to Sporgon 50WP is worrying, as the industry in Britain relies heavily on this product to keep *Verticillium* under control. However, growers have been reporting increases in the amount of *Verticillium* present on their farms despite the use of Sporgon 50WP. This project was commissioned to determine whether Sporgon 50WP effectively controlled two different isolates of *Verticillium* - one from the more sensitive group and one from the less sensitive group.

2. Materials and Methods

2.1 Verticillium isolates.

Two *Verticillium* isolates, isolate 182 and isolate 620, which represented the two major groups of isolates identified during the 1987 HDC-funded survey (See report M 14b) were chosen for this study. The fungicide-resistance profiles of both isolates, in response to increasing concentrations of prochloraz manganese, were determined prior to the inoculation of crop in order to demonstrate the *in vitro* responses of the isolates being used in the study (Figure 1). Isolate 182 was more resistant to prochloraz manganese and was capable of some growth at concentrations of 20 and 50 ppm. Isolate 620 was more sensitive to this fungicide and was inhibited totally at these concentrations.

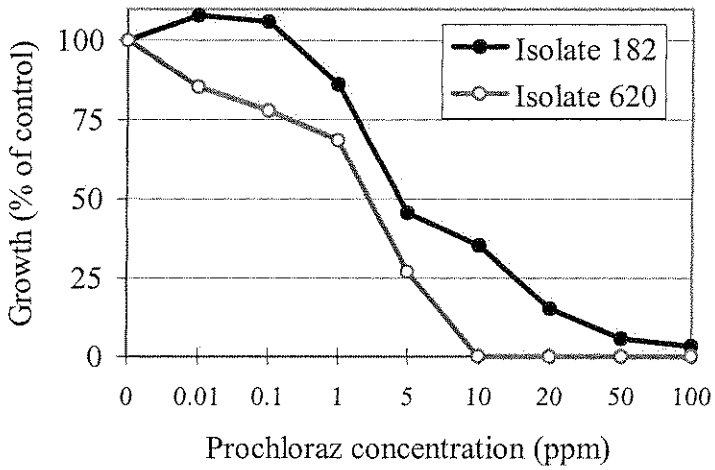

Figure 1. *In vitro* response to prochloraz (a.i. in Sporgon 50WP) of two *Verticillium* isolates

2.2 Inoculum

Verticillium inoculum in the form of a spore suspension was prepared on the day when casing was to be inoculated. Spores were washed from a pure culture of each isolate and the concentration of the spore suspension was calculated using a counting slide (Haemocytometer). The spore suspension was diluted so that a 100 ml volume per tray delivered in the region of 1 million spores /m^2. Spore suspensions were applied to casing on Day 4 after casing using a separate sterilized wash bottle for each isolate.

2.3 Sporgon 50WP treatments

Sporgon 50WP was applied to casing using two different rates of application. These consisted of the standard application as follows:

- 120 grams in 180 litres of water for 100 m^2 of mushroom bed area, applied on Day 3 after casing and after the first flush (Sp 180)

A second treatment consisted of applying the same amount of product but reducing the volume of water to 90 litres/100 m^2 as follows:

- 120 grams in 90 litres of water for 100 m^2 of mushroom bed area, applied on Day 3 after casing and after the first flush (Sp 90)

2.4 Crop details

Compost produced by the HRI Mushroom Unit (compost batch 18/98) was used for this experiment. Wooden trays, measuring 91cm x 61cm x 17cm (l x b x h) were filled with 50 kg of spawned (Sylvan A12) compost and spawn run for 17 days at 25°C. Trays were

cased with a 4-5 cm layer of black peat/sugar beet lime casing (Tunnel Tech English) and case-run at 25°C. The first of two Sporgon 50WP doses was applied to the relevant trays on Day 3 after casing. *Verticillium* inoculum was applied to relevant trays on Day 4. The crop was aired on Day 7-8 over a three day period, by which time the air temperature was reduced to 18°C. Harvested mushrooms were classified as either healthy or spotted. Pieces of bubble were also harvested at intervals during the crop. Control plots were picked before diseased plots to minimise the chances of cross contamination. The first flush was harvested from Day 17 to 21; the second dose of Sporgon 50WP was applied to relevant trays on Day 21 with water being applied to control trays; the whole crop was watered on Days 22 and 23 and the second flush was harvested from Day 24 to 28. Two flushes were harvested in total.

2.5 Determination of prochloraz manganese levels in casing

Samples of casing were removed on Days 4, 14, 20, 21 and 28 after casing. Five cores of casing (26 mm diameter, 50 mm approx. deep), were taken from each tray on each sampling day. The cores were split in half transversely to give 'top' and 'bottom' sub-samples which were pooled together and then frozen (-15°C) until analysed. After defrosting the samples were weighed and mixed. Dry weights were determined by drying samples of casing to constant mass in a microwave oven. Residues were extracted from casing (20 g) with methanol (60 ml, hplc grade) by tumbling end-over-end for 1 hour. The extracts were filtered through filter paper (Whatman No. 5) before further analysis. Prochloraz manganese content was analysed by high performance liquid chromatography using a Spectra Physics SP8810 pump, Cecil CE1200 uv detector and a 250 x 4.6 mm Spherisorb C8 column. The mobile phase was methanol:acetonitrile:water (60:20:20). The flow rate was set at 1.5 ml/min giving a retention time of 4.1 minutes. Detection was by uv absorbence at 220 nm. Analytical efficiencies were assessed by fortifying untreated casing samples with standard fungicide solutions in methanol at 5 - 100 mg a.i./kg dry casing. Recoveries were always in the range of 90-110% and results were not corrected for analytical loss.

2.6 Statistical analysis

Four replicate trays were prepared for each of nine treatments which consisted of three inoculation treatments (isolate 182, isolate 620, None), each receiving three fungicide treatments (120 g Sporgon 50WP in 180 litres water/100 m^2, 120 g Sporgon 50WP in 90 litres water/100 m^2, None). Trays were positioned in stacks, in a growing room, according to a trojan square design which takes into account possible sources of variation within a three dimensional space. Data were analysed by ANOVA. The experimental design and data analysis were devised, and carried out by HRI Biometrics Department.

3. Results and Discussion

3.1. Incidence of spotted mushrooms

When no Sporgon 50WP had been applied, between 8-12% of the total mushroom yield developed *Verticillium* spotting symptoms as a result of inoculation with either *Verticillium* 182 or *Verticillium* 620. However, when Sporgon 50WP had been applied, there was a significant reduction in the spotting symptoms due to either isolate to between 2 and 3% of the total yield (Figure 2; see Appendix for statistical analysis)). There was no appreciable reduction in the level of spotting recorded when Sporgon 50WP had been applied in 90 litres of water/100 m^2 bed area under the conditions of this experiment. Although isolate 620 is more sensitive to Sporgon 50WP *in vitro* (Figure 1), it was still capable of causing spotting symptoms in a crop, and was thus not totally controlled. Following the use of Sporgon 50WP, there was little difference between the two isolates in the level of control obtained.

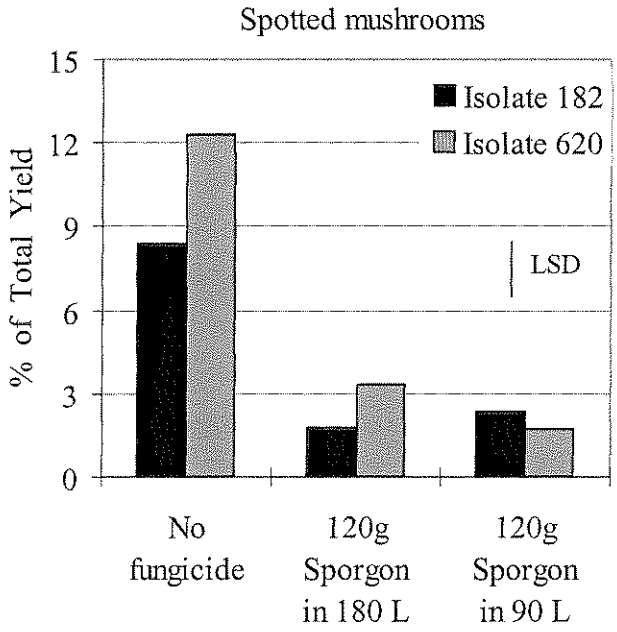

Figure 2. Yield of spotted mushrooms over 2 flushes as % of total yield.

3.2 Incidence of dry bubble

There was a significant difference in the amount of dry bubble which developed due to Isolate 182 or Isolate 620 in the absence of any Sporgon 50WP application. Isolate 620 produced 678 pieces of bubble compared with 335 pieces produced by isolate 182 (Figure 3; See Appendix for statistical analysis). In two further crops this trend was repeated which strongly suggests that Isolate 620 is the more aggressive pathogen of the two. When Sporgon 50WP had been applied, there was a significant reduction in the amount of bubble which developed but levels were similar for both isolates although there was a greater reduction in the number of *Verticillium* 620 bubble pieces than *Verticillium* 182 bubble pieces. This indicates that neither isolate was totally controlled by Sporgon, under

the conditions of this experiment, where inoculum levels were moderately high at 1 million spores/m^2. Isolate 182, although less aggressive as a pathogen, is more tolerant of Sporgon 50WP and therefore control with Sporgon 50WP will be more difficult, whereas Isolate 620, although more sensitive to Sporgon 50WP, is a much more aggressive pathogen which means that is more difficult to contain, and therefore control, even with Sporgon 50WP, to which it is reasonably, but not totally, sensitive to.

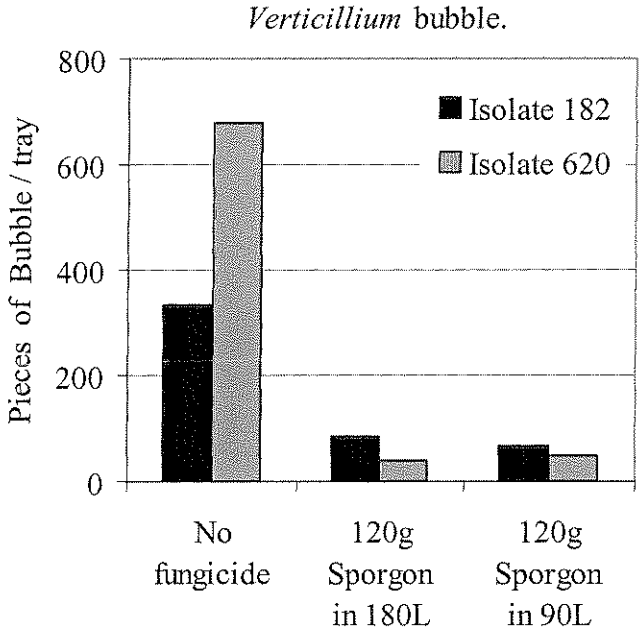

Figure 3. Number of bubble pieces harvested over 2 flushes.

3.3 Prochloraz manganese levels in casing

Prochloraz manganese (a.i. in Sporgon 50WP) was recovered from casing throughout the duration of the crop but the levels present dropped significantly during the crop for both methods of Sporgon 50WP application (Figure 4, see Appendix for statistical analysis). A standard 120 g dose of Sporgon 50WP to 100 m^2 applies the equivalent of 55 g active ingredient/100 m^2. Following a standard application in 180 litres of water /100 m^2, the amount of active ingredient recovered was less than this at 34g/100 m^2 (Day 4) whereas when Sporgon 50WP had been applied in only 90 litres of water /100 m^2, the amount of active ingredient recovered was more than expected at 63 g/100 m^2 (Day 4). The reason for these differences probably reflect difficulties in applying a set volume of liquid to a set area of mushroom bed. The greater the volume of water to be applied then the greater the chance that some of it will drip through, with a corresponding loss of some active ingredient. The smaller the volume to be applied, the more difficult it is to ensure even application and also the more likely it is that a larger volume will be applied. Applying Sporgon 50WP in 90 litres of water /100 m^2 resulted in a greater proportion of the active ingredient being retained in the top layer of casing. This may give better control initially to disease development but the falling levels of active ingredient over time would reduce this benefit.

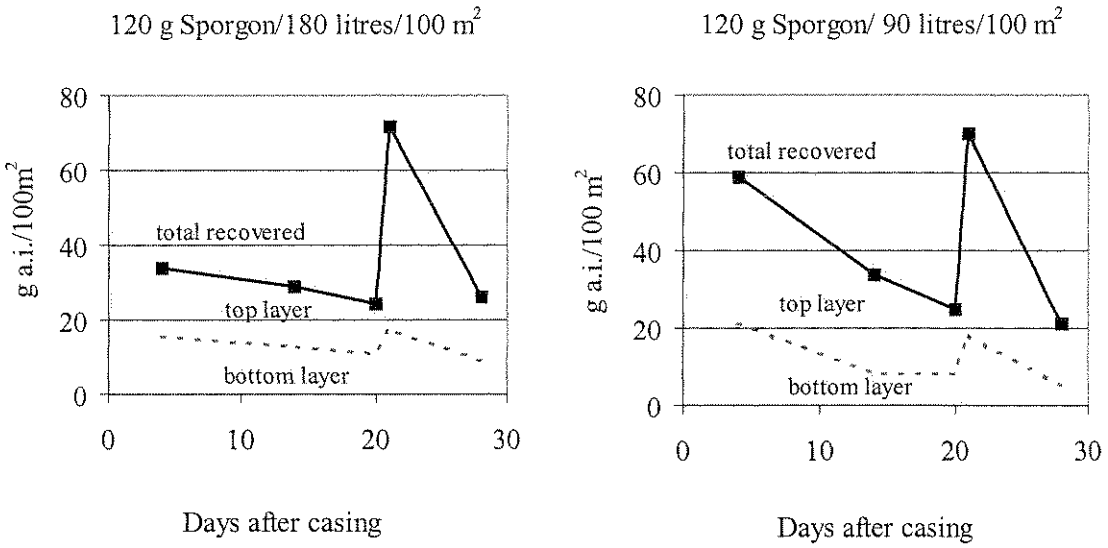

Figure 4. Levels of prochloraz (a.i. in Sporgon 50WP) in top and bottom layers of casing.

By the end of the first flush, the amount of active ingredient recovered had dropped for both Sporgon 50WP treatments to 22-25 g /100 m^2 compared with the 34-63 g/100 m^2 recovered after application. Following the second application at the end of the first flush, levels increased to 70-78 g/100 m^2 but these had dropped again to 36 g/100 m^2 by the end of the second flush. Since no further applications are permitted, the amount of active ingredient in casing in the third and subsequent flushes is much reduced. As a result of this, diseases control by Sporgon 50WP at this time would be expected to be less effective.

4. Conclusions

- Two isolates of *Verticillium*, which were either weakly resistant to Sporgon 50WP (Isolate 182) or moderately sensitive (Isolate 620), were significantly, but not totally, controlled by the use of Sporgon 50WP.

- Isolates of *Verticillium* differ in their sensitivity to Sporgon 50WP which may affect the level of control that can be expected from this fungicide. However, in this study, the more sensitive isolate (Isolate 620) proved to be a more aggressive pathogen causing twice as much bubble, when no Sporgon 50WP was applied, than the more resistant isolate (Isolate 182). This aggressivity cancelled out the benefit of its fungicide sensitivity when Sporgon 50WP was applied.

- Levels of Sporgon 50WP active ingredient in the casing dropped significantly following each application with only 25% (approx.) remaining by the end of the second flush. This would significantly reduce the level of disease control that could be expected.

5. References

Desrumeaux, B., Sedeyn, P, Webrouck, A. & Lannoy, P. (1998). Resistence de la moele seche au sporgon (*Verticillium fungicola* var *fungicola*). *Le bulletin de la Federation Nationale des Syndicats Agricole de Cultivateurs de champignons, Nouvelle serie* **77**, 677-681.

Draght, J.W., Geels, F.P., De Bruijn, W.C. & Van Griensven, L.J.L.D. (1995). Intracellular infection of the cultivated mushroom *Agaricus bisporus* by the mycoparasite *Verticillium fungicola* var *fungicola*. *Mycological Research* **100**, 1082-1086.

Geels, F.P. (1996). Gevoeligheid voor Sporgon van recent geisoleerde stammen van *Verticillium fungicola* var *fungicola*. *De Champignoncultuur* **40**, 401-406.

Grogan, H.M., Gaze, R.H., Amey, R. & Scruby, A. (1998). Survey of fungicide resistance in the mushroom pathogen *Verticillium fungicola* and *Mycogone perniciosa*. *HDC Project Report M 14b* 18pp.

6. Appendix

Statistical analyses of data presented in the text (5 pages)

Genstat 5 - Release 4.1 Created on:16/07/99

03.....

***** Analysis of variance *****

(Figure 2)

Variate: %dyield Percentage diseased by yield (total)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.	
layer stratum	3	7.769	2.590	1.34		
layer.*Units* stratum						
isolate	2	180.992	90.496	46.66	<.001	***
fungicid	2	226.098	113.049	58.29	<.001	***
isolate.fungicid	4	142.672	35.668	18.39	<.001	***
Residual	23(1)	44.605	1.939			
Total	34(1)	591.478				

* MESSAGE: the following units have large residuals.

layer Bottom *units* 1 -3.41 s.e. 1.11

***** Tables of means *****

Variate: %dyield Percentage diseased by yield (total)

Grand mean 3.45

isolate	Uninoculated	Isolate 182	Isolate 620
	0.42	4.16	5.77

fungicid	Nil	S 90	S 180
	6.97	1.36	2.01

isolate \ fungicid	Nil		S 90		S 180
Uninoculated	0.26	=	0.06	=	0.94
Isolate 182	8.37	>	2.33	=	1.78
Isolate 620	12.29	>	1.71	=	3.32

LSD = 2.037

Data for Fig 2.

*** Standard errors of differences of means ***

Table	isolate	fungicid	isolate fungicid
rep.	12	12	4
d.f.	23	23	23
s.e.d.	0.569	0.569	0.985
LSD (sed x t) =	1.177	1.177	2.037

(Not adjusted for missing values)

t(23 df; P ≤ 0.05) = 2.069

***** Missing values *****

Variate: %dyield Percentage diseased by yield (total)

Unit	estimate
15	0.23

Max. no. iterations 2

Missing values: 1

```
204
205  for dy=bubblep[1,2],tbubblep
206
207  anov [fprob=y; des=acl] dy
208  daplot me=hi,fi,no
209  akeep terms=isolate*fungicide; means=mtab[1...3]
210  calc btmtab[1...3] = mtab[1...3]**2-0.375
211  print btmtab[1...3]
```

Genstat 5 - Release 4.1 Created on:16/07/99

13.....

***** Analysis of variance *****

Variate: tbubblep Total number of bubble pieces (sqrt transform) (Figure 3)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.	
layer stratum	3	71.993	23.998	5.65		
layer.*Units* stratum						
isolate	2	1017.271	508.636	119.67	<.001	***
fungicid	2	780.400	390.200	91.80	<.001	***
isolate.fungicid	4	483.615	120.904	28.45	<.001	***
Residual	23(1)	97.759	4.250			
Total	34(1)	2358.887				

* MESSAGE: the following units have large residuals.

layer Top *units* 4 4.02 s.e. 1.65

***** Tables of means *****

Variate: tbubblep Total number of bubble pieces (sqrt transform)

Grand mean 8.47

isolate	Uninoculated	Isolate 182	Isolate 620
	0.99	11.56	12.86

fungicid	Nil	S 90	S 180
	15.04	4.86	5.50

isolate \ fungicid	Nil		S 90		S 180
Uninoculated	1.26	=	0.35	=	1.35
Isolate 182	18.11	>	7.47	=	9.10
Isolate 620	25.75	>	6.77	=	6.05

LSD = 3.017

For back transformed data: PTO →

*** Standard errors of differences of means ***

Table	isolate	fungicid	isolate fungicid
rep.	12	12	4
d.f.	23	23	23
s.e.d.	0.842	0.842	1.458
LSD (sed x t)	1.742	1.742	3.017

(Not adjusted for missing values)

t(23df, p≤0.05) = 2.069

***** Missing values *****

Variate: tbubblep Total number of bubble pieces (sqrt transform)

Unit	estimate
15	-1.00

Max. no. iterations 2

Missing values: 1

btmtab[1]

isolate	
Uninoculated	0.60
Isolate 182	133.17
Isolate 62	164.88

btmtab[2]

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fungicid

Nil	225.83
S 90	23.25
S 180	29.84

Back transformed mean table (BTMtab)

btmtab[3]

fungicid / isolate	Nil	S 90	S 180
Uninoculated	1.2	-0.3	1.4
Isolate 182	327.5	55.4	82.4
Isolate 620	662.7	45.4	36.2

Data for Figure 3.

```
214
215  endjob

******** End of design for fungicide rate trial - Helen Grogan - July 1998.
Maximum of 34001 data units used at line 213 (3233609 left)
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(Figure 4)

***** Analysis of variance *****

Variate: totairec Total weight of active ingredient (log transform)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.	
sblock stratum	3	2.0430	0.6810	1.59		
sblock.splot stratum						
sfungici	2	129.6331	64.8166	151.37	<.001	***
Residual	5(1)	2.1410	0.4282	1.78		
sblock.splot.*Units* stratum						
stime	4	11.9465	2.9866	12.41	<.001	***
sfungici.stime	8	13.5518	1.6940	7.04	<.001	***
Residual	32(4)	7.7035	0.2407			
Total	54(5)	161.8443				

* MESSAGE: the following units have large residuals.

sblock 1	splot 1.00	*units* 3	1.031	s.e. 0.358
sblock 1	splot 1.00	*units* 5	-0.964	s.e. 0.358
sblock 2	splot 26.00	*units* 3	-0.999	s.e. 0.358
sblock 2	splot 26.00	*units* 5	0.998	s.e. 0.358

***** Tables of means *****

Variate: totairec Total weight of active ingredient (log transform)

Grand mean 2.543

sfungici	Nil	S 180	S 90
	0.465	3.533	3.631

stime	Day 4	Day 14	Day 20	Day 21	Day 28
	2.211	2.558	2.607	3.321	2.018

sfungici	stime	Day 4	Day 14	Day 20	Day 21	Day 28
Nil	} N.S.	-0.981	0.765	1.391	1.435	-0.286
S 180		3.532 =	3.376 =	3.210 <	4.275 >	3.272
S 90		4.081 =	3.533 =	3.220 <	4.251 >	3.067

For back transformed data: PTO →

*** Standard errors of differences of means ***

Table	sfungici	stime	sfungici stime	
rep.	20	12	4	LSD
s.e.d.	0.2069	0.2003	0.3730	} 0.762
d.f.	5	32	29.48	
Except when comparing means with the same level(s) of sfungici			0.3469	
d.f.			32	
LSD	0.532	0.408	0.707	

(Not adjusted for missing values)

t values

d.f	t
5	2.571
32	2.037
29.48	2.044

***** Missing values *****

Variate: totairec Total weight of active ingredient (log transform)

Unit	estimate
9	3.859
21	3.311
33	2.998
45	4.030
57	2.845

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Max. no. iterations 5

Missing values: 5

btm2tab[1]

sfungici	
Nil	1.22
S 180	33.85
S 90	37.36

btm2tab[2]

stime	
Day 4	8.75
Day 14	12.53
Day 20	13.18
Day 21	27.30
Day 28	7.15

Back transformed mean table (btm2tab)

btm2tab[3]

sfungici / stime	Nil	S 180	S 90
Day 4	0.00	33.82	58.82
Day 14	1.77	28.87	33.84
Day 20	3.64	24.40	24.66
Day 21	3.83	71.53	69.83
Day 28	0.38	25.98	21.11

Data for Figure 4

427
428 endjob

******** End of design for fungicide rate trial - Helen Grogan - July 1998.
Maximum of 33236 data units used at line 426 (3234374 left)