Contract Report M1b

Control of *Trichoderma* by fungicide treatment of spawn

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by

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CONTENTS

Relevance to growers and practical application

Application

Carbendazim applied to spawn (in the form of Bavistin DF) was successful in controlling the development of compost *Trichoderma* following inoculation with *T. harzianum* spores in an earlier experiment (HDC Contract M1a). A high degree of spawn protection can be obtained when 0.23g Bavistin DF is bulked up in 20g chalk carrier and used to treat 1kg spawn (Figure 6). At a spawning rate of 8 litres/tonne of compost, 1.1g Bavistin DF bulked up in 96g chalk carrier would be required to treat 8 litres of spawn (4.8kg). Greater spawn protection can be acquired using a higher concentration of Bavistin DF but earlier results suggest that carbendazim residue levels might be too high so the lower rate is recommended at this point. Subsequent residue data contradict the earlier findings and further work is required to clarify the situation before off label approval can be obtained.

Summary

The objectives of the research presented in this report were as follows: (1) to assess the aggressivity of two compost isolates of *T. harzianum*; (2) to determine the optimum quantities/kg spawn of both fungicide and chalk carrier required to give a high degree of protection to the spawn against *T. harzianum* and (3) to determine the fungicide residue levels in first flush mushrooms with a view to satisfying the requirements for a future off-label application.

In laboratory tests, visible fungicidal halos developed around the majority of spawn grains

which had been treated with Bavistin DF at a rate of 0.23g diluted in 20g chalk carrier per kg of spawn. Tests also indicated that higher and lower quantities of chalk carrier could reduce the effectiveness of the fungicide.

Laboratory and cropping experiments have indicated that the aggressivity of isolates can vary between isolates and can also be lost over a very short time span. The underlying reasons for this are not yet fully understood.

Despite the loss of aggressivity with respect to *Agaricus* growth both *T. harzianum* isolates T7 and T32 proceeded to become established in inoculated compost which had no fungicidal protection but they failed to reach levels known to reduce *Agaricus* yields. The application of Bavistin DF at rates of 0.12g, 0.18g and 0.23g/kg spawn dramatically inhibited the growth of both isolates within the compost (Figure 9) although isolate T32 was more prolific.

Carbendazim residues were not detected in first flush mushrooms derived from spawn which had been treated with 0.12g to 0.23g Bavistin DF/kg spawn. These findings contradict those obtained for a similar experiment reported on in HDC Contract Report M1a. This discrepancy needs to be clarified prior to recommending a rate for off label approval.

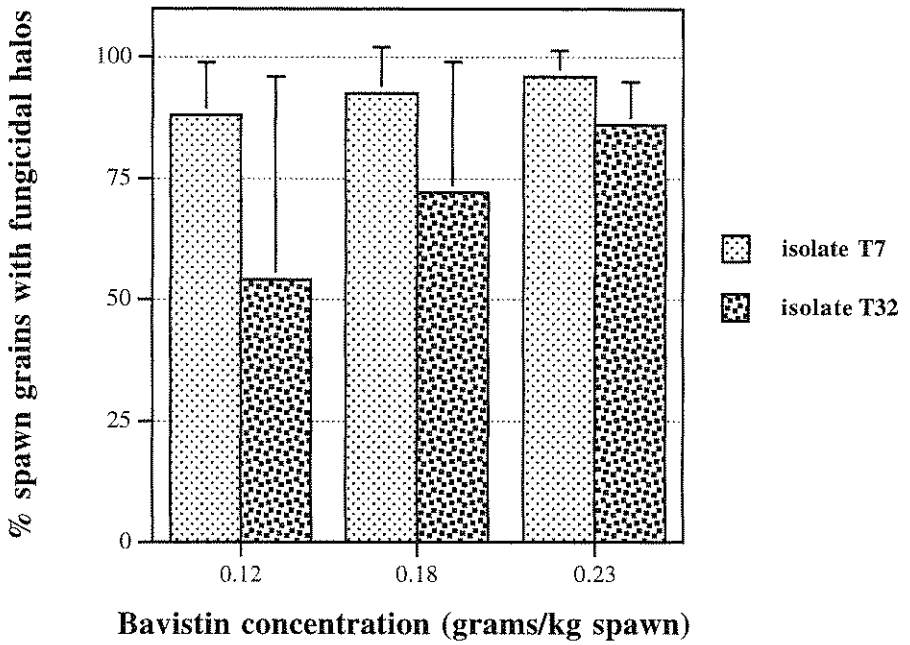

Figure 6. Effect of increasing Bavistin concentration (in 20g chalk carrier) on the frequency of fungicidal halos around treated spawn grains in the presence of *T. harzianum* isolates T7 or T32; mean + standard deviation.

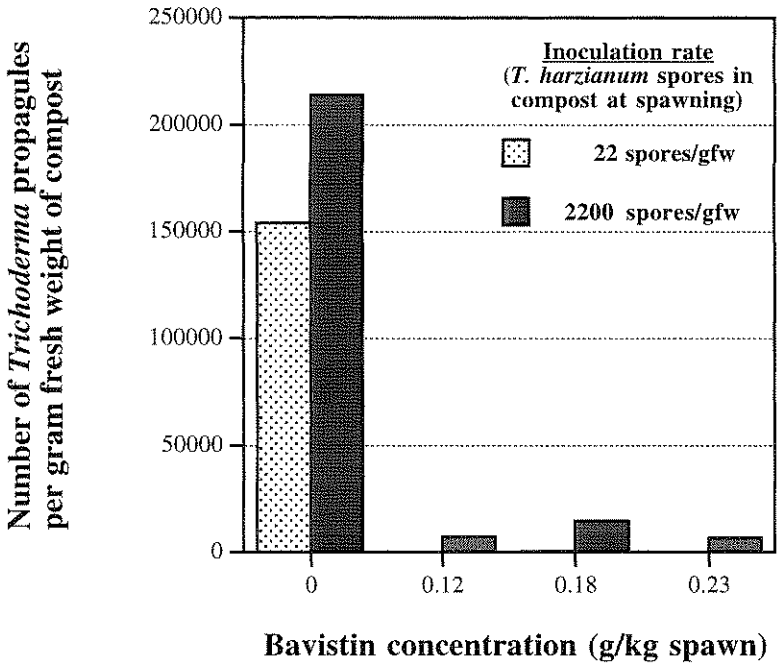

Figure 9. Presence of *Trichoderma* propagules in compost (after four flushes) using spawn treated with different concentrations of Bavistin and inoculated with *T. harzianum* spores at two concentrations. Values represent overall means of treatments (see Appendix for data and statistical analyses).

RESEARCH REPORT

1. Introduction

Trichoderma harzianum remains a serious weed mould of mushroom cultivation ever since the initial devastating outbreaks in 1985/86 (Doyle, 1991). It has also now been recorded in Canada (Rinker, 1994), Australia and more recently the United States of America where it is currently causing severe problems for some growers.

Four strains of *T. harzianum* have been recognised using molecular techniques and have been designated Th1, Th2, Th3 and Th4. Strain Th2 is the one most frequently associated with outbreaks in the British Isles (Muthumeenakshi *et. al.*, 1994) but a fourth strain, Th4, has been associated with the outbreaks in the USA and Canada.

Epidemiological studies have shown that compost colonisation by *T. harzianum* can be achieved at relatively low levels of inoculation. It also seems that *Agaricus* grain spawn in or on the compost provides a readily available carbohydrate source from which compost colonisation can then proceed (J. Fletcher, unpublished). An earlier HDC Research Contract, M1a, (Grogan & Fletcher, 1993) reported on preliminary experiments to control compost *Trichoderma* using fungicides and concluded that significant control could be achieved by the application of Bavistin DF fungicide (active ingredient: carbendazim) to spawn. Only one concentration of fungicide was used which was diluted in a given quantity of chalk carrier.

Carbendazim residue analysis indicated that residues were detectable but below the maximum residue level (MRL) allowed by the Pesticide Safety Directorate. The research presented in this report was commissioned to examine further the effectiveness of Bavistin DF applied to

spawn in controlling compost *Trichoderma* over a range of concentrations of both fungicide and carrier while bearing in mind the maximum residue level that would be acceptable for an off-label application.

2. Materials and Methods

2.1 *Trichoderma harzianum* isolates

Two *T. harzianum* isolates, designated T7 and T32, were used in all experiments. Both were designated as Th2 strains according to molecular techniques and both isolates were obtained from compost which had been severely colonised by *Trichoderma*. Isolate T7, which was used in a previous HDC funded experiment (Report M1a); appeared to be less aggressive than it was originally in terms of compost colonisation so a second, more aggressive, isolate was included in the experimental work presented in this report.

2.2 Carbendazim

The commercial fungicide Bavistin DF (BASF Plc) was used as a source of carbendazim. The "Dry Formulation" (DF) was used in preference to any other because the manufacturers are believed to be concentrating on this formulation for the future.

2.3 Carbendazim application to spawn

Quantities of Bavistin DF ranging from 0.06 to 0.29 grams/kg spawn were bulked up by incorporation of the product into a chalk carrier. Three quantities of carrier were investigated, 10, 20 and 30g/kg spawn, to determine the level which gave the best overall distribution of product on the spawn grains.

2.4 Compost

A commercial compost, made from wheat-straw mixed with pig, horse and poultry manure, (Table 1) was used in the cropping experiment. A small quantity of compost from a second commercial farm was used for the tube bio-assay experiment to assess the agressivity of the *Trichoderma* isolates.

Table 1. Analyses of compost used in cropping experiment

Oven Dry Matter	26.6%
pH (Dry)	6.9%
pH (Fresh)	7.3%
Total Nitrogen	2.45%
Ammonium - N	3050
Total Ash	18.8%
Water Soluble Carbohydrates	0.7%
Enzymatic Starch	<0.1%
Sugar (Luff Schoorl)	0.8%

3. Experimental Procedures

3.1 Tube bio-assay of *Trichoderma harzianum* aggressivity

Three healthy spawn grains and three spawn grains inoculated with *T. harzianum* isolate T7 or T32 were placed in the bottom of a universal tube (9 cm long, 2.1 cm diameter) which was then filled with compost. The lid was lightly screwed in place to allow air exchange to occur. Twenty replicate tubes were prepared for each of the isolates used. The experiment

was carried out twice prior to the cropping experiment and once afterwards on which occasion only eight replicate tubes were prepared. The tubes were incubated for 3 weeks at 25°C after which time they were assessed for colonisation by *Agaricus* mycelium and *T. harzianum* as indicated by the presence of green sporulating areas. Aggressivity was scored on a scale of 0-3 where 0 = no compost colonisation, 1 = lower third of compost colonised by *T. harzianum,* 2 = two-thirds of compost colonised, 3 = compost fully colonised by *T. harzianum*.

3.2 Agar-plate bio-assay of carbendazim activity against *Trichoderma* using Bavistin DF

Healthy spawn grains were treated with Bavistin DF at rates of 0.29, 0.23, 0.18, 0.12 or 0.06g/kg spawn. The fungicide was initially bulked up in a chalk carrier at rates of 10, 20 or 30 grams chalk/kg spawn. The bulked up fungicide was then added to the spawn and gently mixed in thoroughly. One hundred spawn grains from each treatment and 100 untreated spawn grains were then placed onto agar plates which had previously been seeded with 10^4-10^6 spores of *T. harzianum* isolate T7 or T32. Control plates were also set up consisting of untreated spawn grains and Bavistin-treated spawn grains on plates with no *Trichoderma*. After 11 days incubation at 25°C, the presence of fungistatic halos around spawn grains was noted.

3.3 Cropping experiment to evaluate the efficacy of carbendazim in controlling compost *Trichoderma* using Bavistin-treated spawn.

Bavistin-treated spawn was incorporated into commercial compost at the standard rate of 0.5% per tonne of compost which was then filled into wooden trays containing 45 kg. At

spawning, 100 mls of a *Trichoderma* spore suspension (10^4 or 10^6 spores per ml) were sprayed into each 45 kg tray of compost as it was being filled, using two 50 ml syringes. The effective spore concentration in the compost at spawning was 22 or 2200 spores per gram fresh weight of compost corresponding to the 10^4 and 10^6 inoculum concentrations respectively. Eight replicate trays were prepared for each treatment combination (Bavistin rate/*Trichoderma* isolate/inoculum level). Two control treatments, using either Bavistin-treated or untreated spawn in conjunction with no *Trichoderma* inoculum, were also prepared to examine the effects of carbendazim on yield. Four flushes were harvested over a six week period. Prior to cooking-out, compost samples were taken from each tray and analysed for the presence of *Trichoderma* propagules.

3.4 Residue Analysis

Analyses for carbendazim residues were carried out on 500g of first flush mushrooms harvested from trays which had received Bavistin-treated spawn. Mushrooms derived from untreated spawn were also analysed for carbendazim residues. The analyses were carried out by Oxford Analytical Laboratories, Bicester, OX6 7PP, UK, according to good laboratory practise (GLP) standards.

4. Results and Discussion

4.1 Aggressivity of *Trichoderma harzianum* isolates.

The results from the tube bio-assay of *T. harzianum* agressivity are presented in Figure 1. Isolate T7 colonised less than one-third of the compost in the tubes in each of the three

experimental runs although there was considerable variation among the replicates. The growth of *Agaricus* was good (although variable) but this isolate used to be an aggressive compost coloniser, frequently fully colonising the tube of compost and totally inhibiting *Agaricus* growth. Its loss of aggressivity may be related to the age of the isolate along with its preservation and maintenance in the laboratory.

Isolate T32 was the more aggressive of the two isolates used, colonising on average almost two-thirds of the compost in the tubes in two out of the three experimental runs, although considerable variation was also recorded. *Agaricus* growth was also more severely inhibited. In the third run however, which utilised the cultures used in the cropping experiment, there was no colonisation of the compost at all by isolate T32 with good colonisation by *Agaricus* indicating how the ability to aggressively colonise compost can be lost quite quickly.

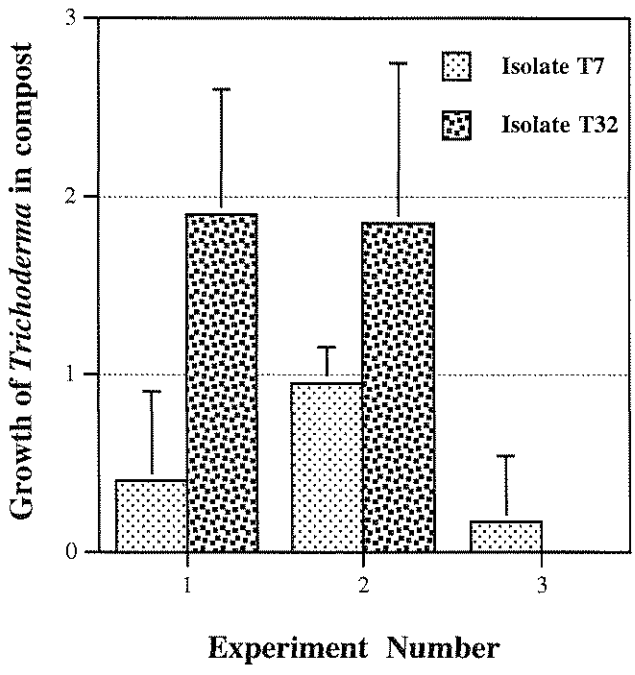

Figure 1. Growth of *T. harzianum* isolates T7 and T32 in tubes of compost on a scale of 0 (no growth) to 3 (tube fully colonised by *T. harzianum)*; mean + standard deviation.

4.2 Carbendazim activity against *Trichoderma harzianum* using Bavistin DF in a chalk carrier.

A number of experiments were carried out to determine the optimum rates of Bavistin DF and chalk carrier required to protect spawn grains from colonisation by *T. harzianum* isolates. The results are presented in Figures 2 to 6.

In the course of the initial exploratory experiments using fungicides on spawn to control *Trichoderma* (HDC Report M1a) only one rate of Bavistin DF, 0.23g/kg spawn, was examined. The residues in first flush mushrooms from this experiment were close to the maximum residue level allowed by the Pesticide Safety Directorate so the range of rates used in the present experiment were not increased significantly. Lower rates were examined to see if they were as effective as the original rate of 0.23g/kg spawn and one higher rate of 0.29g/kg spawn was examined for comparison. Different quantities of fungicide carrier (chalk) were also examined as only one rate, 10g/kg spawn, was used in the initial exploratory experiments (HDC Report M1a).

Rates of Bavistin DF

Spawn grains treated with Bavistin DF at various concentrations showed no signs of any phytotoxic effects when grown in sterile conditions. Figures 2 illustrates how increased spawn protection against *T. harzianum* isolate T7 was obtained with increasing concentrations of Bavistin DF. No protection was given at a rate of 0.06g/kg spawn at which concentration all spawn grains were colonised by *Trichoderma*. After 11 days incubation, *T. harzianum* isolate T32 had colonised many of the spawn grains with very few developing protective fungicidal halos (Figure 3) indicating that this isolate may be more difficult to control.

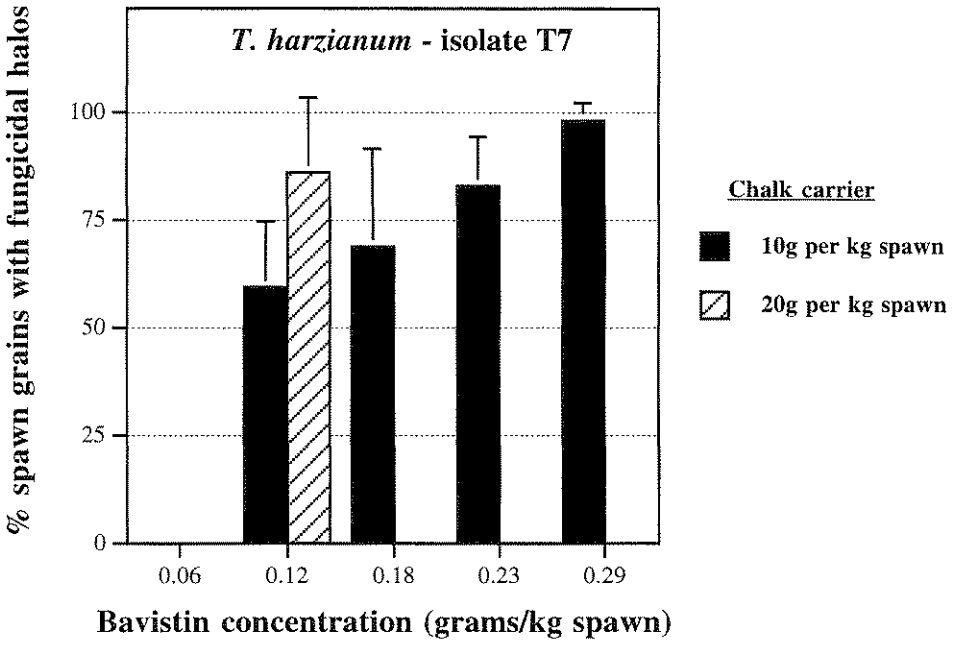

Figure 2. Effect of increasing Bavistin concentration on the frequency of fungicidal halos around treated spawn grains in the presence of *T. harzianum* isolate T7; mean + standard deviation.

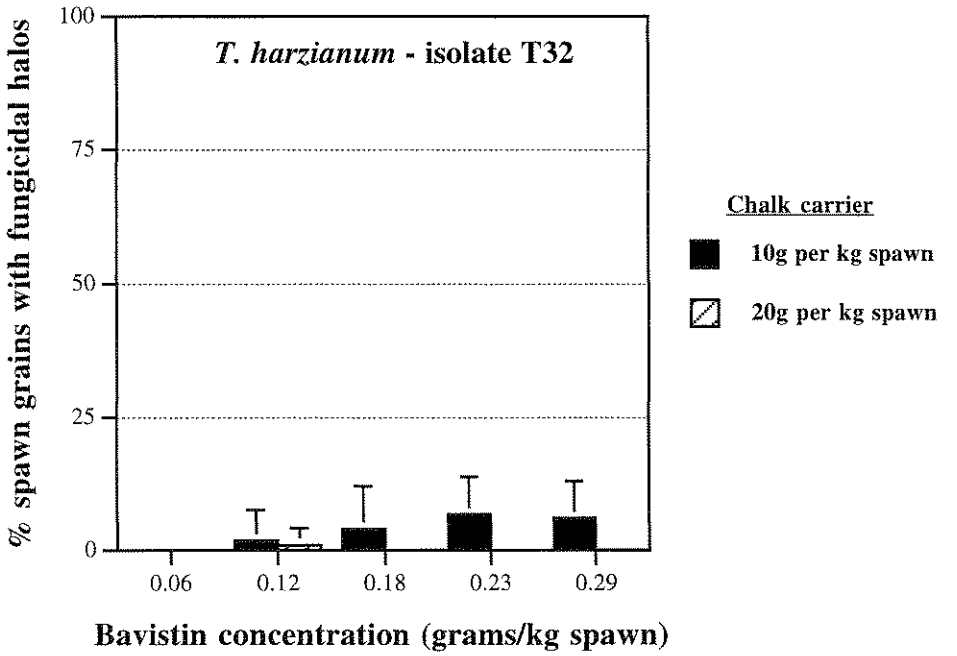

Figure 3. Effect of increasing Bavistin concentration on the frequency of fungicidal halos around treated spawn grains in the presence of *T. harzianum* isolate T32; mean + standard deviation.

Alternatively, it may indicate poor distribution of the fungicide during mixing which is an area requiring further examination.

During this experiment, one Bavistin treatment (0.12g/kg) was repeated using double the quantity of chalk carrier to see if there was any difference in the effectiveness of the fungicide. The results in figure 2 indicated that doubling the quantity of chalk carrier significantly increased the presence of fungicidal halos around the spawn grains and a second experiment was carried out to examine this further.

Rates of chalk carrier

Three rates of chalk carrier were investigated for their effect on the degree of protection given to spawn grains treated with two rates of Bavistin DF. The results, presented in Figures 4 and 5, were not as clearcut as the results in Figure 2 seemed to suggest. At the lower Bavistin concentration (0.12g/kg spawn) fungicidal halos developed around a significant number of spawn grains with no *Trichoderma* colonisation being recorded for either isolate T7 or T32 and with no clear effect related to the quantity of chalk carrier used. At the higher Bavistin concentration (0.24g/kg spawn) there was little difference between the 10g and 20g rates of chalk carrier but the results suggest that 30g of chalk carrier/kg spawn can have a negative effect. It was noticed during the experiment that at this rate, there was always some unmixed chalk remaining at the bottom of the container which would effectively reduce the concentration of fungicide applied to the spawn.

The results of these laboratory experiments indicated that the optimum rate of chalk carrier should be in the region of 20g/kg spawn but further experimentation is advised due to the degree of variation observed.

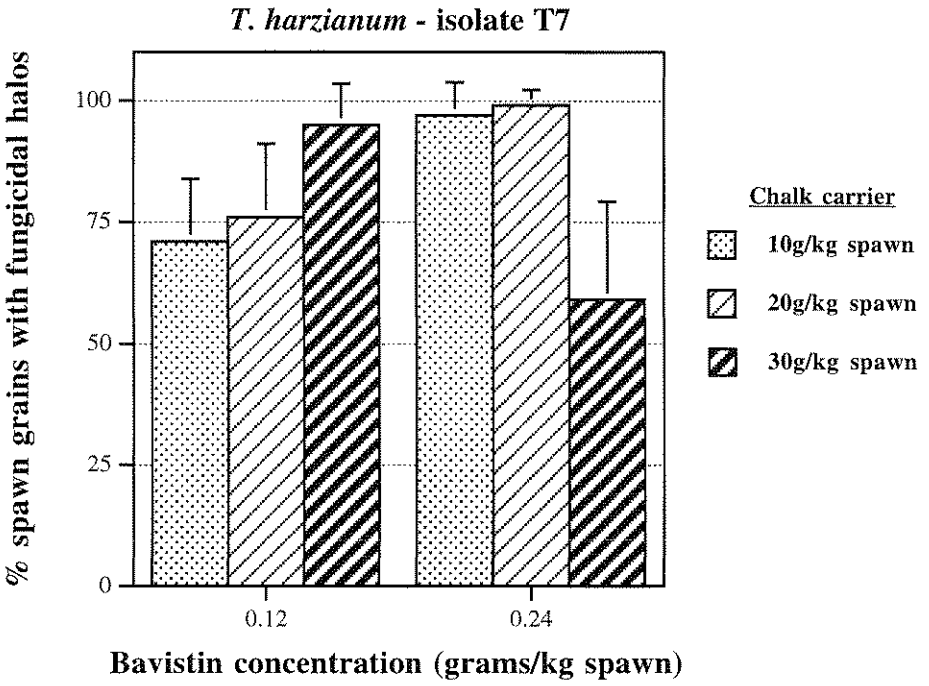

Figure 4. Effect of increasing chalk carrier concentration on the frequency of fungicidal halos around Bavistin treated spawn grains in the presence of *T. harzianum* isolate T7; mean + standard deviation.

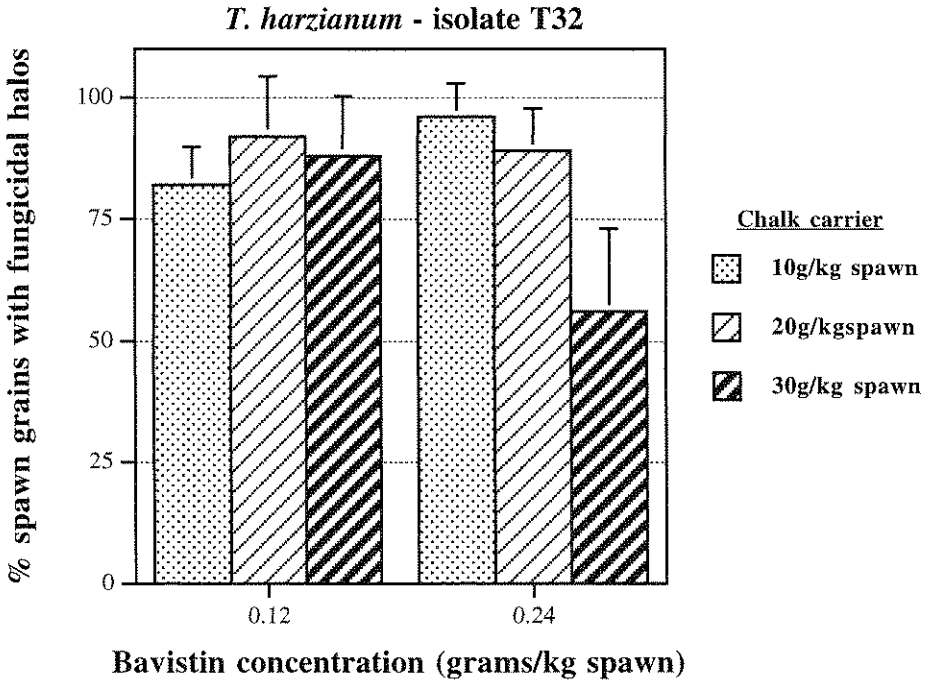

Figure 5. Effect of increasing chalk carrier concentration on the frequency of fungicidal halos around Bavistin-treated spawn grains in the presence of *T. harzianum isolate* T 32; mean + standard deviation.

4.3 Yield effects and *Trichoderma harzianum* population development during a cropping experiment.

A commercial compost was spawned with Somycel 609 spawn, batches of which had been earlier treated with Bavistin DF at rates of 0.12, 0.18 or 0.23g/kg spawn. The fungicide was evenly mixed into 20g of chalk carrier/kg spawn and then gently mixed into the spawn to get an even distribution. One hundred grains of each spawn treatment were placed onto agar plates previously inoculated with spores of *T. harzianum* isolates T7 and T32. The distribution of the fungicide onto the individual spawn grains was assessed by scoring the grains for the presence of fungicidal halos (Plate 1, Appendix). Figure 6 indicates how the best protection was obtained at the rate of 0.23g Bavistin/kg spawn for both isolates. Fungicidal protection was less effective against isolate T32, especially at the lower rates of Bavistin, indicating the more aggressive nature of this isolate as indicated elsewhere.

Yield

There were no significant differences in the yields obtained from various treatments examined in the cropping experiment (Figure 7). Compost which was inoculated with *T. harzianum* isolates T7 and T32, and spawned with non-fungicide treated spawn, failed to develop any visible *Trichoderma* symptoms and yields were as good as for the uninoculated controls. Since neither of the two isolates acted aggressively, the efficiency of the spawn treatments with respect to yeild could not be assessed. This loss of aggressivity was subsequently verified using the tube bio-assay technique described earlier (see results for experiment 3, Figure 1). The application of the fungicide to spawn did not significantly affect the yield indicating that such treatment does not have a fungitoxic effect on the *Agaricus* mycelium.

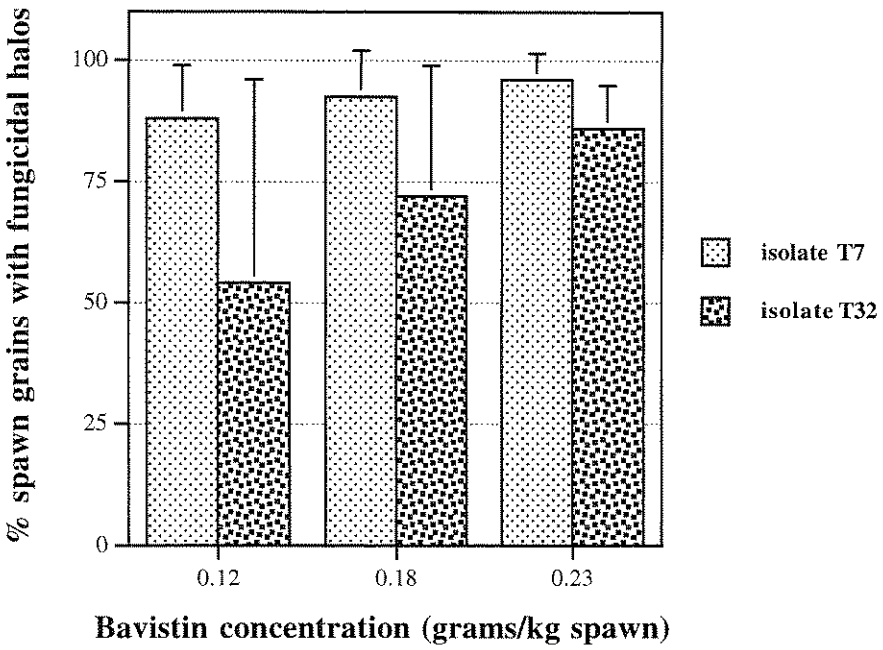

Figure 6. Effect of increasing Bavistin concentration (in 20g chalk carrier) on the frequency of fungicidal halos around treated spawn grains in the presence of *T. harzianum* isolates T7 or T32; mean + standard deviation.

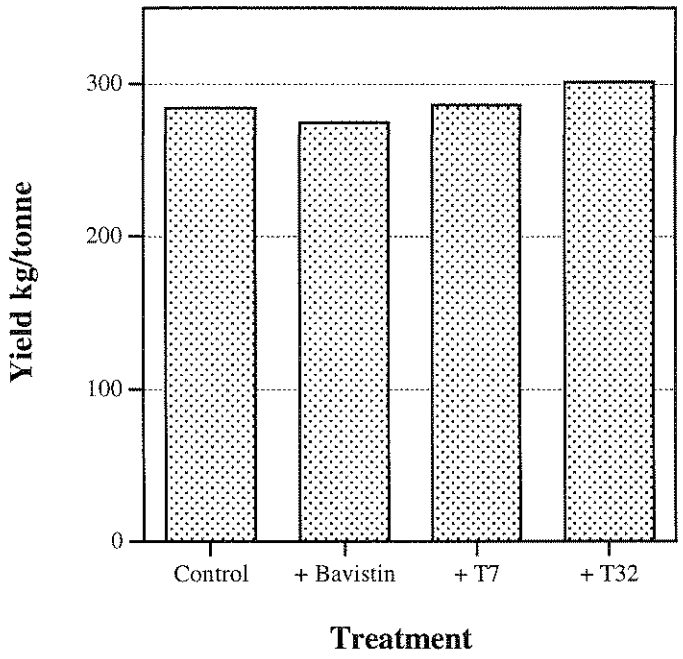

Figure 7. Yield of mushrooms from compost which had been either untreated (Control), spawned with Bavistin-treated spawn (+Bavistin), inoculated with *T. harzianum* isolate T7 (+T7) or inoculated with *T. harzianum* isolate T32 (+T32).

Trichoderma harzianum populations in compost

Although neither of the *T. harzianum* isolates T7 or T32 behaved aggressively during the course of the cropping trial, both organisms grew significantly within the compost and high numbers of propagules were isolated at the end of the crop. In the inoculated control plots 2 x 10^5 propagules/gram fresh weight (gfw) of compost were present but this was considerably less than the 46 x 10^6 propagules/gfw isolated from inoculated compost during HDC Research Contract M1a. In general, isolate T32 was more prolific than isolate T7 (Figure 8) indicating significant differences between these two Th2 isolates although there was a high degree of variation among samples.

The fungicide treatments succeeded in significantly reducing the growth of *Trichoderma* in the compost but the actual rate of fungicide used had little effect (Figure 9) with all three levels used giving significant control. Better control of propagule numbers was achieved when the compost was inoculated with a relatively low concentration of spores. There was a significantly higher number of propagules recovered after the fourth flush from the compost which had been inoculated with the higher concentration of *Trichoderma* spores as might be expected but it was still very much lower than the control plots. The propagule numbers isolated from compost samples during the course of these experiments never reached levels that were associated with detectable crop losses in the previous HDC *Trichoderma* Research Contract M1a. Nonetheless valuable information has been obtained to indicate that there are growth differences in response to fungicide treatments between so called "aggressive Th2" isolates of *T. harzianum* when incorporated into compost.

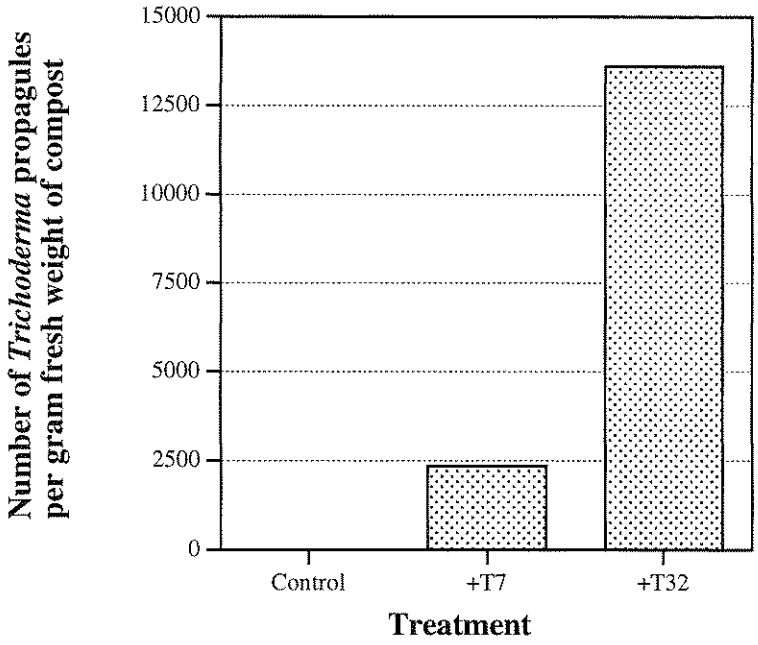

Figure 8. Presence of *Trichoderma* propagules in uninoculated (Control) or inoculated compost (+T7, +T32) after four flushes. Values represent overall means of treatments (see Appendix for data and statistical analyses).

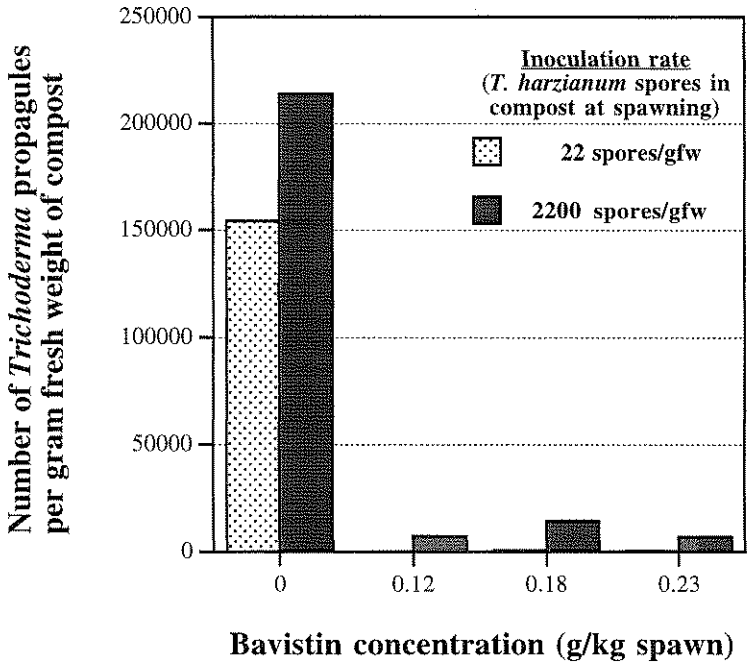

Figure 9. Presence of *Trichoderma* propagules in compost (after four flushes) using spawn treated with different concentrations of Bavistin and inoculated with *T. harzianum* spores at two concentrations. Values represent overall means of treatments (see Appendix for data and statistical analyses).

4.4 Residue data of first flush mushrooms

No carbendazim residues, the active ingredient in Bavistin DF, were found in the first flush mushrooms analysed from each fungicide treatment. This result was contrary to what was expected based on the residue data from the earlier HDC *Trichoderma* Research Project M1a where residues of 0.93mg/kg mushrooms were detected in mushrooms grown from spawn treated with Bavistin DF at a rate of 0.23g/kg spawn (Table 2). The analyses were carried out by different laboratories and raise a question over the standardisation of residue detection methods. Recent discussion with dutch scientists has indicated a similar problem with regards to the detection of prochloraz manganese (Sporgon) residues where different results were recorded for the same samples but which where analysed using different methods or in different laboratories.

Table 2 Carbendazim residues in first flush mushrooms

	Residue levels (mg/kg)*	
Spawn treatment with Bavistin DF	Previous HDC Research Contract M1a (September 1993)	Current HDC Research Contract M1b
0.23g/Kg Spawn	0.93	ND**
0.18g/Kg Spawn		ND
0.12g/Kg Spawn		ND
0g/Kg Spawn	ND	ND

* Maximum residue level (MRL) accepted by Pesticide Safety Directorate = 1 mg/kg

** ND not detected

In view of the closeness of one set of residue data to the MRL it is advisable to carry out further residue analyses, preferably by two laboratories simultaneously. Accurate residue data is essential before any off-label approval can be obtained.

5. Conclusions

- *Trichoderma harzianum* populations in compost can be controlled by treating spawn grains with Bavistin DF at rates of between 0.12g and 0.23g in 20g chalk carrier per kg of spawn.

- Due to a discrepancy in carbendazim residue analysis between two different analytical laboratories further residue analysis must be done to clarify the situation prior to seeking off label approval.

- Laboratory and cropping experiments indicate that the different *T. harzianum* isolates vary in their aggressiveness and that aggressiveness can be lost over a relatively short time span.

6. References

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7. Appendix

Statistical Data for Figure 8

Variable: *Trichoderma* propagule counts converted to their corresponding 10^{th} root value in order to stabilize their variances.

Source of variation: *Trichoderma* isolates.

Data for Figure 8. Presence of *Trichoderma* propagules in uninoculated (control) or inoculated compost (+T7, +T32) after four flushes. Analysis of variance carried out on 10^{th} root transformed data.

	Control	+T7	+T32
Transformed mean	0.692	2.173	2.590
Back transformed mean (no. propagules/gfw)	(0.025)	(2347)	(13,583)
No. of replicates	8	64	64

P = 0.003 (**)

Standard error of differences of transformed means:

8 and 64 replicate comparison 0.2883

64 and 64 replicate comparison 0.1359

Statistical Data for Figure 9

Variable: *Trichoderma* propagule counts transformed to their corresponding 10^{th} root value in order to stabilize their variances.

Source of variation: Inoculum concentration and fungicide concentration.
Presence of *Trichoderma* propagules in compost inoculated with two concentrations of *Trichoderma* spores and using spawn treated with different concentrations of Bavistin DF. Analysis of variance carried out on 10^{th} root transformed data. Back transformed means are in brackets.

Inoculum concentration	10^4 spores/ml		10^6 spores/ml	
Bavistin DF concentration (g/kg spawn)	(22 Spores/gfw compost)			
0	3.302	(154,088)	3.412	(213,840)
0.12g	1.275	(11)	2.434	(7,298)
0.18g	1.884	(563)	2.605	(14,390)
0.23g	1.721	(227)	2.417	(6,804)
Number of replicates	16		16	

P = 0.0416 (*)

Standard error of differences of transformed means = 0.2591